

**Advanced Star Polymers:
From Synthetic Developments to
Biomedical Applications**

By

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June 2020

*Submitted in total fulfillment of the requirements
for the degree of Doctor of Philosophy*

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Abstract

The developments in polymer synthesis and their applications over the last 100 years have had a profound effect on the world as we know it. While polymers abound in our everyday lives, significant advances continue to be made in the field of polymer chemistry. The first reversible deactivation radical polymerization (RDRP) method was discovered in the 1980s, enabling the synthesis of polymers with tailored molecular weights and advanced architectures. Reversible addition-fragmentation chain transfer (RAFT) polymerization is one type of RDRP that has shown exceptional promise for the synthesis of advanced materials, with recent developments in photo-mediated RAFT polymerization leading to unprecedented control of polymer size and architecture.

Star polymers are one type of advanced architecture readily synthesized by RDRP and other techniques for a range of applications. Many of the recent advances in RAFT polymerization have yet to be applied in advanced architectures such as stars. Thus, the objective of this thesis is to investigate the synthesis of star polymers and their applications, with focus on stars synthesized by photo-mediated RAFT polymerization and N-carboxyanhydride ring-opening polymerization (NCA ROP).

We first investigated the synthesis of star polymers via photoiniferter RAFT polymerization using a core-first approach. Multifunctional cores were synthesized for star synthesis via RAFT polymerization and early results demonstrated the highly “living” nature of the star polymers. This highly living nature allowed us to synthesize ultra-high molecular weight (UHMW) star polymers with molecular weights in excess of 20 MDa, the largest reported for any RDRP techniques to date.

Although photoiniferter RAFT polymerization resulted in highly living stars, it does suffer from two significant limitations. The polymerizations are sensitive to oxygen and can only be mediated by a small portion of the electromagnetic spectrum. Photoinduced energy/electron transfer (PET)-RAFT polymerization can overcome these limitations by employing a photoredox catalyst or photosensitizer. We synthesized a novel self-assembled photocatalyst with broadband and near-infrared (NIR) absorbance, facilitating polymerization across the UV, visible light, and NIR regions. Using this photocatalyst we reported the first NIR-mediated RDRP in aqueous conditions and NIR-mediated star polymerization.

Finally, the application of star polymers with degradable polypeptide segments was investigated for lysosomal escape of a model drug *in vitro*. Endo-lysosomal escape of therapeutics to reach their site of action remains a challenge in the field of nanoparticle drug delivery. Star polymers have exceptional potential in drug delivery stemming from their nanometer size and compact, unimolecular structure that allows for functionalization of distinct core, arm, and peripheral regions. Dye-loaded polypeptide star polymers with PEG-brush coronas were biocompatible, demonstrated successful internalization by cells, and allowed the dye to escape the lysosome due to enzymatic degradation of the polypeptide arms while a nondegradable control star accumulated in the lysosome, demonstrating the advantage of polypeptide stars for drug delivery.

Together, these results mark exciting advances in the synthesis of star polymers and their applications. The versatility and exquisite control of photo-mediated RAFT polymerizations are demonstrated, while the advances reported in this thesis show great potential for numerous biomedical applications including biomaterials, cell surface modification, and drug delivery.

Declaration of Authorship

This is to certify that:

- i. the thesis comprises only their original work towards the PhD except where indicated in the preface;
- ii. due acknowledgement has been made in the text to all other material used; and
- iii. the thesis is fewer than 100 000 words, exclusive of tables, maps, bibliographies and appendices

Stephanie Nadine Allison

June 2020

Preface

i. The text on pages 1-47 includes excerpts from “Star polymers by RAFT polymerization (book chapter)”, G Moad (Ed). *Handbook of RAFT Polymerization: 2nd Edition*. Wiley Publishing. *Submitted* and “Polypeptides via ring opening polymerization of α -amino acids: Advances in synthesis, architecture and applications”, *Chemical Society Reviews*, **2020**. DOI: 10.1039/c9cs00738e

- All the writing in the Introduction is my own
- Copies of the original publications have been attached at the end of the thesis

ii. The text on pages 76-97 is from “Highly living stars via core-first photo- RAFT polymerization: Exploitation for ultra-high molecular weight star synthesis”, *ACS Macro Letters*, **2019**, 8: 1291-1295.

- All the results in this publication are my own work
- Dr Fatemeh Karimi and Dr Thomas McKenzie assisted with the synthesis of multifunctional cores
- A copy of the original publication has been attached at the end of the thesis

iii. The text on pages 102-122 is from “From UV to NIR: A full spectrum metal-free photocatalyst for efficient polymer synthesis in aqueous conditions”, *Angewandte Chemie International Edition*, **2020**, DOI: 10.1002/anie.202007196.

- All the results in this publication are my own work
- Min Liu performed scanning and transmission electron microscopy.
- Dr Qiang Fu assisted with the self-assembly of the photocatalyst and RAFT polymerizations
- Previously reported photocatalysts CTF, FAT-1, and g-C₃N₄ were provided by the lab of Prof Junwang Tang at University College London
- A copy of the original publication has been attached at the end of the thesis

iv. The text from pages 128-149 is from “Polypeptides stars for improved lysosomal escape” (In preparation).

- All the results in this publication are my own work
- Dr Laura FitzGerald performed the lysosomal escape assay
- Wang Yin performed the cell viability experiment

Acknowledgements

The phrase “it takes a village” has never rung so true for me before. This thesis would not have been possible without the help of countless people. The past 4.5 years have been a rollercoaster filled with amazing labmates, exciting results, research setbacks, incredible research experiences, and the occasional malfunctioning GPC. I would not have made it this far without the support of so many people.

First and foremost, I would like to thank my supervisor Prof Greg Qiao. You agreed to take me on as a student despite having no previous experience in polymer or chemical synthesis and I am so grateful you did. I would also like to thank you for your support in pursuing research experiences outside of our lab. Throughout my PhD I attended five conferences, Polymer Summer School, visited research institutes across Germany, and completed a research stay at the Massachusetts Institute of Technology, none of which would have been possible without your support. I would like to thank my supervisory committee members Prof Wei Duan and Prof Frank Caruso. I have appreciated your guidance, feedback, and advice over the past few years. I would also like to thank Jodie Salmons and Louise Baker for your help with the administrative side of my PhD studies.

I am so grateful to the past and present members of the Polymer Science Group who were so supportive throughout my PhD. I had a so much fun with you all at our end of year parties, conferences, and many campus BBQs. Thank you to all the great post docs in the group: Paul Gurr for keeping the lab running and suffering alongside me and the Dees, Steven Shirbin, Tom McKenzie, Mitch Nothling, and Qiang Fu for their knowledge and assistance, and Joel Scofield for always being happy to help with any and all issues. Alicia, Fatemeh, Bingxin, and Amin – I feel so lucky to have progressed through my PhD with you! I don’t know how I would have done it without you. Wenlian, Min, Nick, and Hadi – thank you for being awesome labmates, collaborators, and all-round great people. Thanks for joining our group Ross, it was great to have another Canadian in my corner.

I would like to thank Prof Bob Langer, Prof Dan Anderson, and Dr Arijit Basu for your mentorship and supervision during my research stay at MIT. I learned so much and met so many amazing people. Joe, Crystal, Dane, Ben, Alby, Maria, and Subha – you made my stay so much fun in and out of the lab. Thank you to Arnab and Rajit for all your help in the lab, I wouldn’t have got anything done without all your guidance. A big thanks to the other hundred members of

the lab for helping me and being so welcoming. I had an absolute blast spending time with you at softball, conferences, and in Hawaii.

I would like to thank the Melbourne School of Engineering, the Len Stevens family, the Australian Nanotechnology Network, and the Royal Australian Chemical Institute for funding that allowed me to complete my research stay at MIT, as well as the Bayreuth-Melbourne Polymer/Colloid Network that supported my trip to Germany.

Finally, I would like to thank my friends and family. My husband Andrew, who went on this crazy adventure and moved to Australia with me. I can't put into words how much I appreciate all the times you listened to me both rejoice and complain about reactions and polymerizations that you really weren't interested in. I couldn't have done this without you. Thank you to my parents, Trina and Steve, for your constant support and encouragement. Thank you to my sister who came to visit me twice! I had a blast adventuring around Australia with you. I would also like to thank my in-laws, who are so encouraging even when I felt like I would never finish. To all the new friends I have made in Melbourne, thank you for making my time here so enjoyable, and providing an often-needed break from studies.

Publications

Publications Included in this Thesis

Book Chapter

Chapter 1

S Allison-Logan, F Karimi, MD Nothling, GG Qiao. Star polymers by RAFT polymerization (book chapter). G Moad (Ed). *Handbook of RAFT Polymerization: 2nd Edition*. Wiley Publishing. *Submitted*.

Peer-review Journal Papers

Chapter 1

A Rasines Mazo, **S Allison-Logan**, F Karimi, NJ Chan, W Qiu, NM O'Brien-Simpson, GG Qiao. Polypeptides via ring opening polymerization of α -amino acids: Advances in synthesis, architecture and applications. *Chemical Society Reviews*, **2020**. DOI: 10.1039/c9cs00738e

M Nothling, Q Fu, A Rehyani, **S Allison-Logan**, J Zhu, M Kamigaito, C Boyer, GG Qiao. Progress and perspectives beyond traditional RAFT polymerization. *Advanced Science*, **2020**. DOI: 10.1002/advs.202001656

Chapter 2

S Allison-Logan S, F Karimi, Y Sun, TG McKenzie, MD Nothling, G Bryant, GG Qiao. Highly living stars via core-first photo- RAFT polymerization: Exploitation for ultra-high molecular weight star synthesis. *ACS Macro Letters*, **2019**, 8: 1291-1295.

Chapter 3

S Allison-Logan, Q Fu, Y Sun, M Liu, J Tang, GG Qiao. From UV to NIR: A full spectrum metal-free photocatalyst for efficient RAFT polymerization in aqueous conditions. *Angewandte Chemie International Edition*, **2020**. DOI: 10.1002/anie202007196

Chapter 4

S Allison-Logan, L FitzGerald, W Yin, W Duan, A Johnston, GG Qiao. *In preparation*

Other Publications

A Reyhani, **S Allison-Logan**, H Ranji-Burachaloo, TG McKenzie, G Bryant, GG Qiao. Synthesis of ultra-high molecular weight polymers by controlled production of initiating radicals. *Journal of Polymer Science, Part A: Polymer Chemistry*, **2019**, 57: 1922-1930.

J Collins, TG McKenzie, MD Nothling, **S Allison-Logan**, M Ashokkumar, GG Qiao. Sonochemically initiated RAFT polymerization in organic solvents. *Macromolecules*, **2019**, 52(1): 185-195.

Conference Proceedings

S Allison-Logan, F Karimi, Y Sun, TG McKenzie, M. Nothling, G Bryant, and GG Qiao. Highly “living” star polymers via photo-mediated RAFT polymerization. *Poster Presentation*. 15th US-Japan Symposium on Drug Delivery Systems. Maui, USA, 14-18 December 2019.

S Allison-Logan, F Karimi, Y Sun, TG McKenzie, M. Nothling, G Bryant, and GG Qiao. Highly living stars via core-first photo-RAFT: Exploitation for UHMW star synthesis. *Oral Presentation*. 37th Australasian Polymer Symposium, Sunshine Coast, Australia, 10-13 November 2019.

S Allison-Logan, F Karimi, Y Sun, TG McKenzie, M. Nothling, G Bryant, and GG Qiao. Highly living stars via core-first photo-RAFT: Exploitation for UHMW star synthesis. *Oral Presentation*. PolymerVic 2018, Parkville, Australia, 21-22 November 2018.

S Allison-Logan, F Karimi, TG McKenzie, M. Nothling, G Bryant, and GG Qiao. Synthesis of UHMW star polymers. *Oral Presentation*. World Polymer Congress Macro18, Cairns, Australia, 1-5 July 2018.

S Allison, S Shirbin, TG McKenzie, GG Qiao. Polypeptide nanocarriers for cancer therapy. *Poster Presentation*. 36th Australasian Polymer Symposium, Lorne, Australia, 20-23 November 2016.

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List of Abbreviations

AIBN	Azobisisobutyronitrile
ATRP	Atom transfer radical polymerization
BASP	Brush arm star polymer
BLG	γ -Benzyl L-glutamic acid
BTPA	2-(n-butylthiocarbonylthio) propionic acid
CCS	Core crosslinked star
CD	Cyclodextrin
CL	Crosslinker
CRP	Controlled/living radical polymerization
CTA	Chain transfer agent
Da	Dalton
DAPI	4',6-diamino-2-phenylindole
DCC	Dicyclohexyl carbodiimide
DCM	Dichloromethane
DLS	Dynamic Light Scattering
DMA	Dimethylacrylamide
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DP	Degree of polymerization
EDCI	1-ethyl-3(3-dimethylaminopropyl)carbodiimide
EDTA	Ethylenediaminetetraacetic acid
FCS	Fetal calf serum
FBS	Fetal bovine serum
GPC	Gel permeation chromatography
HBr	Hydrogen bromide
HCl	Hydrogen chloride
kDa	Kilodalton
LAM	Less activated monomer
LED	Light emitting diode
LS	Light scattering

M _n	Number-averaged molecular weight
M _w	Weight-averaged molecular weight
MADIX	Molecular design by the interchange of xanthates
MAM	More activated monomer
MBA	Methylenebis(acrylamide)
MBC	Minimum bactericidal concentration
MI	Macroinitiator
MIC	Minimum inhibitory concentration
MM	Macromonomer
MSA	Methanesulfonic acid
MW	Molecular weight
NCA	N-carboxyanhydride
NHS	N-hydroxysuccinimide
NIR	Near-infrared
NMP	Nitroxide-mediated polymerization
NMR	Nuclear magnetic resonance
OEG	Oligoethylene glycol
PAMAM	Poly(amidoamine)
PBLG	Poly(γ -benzyl L-glutamic acid)
PBS	Phosphate buffered saline
PDMA	Poly(dimethylacrylamide)
PEG	Poly(ethylene glycol)
PEGA	Poly(ethylene glycol) methyl ether acrylate
PET	Photoinduced electron/energy transfer
PFPA	Pentafluorophenyl acrylate
PLG	Poly(L-glutamic acid)
PLL	Poly(L-lysine)
PPEGA	Poly(PEGA)
PZLL	Poly(ϵ -carbobenzyloxy-L-lysine)
RAFT	Reversible addition fragmentation chain transfer
RI	Refractive index
RDRP	Reversible deactivation radical polymerization
ROMP	Ring-opening metathesis polymerization

ROP	Ring-opening polymerization
SA-TCPP	Self-assembled 5,10,15,20-tetra (4-carboxy-phenol) porphyrin
SEM	Scanning electron microscopy
SLS	Static light scattering
tBA	Tert-butyl acrylate
TEA	Triethylamine
TEOA	Triethanolamine
TEM	Transmission electron microscopy
THF	Tetrahydrofuran
TCPP	5,10,15,20-tetra (4-carboxy-phenol) porphyrin
TTC	Trithiocarbonate
UV	Ultraviolet
VBA	Vinyl benzaldehyde
VBC	4-vinylbenzyl chloride
ZnTPP	Zinc tetraphenylporphyrin

Chapter 1

Introduction

1.1 Polymerization Overview

Polymers as we know them today were first proposed by Staudinger in 1920.^{1,2} Despite initial skepticism from the chemistry community, Staudinger's theory that high molecular weight macromolecules could be formed from covalently linked small molecules gained widespread acceptance by the 1930s. Commercial production of numerous polymers including polystyrene, polyesters, and Nylon began in the 1920s and 1930s, and further expanded following the end of World War II.^{3,4} Today, polymers are ubiquitous in society, accounting for many of the materials used in our homes, cars, and clothing.

In comparison, the synthesis and application of polymers with advanced architectures is relatively unexplored. The development of controlled polymerizations was essential to target specific molecular weights, reduce dispersity, and control molecular architecture. First possible using techniques such as anionic polymerization and ring-opening polymerization (ROP), controlled radical polymerizations have become a leading method for the synthesis of advanced architectures. Numerous architectures exist, including but by no means limited to, star, brush, hyperbranched, and surface-initiated polymers, as shown in Figure 1.1.

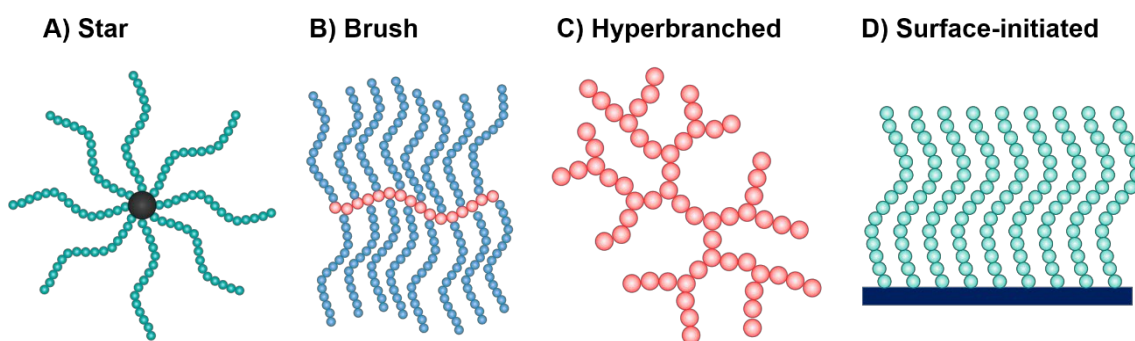


Figure 1.1 Examples of advanced architectures possible when using controlled polymerizations. Examples include (A) star, (B) brush, (C) hyperbranched, and (D) surface-initiated polymers.

1.2 Star Polymers

Star polymers are a class of branched polymers in which three or more linear arms extend outwards from a central core. The dense, compact nature of star polymers can result in unique

chemical, thermal, and rheological properties compared with their linear counterparts.⁵ In addition, the unique architecture of star polymers results in distinct core, arm, and peripheral regions that offer great versatility for customized structures. As shown in Figure 1.2, each region of the star can be altered and optimized, enabling endless possibilities.

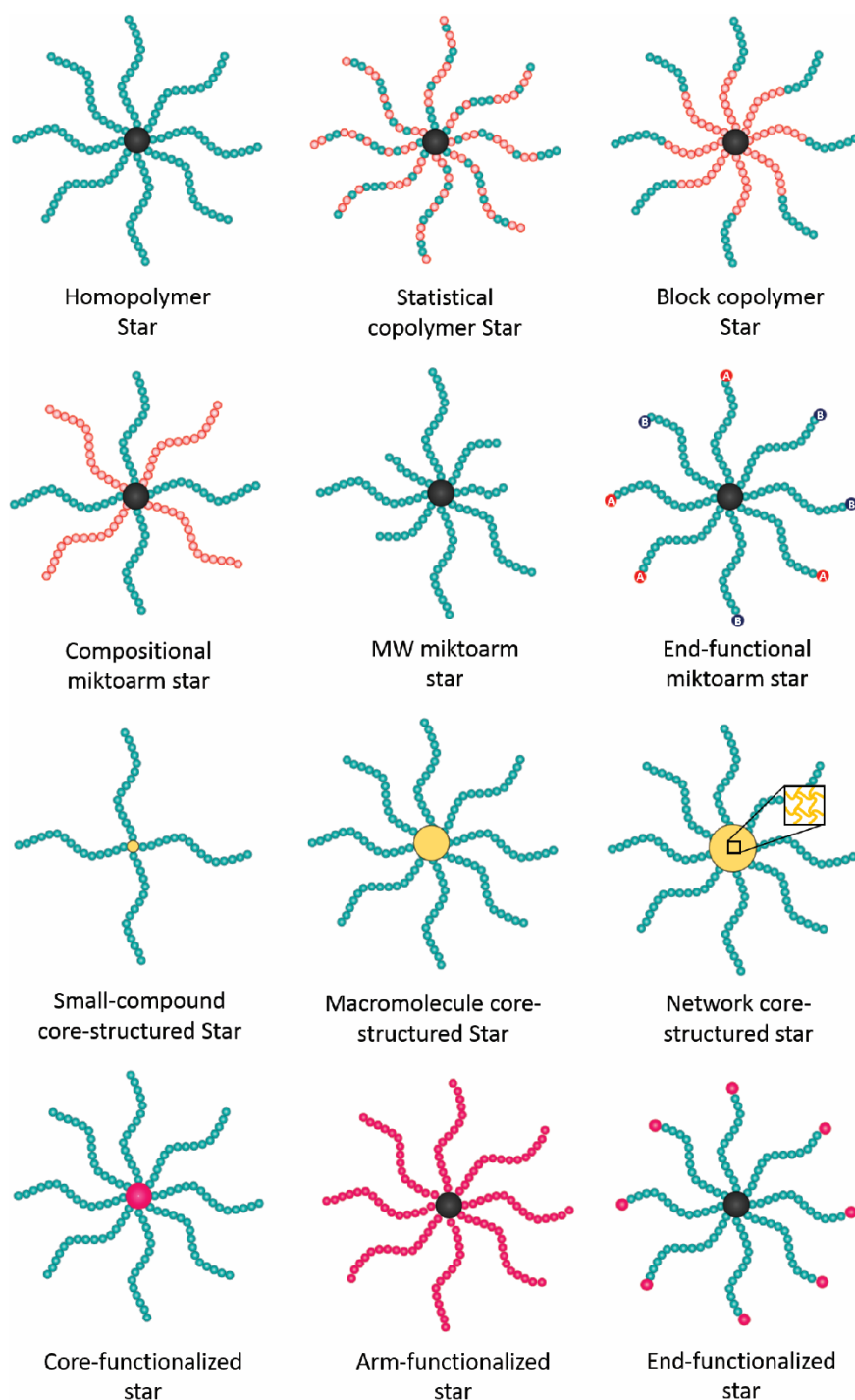
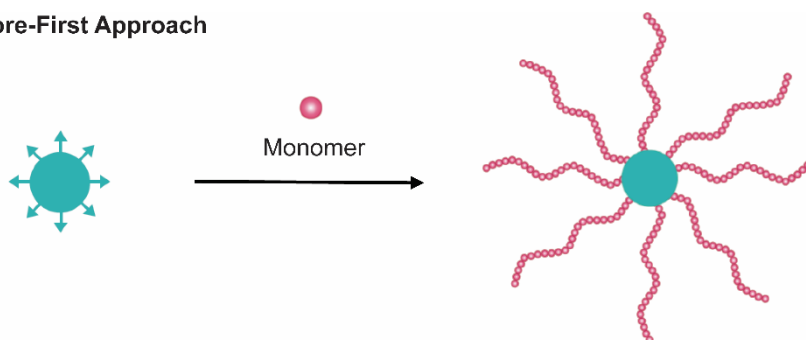


Figure 1.2 An illustration of potential star polymer structures including stars with varied composition and sequence of arm polymer, possible miktoarm and core structures, and different region of functionalization.

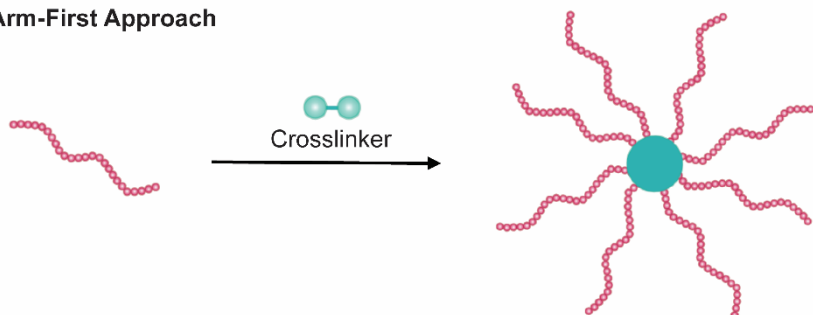
1.3 Synthetic Approaches for Star Polymer Synthesis

Three distinct approaches are possible for the synthesis of star polymers, known as the core-first, arm-first, and grafting-onto approaches (Figure 1.3). The general approaches including their advantages and limitations will be introduced here, with any polymerization technique-specific properties discussed more thoroughly under their respective sections.

A) Core-First Approach



B) Arm-First Approach



C) Grafting-On Approach

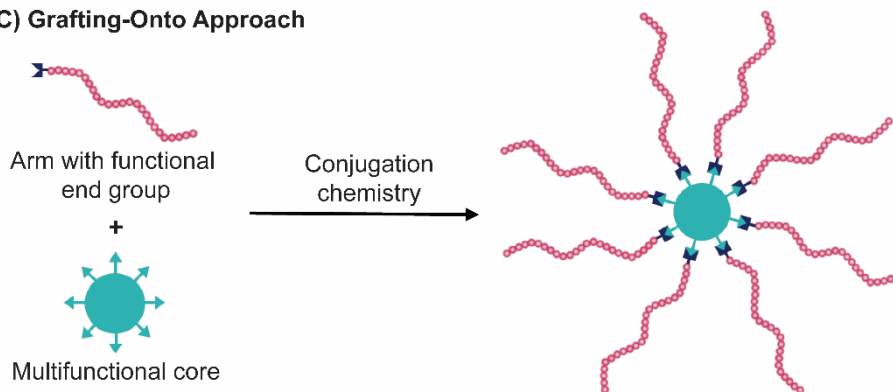


Figure 1.3 (A) Core-first, (B) arm-first, and (C) grafting-onto approaches for the synthesis of star polymers.

1.3.1 Core-First Approach

The core-first approach requires a multifunctional initiator from which arms can be polymerized. Any molecule that contains 3 or more initiation sites can serve as a core, enabling the use of a wide array of compounds and a great amount of freedom.

When using the core-first approach, the number of arms is equal to the number of initiating sites and is therefore known prior to polymerization (assuming 100% initiation). This varies greatly from the arm-first approach, where the number of arms cannot be known until after polymerization and is not easily controlled. To achieve star polymers with low molecular weight dispersities, all initiating sites must have equal reactivity and 100% initiation efficiency. It is also important that the rate of initiation is greater than the rate of propagation and that either no or minimal chain termination occurs, both of which result in arms that contain different numbers of repeat units.⁶ The primary advantages of the core-first approach are high yields and facile purification, as low molecular weight monomers are easily separated from the resulting star polymers.⁵

The core-first approach does suffer from some limitations. Star arms cannot be directly analyzed unless cleaved from the core. The synthesis of star polymers containing more than one type of arm, known as miktoarm stars, can be difficult as different initiating groups are required, although it has been demonstrated.⁷⁻¹¹ Star polymers synthesized *via* the core-first approach generally have a smaller core domain and lower number of arms than star polymers synthesized using the arm-first approach, however the use of hyperbranched cores such as dendrimers has somewhat alleviated these limitations.

Perhaps the most challenging aspect of the core-first approach is the synthesis of the multifunctional core. Cores should have the same number of initiating sites to ensure the resulting star polymers have the same number of arms, arm molecular weight, and total molecular weight, any of which could impact the star properties. Purification is generally required after synthesis of the core, inhibiting one-pot star polymer synthesis.

1.3.2 Arm-First Approach

The arm-first approach to star polymer synthesis requires the crosslinking of linear arms using di- or multi-functional crosslinking or coupling agents.^{12,13} The arm-first approach generally leads to stars with an overall greater number of arms, larger cores, and higher molecular weights than the core-first approach. Unlike the core-first approach, where the molecular weight of the core is

often negligible, the arm-first approach results in a core that is a crosslinked network and typically represents approximately 30% of the star polymer's total molecular weight.⁵ Star polymers synthesized using the arm-first approach are often referred to as core-crosslinked stars (CCS), distinguishing them from stars synthesized using the core-first or grafting-onto approaches.

The arm-first approach offers several advantages over the core-first approach. Most importantly, the arm-first approach does not require synthesis of a multifunctional core, enabling one-pot synthesis. Unlike the core-first approach, the polymer arms are easily characterized prior to crosslinking. The unique nature of the core resulting from the arm-first approach lends itself to further functionalization, including the loading, conjugation, or confinement of functional compounds.¹⁴⁻¹⁶ In addition, miktoarm star polymers are more easily synthesized using the arm-first approach.

There are however some disadvantages. Although a greater number of arms can be achieved with the arm-first approach, the number of arms can only be determined after synthesis and controlling the number of arms and star molecular weight is difficult. Star synthesis may require the conjugation of crosslinking or polymerizable functional groups to the terminal end of linear polypeptide chains, which can lead to issues. Any arms not successfully functionalized will not be able to become part of the star. Even with 100% conjugation efficiency, or the use of functional initiators, complete conversion of arms to star polymers is difficult due to steric hindrance. Star polymers can also be difficult to purify if not all arms are incorporated into the star polymer, since the arms are of a high molecular weight and have similar properties to the star.⁵ The most simple forms of purification include precipitation and dialysis, although preparative thin layer chromatography and preparative gel permeation chromatography can be performed if necessary. Finally, star-star coupling can also occur if the growing cores of separate stars come into contact.

1.3.3 Grafting-onto Approach

A third method of star polymer synthesis is known as the grafting-to approach and involves the conjugation of arms onto a functional core (Figure 1.3C). This approach allows for excellent characterization as both the core and arm can be analyzed prior to conjugation. It is important to note that although the linear arms can be synthesized *via* polymerization techniques, the actual formation of the star is a result of chemical conjugation. “Click” reactions are commonly used for the conjugation step due to their efficiency and speed.¹⁷⁻¹⁹

Like the core-first approach, a multifunctional core is employed and the number of possible arms is pre-determined. Because the stars are synthesized via coupling instead of polymerization

reactions, star-star coupling is unlikely. However, ensuring 100% conjugation can be difficult. Achieving quantitative conjugation becomes more difficult as the number of arms and the molecular weight of arms increases due to congestion at the core.²⁰ As mentioned with the arm-first approach, any unconjugated arms can be difficult to separate from the resulting star.

Table 1.1 A summary of the advantages and disadvantages of the core-first, arm-first, and grafting-onto approaches for star polymer synthesis.

Synthetic Approach	Advantages	Disadvantages
Core-First	• Pre-defined number of arms • High yields • Easy purification	• Requires multifunctional initiator • Difficult to synthesize miktoarm stars • Difficult to characterize arms
Arm-First	• One-pot synthesis • Large core • Facile synthesis of miktoarm stars	• Difficult purification if low conversion • Cannot readily control arm number or star molecular weight
Grafting-Onto	• Characterization of core and arms possible prior to conjugation • Conducive to synthesis of miktoarm stars • No star-star coupling	• Requires multifunctional core • Quantitative arm conjugation is difficult • Difficult purification if low conjugation

1.4 Polymerization Methods for Star Synthesis

Star polymers can be synthesized using numerous polymerization techniques with the most common being ring-opening polymerization (ROP), ring-opening metathesis polymerization (ROMP), and the reversible-deactivation radical polymerization (RDRP) methods atom transfer radical polymerization (ATRP), nitroxide-mediated living radical polymerization (NMP), and reversible addition fragmentation-deactivation chain transfer (RAFT) polymerization.

This thesis will focus on the synthesis of star polymers via RAFT polymerization and NCA ROP. These techniques, along with recent developments, are therefore presented in greater detail in subsequent sections.

1.4.1 RAFT Polymerization

RAFT polymerization was developed in Australia and first reported in 1998.²¹ RAFT polymerization offers exceptional control over polymer molecular weights and dispersities. Like other RDRP techniques, the “lifetime” of propagating chains is extended by establishing a dynamic equilibrium between active, propagating chains and dormant chains. In RAFT polymerization, this equilibrium is maintained through use of a thiocarbonylthio chain transfer or RAFT agent, which facilitates reversible deactivation through degenerate chain transfer (Figure 1.4).

RAFT polymerization can control a wide range of vinyl monomers with differing reactivities by altering the structure of the RAFT agent R- and Z-groups.²² The R-group, or fragmenting group, is a good homolytic group and cleavage of this C-S bond forms the active carbonyl radical for propagation. The Z-group is a poor leaving group that stabilizes the intermediate radical, modifying the addition and fragmentation rates. The structure of the Z-group therefore determines the suitability of the RAFT agent for a particular monomer species and the class of the RAFT agent (e.g. trithiocarbonate, xanthate, dithiobenzoate, dithiocarbamate).

Traditional RAFT Process

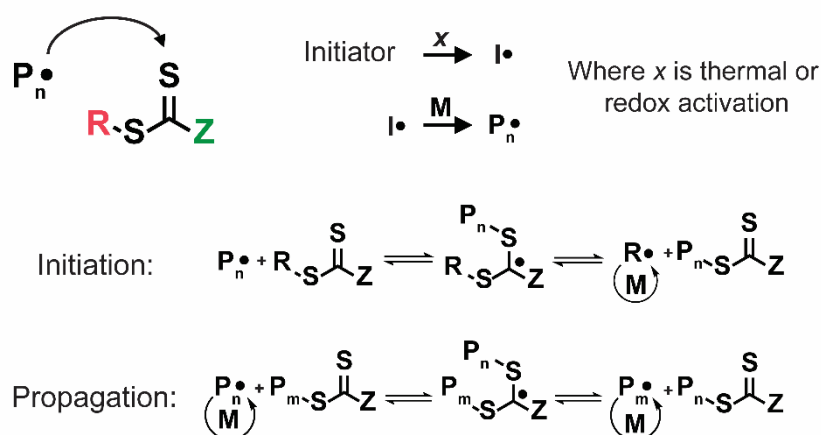


Figure 1.4 Traditional RAFT polymerization is initiated by a radical generated from thermal or redox activation. The dynamic equilibrium between the dormant chains and the active chain is necessary to maintain living characteristics.

1.4.1.1 Photo-mediated RAFT polymerization

Mechanisms and applications of RAFT polymerization have been studied worldwide since its discovery. Arguably, some of the most important advances have centred on novel mechanisms of initiating and mediating RAFT polymerization, leading to spatial and/or temporal control and precision polymers. Traditional RAFT polymerization employed radical sources generated by redox or thermal activation, which results in a high initial concentration of radicals that can lead to undesired termination reactions. In addition, polymerization could not be mediated by external stimuli.

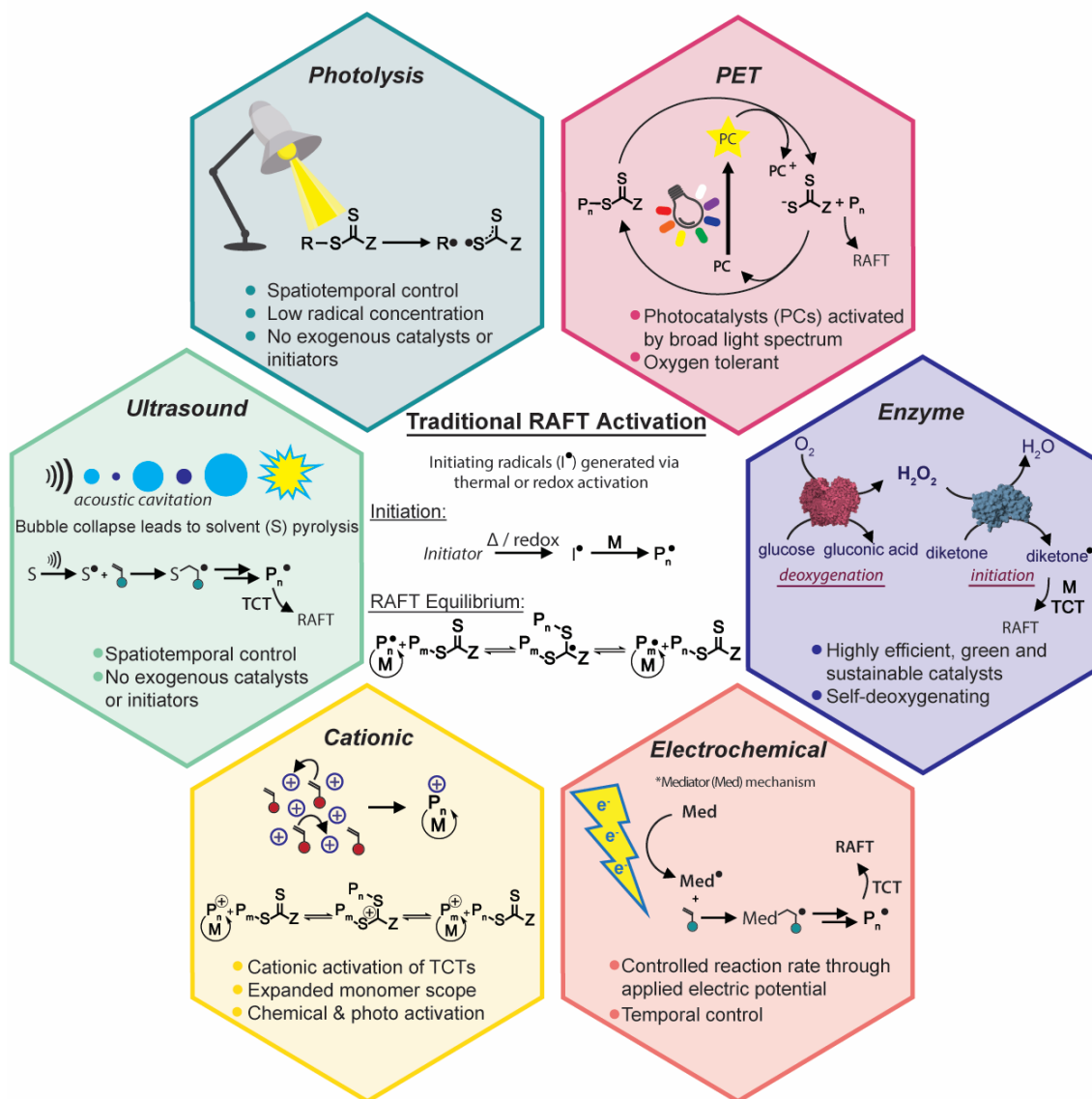


Figure 1.5 Traditional initiation employed radicals generated *via* thermal or redox activation (centre). Here, we present “non-traditional” mechanisms of initiation developed over the past decade including the use of visible light, ultrasound, enzymes, and cationic and electrochemical processes.

Novel initiation and mediation stimuli include light, ultrasound, enzymes, and electrochemical and cationic mediators (Figure 1.5). These mechanisms, as well as emerging environments, technologies, and applications of RAFT polymerization, have been eloquently reviewed by Nothling and coworkers.²³ Light- or “photo”-mediated RAFT polymerizations are currently the most developed and studied of these novel initiation and mediation technique and offer unparalleled spatiotemporal control and accessibility. Photo-mediated RAFT polymerization can be further divided into two mechanisms; i) Direct photolysis or photoiniferter RAFT and ii) photo-induced electron/energy transfer (PET)-RAFT polymerizations.

Photoiniferter RAFT polymerization is initiated by direct photolysis of the RAFT agent and does not require any exogenous radical sources or catalysts. The concept of iniferter polymerizations was first developed by Otsu, wherein a single molecule could act as an *initiator-transfer agent-terminator (iniferter)*.²⁴ Homopolymerization of styrene and methyl methacrylate monomers was performed using disulfide iniferters at 60°C, with block polymerization from the isolated polymers performed under UV irradiation. This seminal work marked the first example of radical polymerizations performed with a limited degree of control.^{24,25}

Following the development of RAFT polymerization, first reported using thermal radical sources, interest developed in the possibility of photo-mediated RAFT polymerization. Early works were performed under UV irradiation,²⁶⁻²⁹ which was already known to activate thio-containing compounds and thereby initiate radical polymerizations,^{24,30,31} although degradation of the RAFT agent under prolonged UV irradiation remained a challenge. The absorption band of RAFT agents from approximately 300-350 nm corresponds to the spin-allowed π - π^* electronic transition, resulting in a photoexcited species (Figure 1.6). Irradiation with UV causes β -fragmentation of the photoexcited species, cleaving the C-S bond between the R-group carbon and the sulfur adjacent to the thiocarbonyl group.³² The cleavage generates a stable sulfur-centred radical and a more active carbon-centred radical that can react with the vinyl monomer species to initiate RAFT polymerization.²⁷

The use of visible light to initiate and mediate photoiniferter RAFT polymerization was reported in 2015 by McKenzie et al.³³ In addition to the UV absorbance band, RAFT agents have a secondary absorption band in the visible light region, between 400-550 nm (Figure 1.6).^{34,35} Irradiation with visible light in this region also causes β -fragmentation of the RAFT agent. Blue light (λ_{max} =460 nm) selectively activated the trithiocarbonate spin-forbidden n - π^* electronic transition to generate the photoexcited species, resulting in improved livingness at monomer

conversions greater than 90%, demonstrated using MALDI-ToF and the one-pot synthesis of a pseudo-hexablock copolymer.³³

It is important to note that the absorption band in the visible range is influenced by both the R- and Z-group of the RAFT agent. Xanthates are generally paired with purple light while dithiocarbamates and dithiobenzoates are often most effective when irradiated with green light.³⁶⁻³⁹ The R-group also plays an important role, with secondary trithiocarbonates absorbing in the blue range while tertiary trithiocarbonates are red-shifted and also show activity under green light.^{40,41} Providing correct pairing of the RAFT agent, monomer, and irradiation wavelength, photoiniferter RAFT polymerization shows highly “living” behaviour.

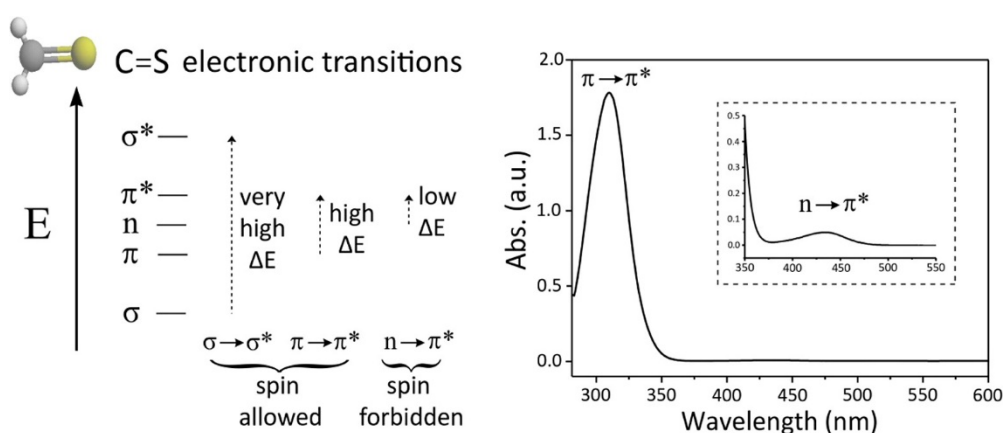


Figure 1.6 Electronic transitions of thiocarbonylthio RAFT agents. Irradiation with UV and visible light causes photoexcitation of the RAFT agent via spin-allowed $\pi\text{-}\pi^*$ and spin-forbidden $n\text{-}\pi^*$ electronic transitions, respectively.³³ Reprinted with permission from *Macromolecules* 2015, 48, 12, 3864–3872, Copyright 2015 American Chemical Society.

The improved livingness likely occurs for two reasons. While degradation of thiocarbonylthio compounds under UV irradiation has been frequently reported, studies into the photolytic stability of RAFT agents under visible light irradiation have shown minimal degradation of the RAFT agent.³⁹ In addition, UV irradiation has been shown to directly activate monomers while visible light cannot, leading to a lower concentration of radicals in the reaction mixture when using visible light, resulting in less irreversible termination reactions. Exploiting this highly living behaviour, the Sumerlin group has recently demonstrated photoiniferter RAFT polymerization for the synthesis of ultra-high molecular weight (UHMW) polymers, using a range of monomers in both aqueous and organic solvents.^{42,43}

The PET-RAFT approach proceeds *via* indirect photo-activation of the RAFT agent through use of a photoredox catalyst or photosensitizer. Once activated, the excited photocatalyst/photosensitizer interacts with the RAFT agent through an energy or electron transfer mechanism. The PET-RAFT was first reported by the Boyer group in 2014.⁴⁴ The authors used iridium catalyst *fac*-[Ir(ppy)₃] under blue light irradiation and demonstrated well-controlled polymerizations across range of RAFT agent and monomer classes. Furthermore, this exceptional control was maintained even without prior degassing to remove oxygen.

The benefits of PET-RAFT over photoiniferter are twofold. First, by employing a photocatalyst, the choice of irradiation wavelength is no longer limited by the absorbance of the RAFT agent. Numerous photocatalysts and photosensitizers have been employed for PET-RAFT polymerization, enabling photo-mediation across the entire visible light spectrum.⁴⁵ These compounds are incredibly varied and encompass metalloporphyrins (e.g. zinc tetraphenylporphyrin),⁴⁶⁻⁴⁹ photosynthetic pigments,^{50,51} organic dyes,⁵²⁻⁵⁵ and coenzymes.⁵⁶ Recent studies have focused on the development of broadband- and near-infrared (NIR)-responsive catalysts, which could have exciting applications in solar-driven polymerization and biomedical applications.^{57,58}

The second major benefit of PET-RAFT polymerization is that deoxygenation is generally not required. Oxygen tolerance is often achieved by converting molecular oxygen to a species that can i) be quenched, ii) act as an inert bystander, or iii) be reduced and subsequently act as an initiating radical.^{45,59} In a recent report from the Boyer group, oxygen has actually been shown to have a catalytic effect and increase the rate of polymerization.⁶⁰ The oxygen tolerance of PET-RAFT polymerization means specialized equipment and glassware used for deoxygenation are no longer required. Highlighting the importance of this feature, Chapman and coworkers reported successful PET-RAFT polymerization by undergraduate students in a non-chemistry lab. Finally, oxygen tolerance can also be exploited for low volume PET-RAFT polymerization, allowing polymerization at microlitre volumes that are difficult to degas.^{61,62}

1.4.2 Star Polymers *via* RAFT Polymerization

The versatility of RAFT polymerization readily lends itself to the synthesis of complex architectures such as star polymers. Compatible with an enormous range of monomers and multiple possible synthetic approaches, RAFT polymerization is a leading technique for star polymer synthesis. An overview of recent developments in RAFT polymerization will be presented and the advantages, disadvantages, and major developments in the synthesis of star polymers *via* RAFT polymerization will be discussed.

1.4.2.1 Core-first Approach *via* RAFT Polymerization

As previously mentioned, any molecule that can be functionalized with 3 or more RAFT agents can serve as a core, enabling the use of a wide array of compounds and a great amount of freedom. Cores as varied as cyclodextrins,⁶³ porphyrins,⁶⁴ hyperbranched polymers,⁶⁵ metal-organic polyhedrons (MOPs),⁶⁶ metal-organic macrocycles (MOMs),⁶⁷ and quantum dots⁶⁸ have been used for the synthesis of star polymers, with benzene- and pentaerythritol-based cores among the most commonly used.

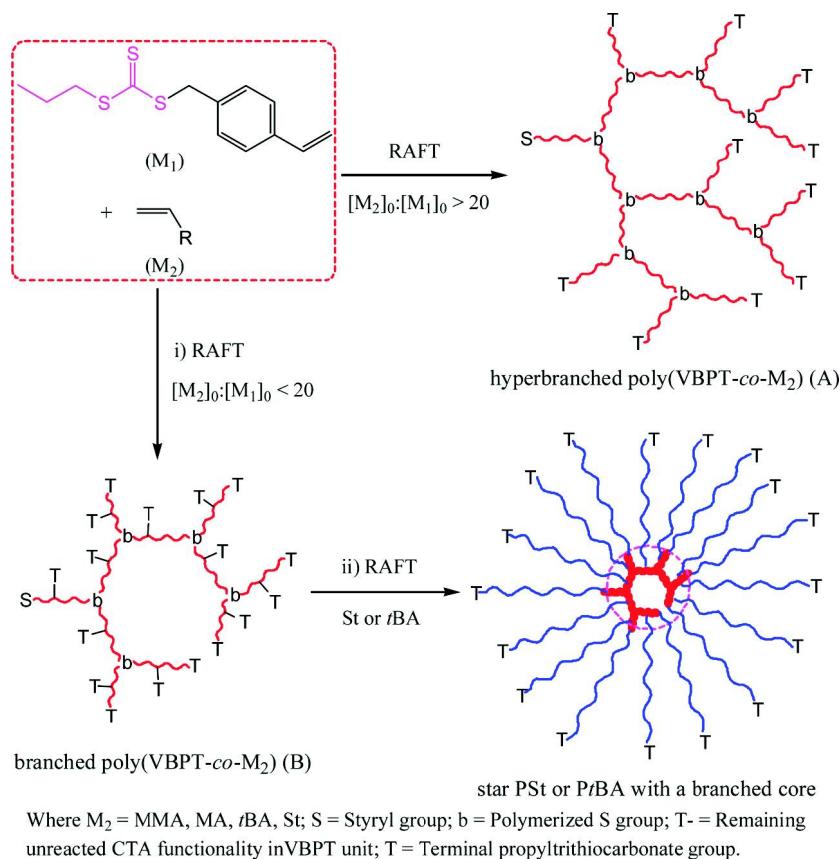


Figure 1.7 Synthesis of hyperbranched cores using a styryl-RAFT monomer (M₁) and styrene or methyl acrylate (M₂). Once the core synthesis was optimized, subsequent polymerization from the core via RAFT resulted in well-defined star polymers.⁶⁹ Reprinted with permission from *Macromolecules* 2011, 44, 7, 2034-2049. Copyright 2011 American Chemical Society.

While the synthesis of cores is most commonly performed through conjugation chemistries, RAFT polymerization has been employed as a facile method of core synthesis. Zhang et al reported the use of self-condensing vinyl polymerization (SCVP)-RAFT to synthesize a core using a polymerizable RAFT agent followed by polymerization from these RAFT agents to synthesize the star arms (Figure 1.7).⁶⁹ To form the hyperbranched macro chain transfer agent

(CTA) core, a RAFT agent containing a styryl group was copolymerized with methyl acrylate or styrene. Branching points occurred when both the styryl and trithiocarbonate groups in one molecule participated in RAFT polymerization. A wide range of conditions were investigated, and the authors concluded that the degree of branching, end group composition, and CTA functionality could be adjusted by altering the RAFT-monomer and comonomer ratios and reaction time. Although initial macroCTA cores had dispersities greater than 2, the authors were able to adjust conditions to achieve cores with dispersities as low as 1.16.

Hyperbranched macroCTA cores with approximately 7, 13, or 56 functional RAFT agents were subsequently used to polymerize styrene or *tert*-butyl acrylate to form star polymers. Theoretical and observed molecular weights were similar and dispersities ranged from 1.21-1.88, indicating controlled star polymers could be achieved using this approach. Subsequent works using RAFT to synthesize hyperbranched cores and subsequent polymerization of arms has further shown the versatility and ease of core-first star polymerization performed using RAFT.⁷⁰⁻⁷²

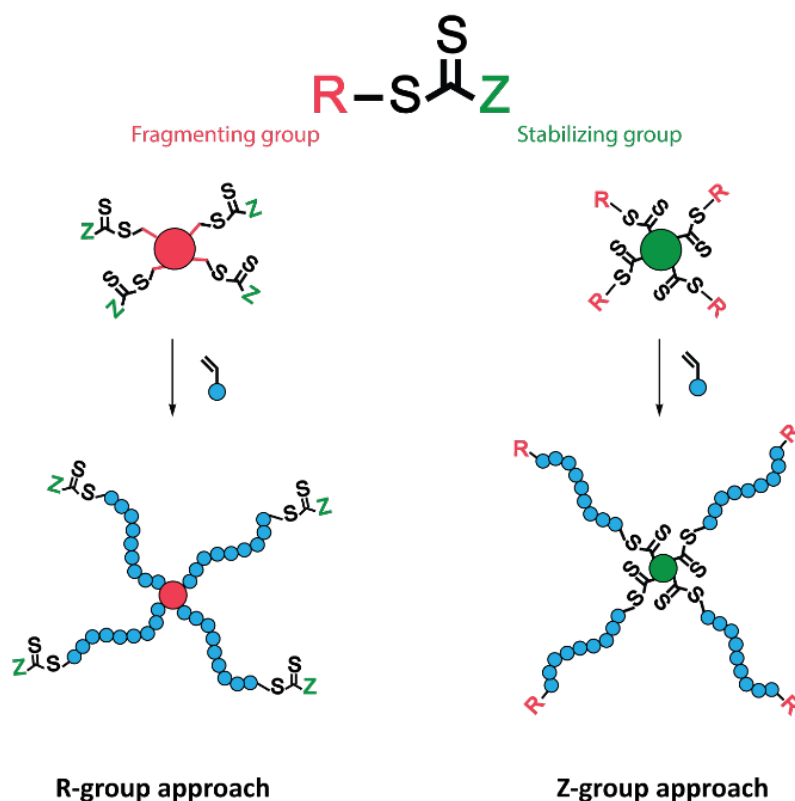


Figure 1.8 RAFT polymerization of star polymers using the core-first R- and Z-group approaches.

While the core-first approach to star polymer synthesis is compatible with a wide range of polymerization techniques, star polymer synthesis *via* RAFT polymerization is unique due to the asymmetrical nature of RAFT agents, which contain a Z- and R-group (Figure 1.8). Which group is conjugated to the core has important implications for the synthesis and functionality of the star polymer and should be considered prior to synthesis of the multifunctional core. Both approaches have advantages and disadvantages which have been extensively studied and will be discussed.^{5,73,74}

1.4.2.1.1 Z-Group Approach

The Z-group approach is used when the Z-group of the RAFT agent is conjugated to the core. The R-group remains at the periphery of the star polymer while the thiocarbonylthio moiety remains at the core. As polymerization proceeds, a linear macroradical is formed away from the star polymer core. Because the propagating radical is not attached to the star core, star-star coupling is prevented. This avoidance of star-star coupling initially made the Z-group approach the preferred method of star polymer synthesis *via* the core-first approach. Termination events can still occur when two macroradicals react, producing a low molecular weight linear byproduct and resulting in the loss of at least one living arm from a star. However, this linear byproduct is much easier to purify from the desired star polymer than the high molecular weight byproducts resulting from star-star coupling. The mechanism of polymerization *via* the Z-group core-first approach is illustrated in Figure 1.9.

The mechanism of polymerization does lead to certain limitations. Macroradical arms must reattach to the core, which becomes more difficult as the molecular weight of the arms and the number of arms is increased.^{63,65} The location of the thiocarbonylthio group in the star core must also be carefully considered. The thiocarbonylthio group is sensitive to nucleophiles and radicals, both of which can cause destruction of the RAFT agent. With the RAFT agent located at the core, this means the destruction of a RAFT agent will lead to the loss of an arm. This could be intentionally exploited if a degradable star is desired or to analyze the arms following star polymer synthesis.^{75,76} It is important to consider prior to synthesis if exposure to nucleophiles and radicals is likely during the intended application of the star polymer.

polymerization. Because the propagating radical is at the star periphery, termination reactions between the growing arms can result in intra-star termination causing two dead arms, or inter-star termination, also known as star-star coupling. Both inter- and intra-star termination reactions will lead to high dispersities, however inter-star termination results in a high molecular weight product up to twice the intended molecular weight that can be difficult to remove.

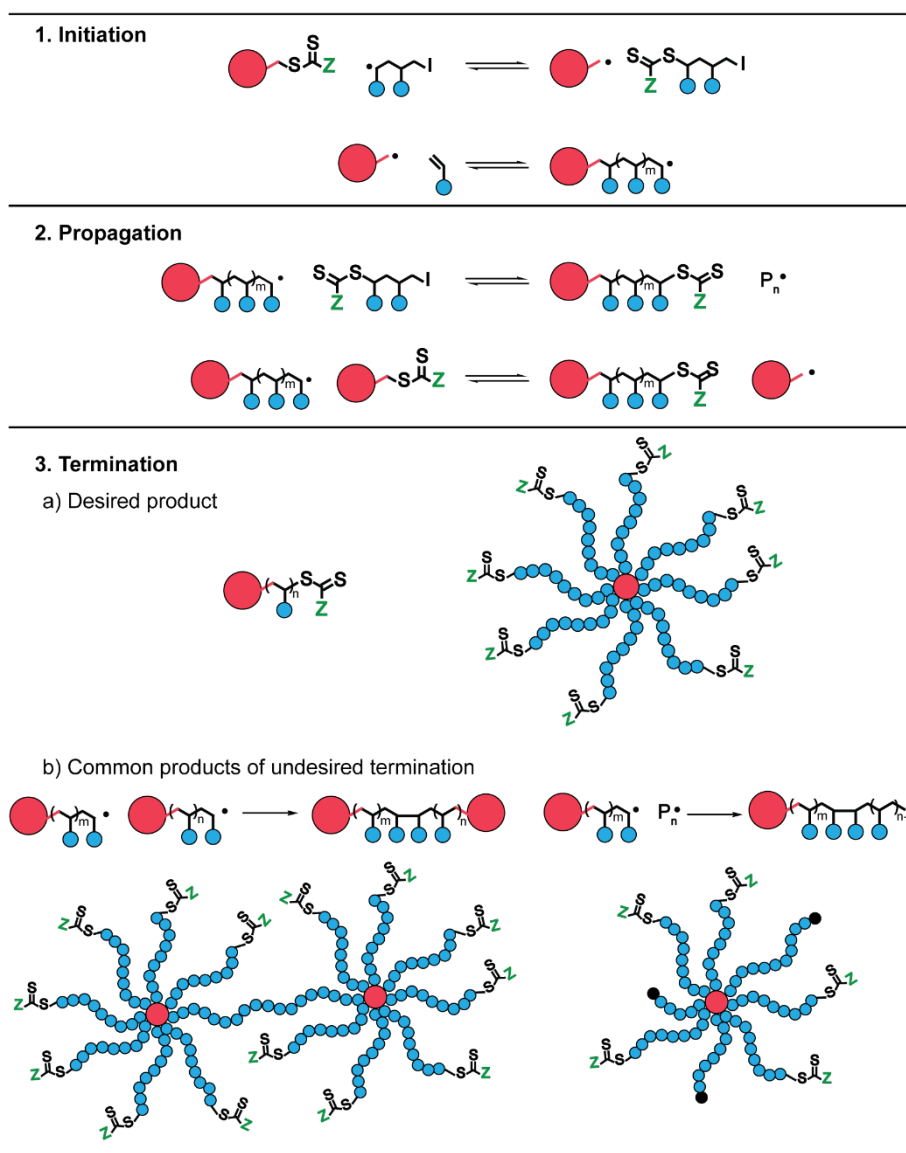


Figure 1.10 RAFT polymerization of star polymers using the R-group core-first approach, where the propagating radical remains at the star periphery. Unwanted termination reactions can result in star-star coupling and dead polymer arms that no longer contain the thiocarbonylthio moiety.

The primary advantage of the R-group approach is that the thiocarbonylthio group remains at the periphery of the star. RAFT agents are often coloured, resulting in coloured products which is often undesired. The location of the RAFT agent at the exterior enables destruction of the RAFT agent to remove colour or to introduce additional functionality. The conversion of RAFT agents to functional groups has been extensively reviewed, with conversion to groups that can participate in click reactions being the most appealing.⁷⁷⁻⁷⁹

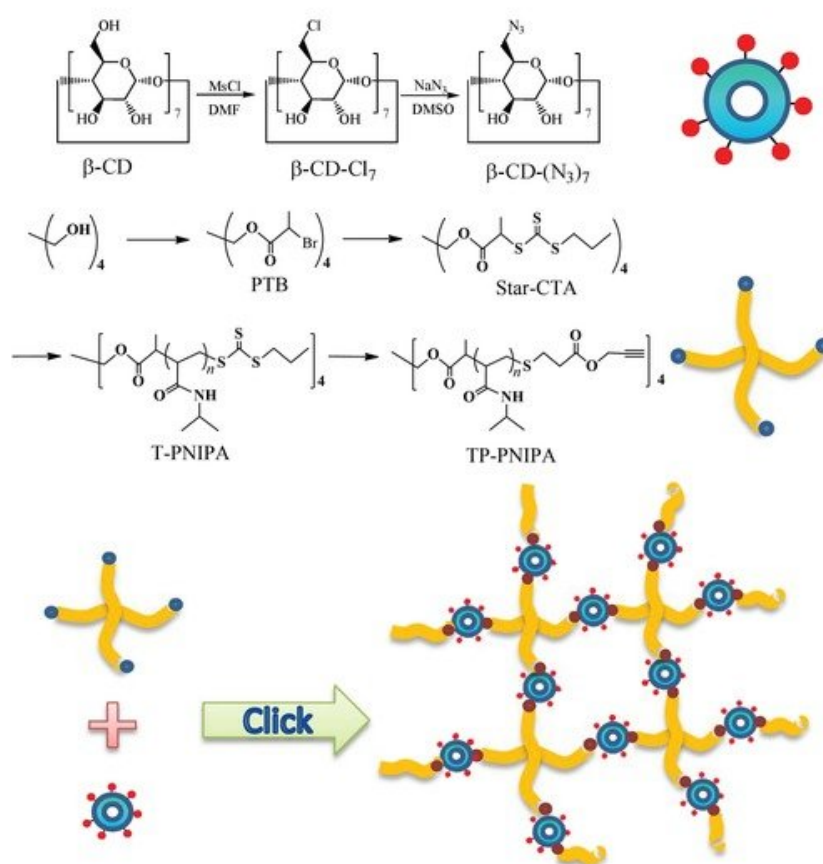


Figure 1.11 Synthesis of star polymers *via* R-group core-first approach using RAFT polymerization. Terminal thiocarbonylthio groups were converted to alkynes, facilitating hydrogel synthesis through click chemistry.⁸² Reprinted with permission from *Polymers* 2016, 8, 93.

The Maynard group exploited the terminal RAFT groups resulting from the R-group core-first approach to synthesize star poly(*N*-isopropylacrylamide)(PNIPAAm)-lysozyme conjugates.⁸⁰ The polymerization of 4-arm PNIPAAm stars was stopped at 52% conversion to limit star-star coupling and ensure retention of the RAFT end group. Using a mechanism previously reported by Perrier et al,⁸¹ the RAFT agents were reacted with a symmetrical azo-initiator containing furan-protected maleimide groups to achieve star polymers with terminal protected maleimide groups.

Once deprotected, the enzyme lysozyme was conjugated to the star polymer *via* thiol-maleimide click chemistry.⁸⁰ More recently, Wang et al synthesized 4-arm PNIPAAm stars and converted the RAFT group to an alkyne *via* one-pot aminolysis and subsequent Michael addition of propargyl acrylate (Figure 1.11).⁸² The 4-arm PNIPAAm star was then reacted with azide-functionalized β -cyclodextrin, forming a temperature-responsive PNIPAAm hydrogel. These examples highlight the ease with which star polymers synthesized using the R-group approach can undergo end-group functionalization.

1.4.2.1.3 Developments in Core-First Star Synthesis

While the R- and Z-group approaches for core-first star polymers has been well studied, there have been few developments that have altered the way the RAFT core-first approach is conducted since its inception. Most reactions are still carried out using thermal initiators such as AIBN and VA-044. Therefore the recent usage of photo-mediated RAFT polymerization for the synthesis of star polymers using the core-first approach has been a welcome and exciting development.

The Boyer group reported the use of PET-RAFT to synthesize 3- and 4-armed stars with photocatalyst zinc tetraphenylporphyrin (ZnTPP).⁴⁶ The star polymers were reasonably well controlled with dispersities ranging from 1.1 to 1.5 and used for screening structure-activity relationships. More recently, the Boyer and Lim groups used PET-RAFT to modify lanthanide-doped upconversion nanoparticles (UCNPs) which have unique photoluminescent properties with potential biomedical applications.⁸³ UCNPs were coated with silica and functionalized with RAFT agent, from which poly (oligoethylene glycol methyl ether methacrylate) (POEGMA) was polymerized. The addition of the POEGMA shell rendered the UCNPs water-soluble without affecting the upconversion emission spectrum of the particles.

1.4.2.2 Arm-First Approach *via* RAFT Polymerization

The arm-first approach *via* RAFT polymerization can be further divided into the macroRAFT and macromonomer routes (Figure 1.12). The macroRAFT method, also referred to as the macro chain transfer agent (macroCTA) method, is most common. Following RAFT polymerization of the arm, the incorporated RAFT agent then acts as the chain transfer agent during the crosslinking polymerization step. This method is dependent on the presence of living RAFT agents in the polymer and achieving near complete monomer conversion. Any dead chains will not be crosslinked and therefore not incorporated into the star polymer, so maintaining livingness of the RAFT end group is essential. Following arm synthesis, crosslinker can be added directly to the reaction mixture enabling a facile, one-pot approach to obtain well-defined star polymers. The macroRAFT approach can also be used with arms synthesized through alternative polymerization

techniques provided a RAFT agent is incorporated into the arms prior to crosslinking, allowing RAFT star polymerization of arms synthesized using other polymerization techniques.

The macromonomer route was first demonstrated by the Matyjaszewski group using ATRP and requires an arm with a polymerizable vinyl end group.⁸⁴ A small molecule RAFT agent is used and the arm is incorporated into the star as a macromonomer during polymerization. The macromonomer route allows for a lower number of initiating sites than the macroinitiator approach, reducing the likelihood of star-star coupling.⁸⁴

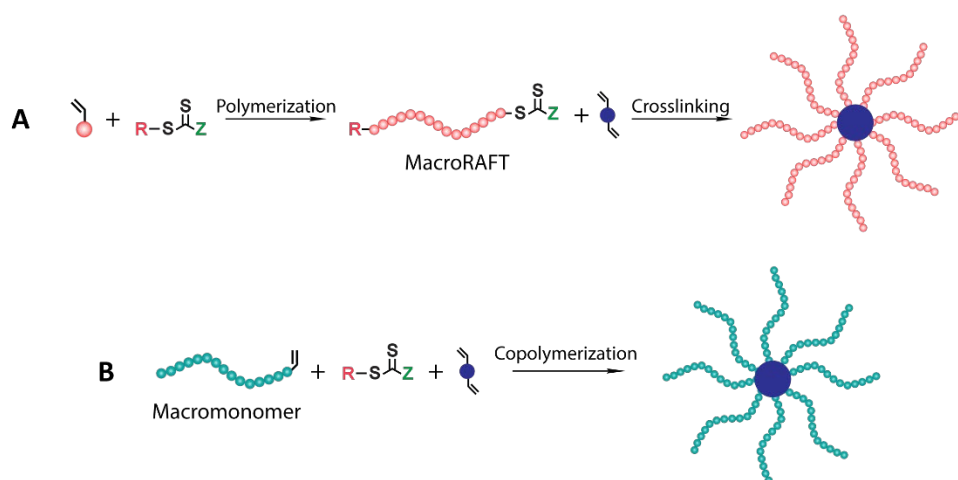


Figure 1.12 Synthesis of star polymers using the arm-first approach *via* RAFT polymerization can be performed using the A) macroRAFT and B) macromonomer approaches.

The An group used commercially available 2 kDa PEG methacrylate (PEGMA), highlighting that synthesis of a macroRAFT agent either through RAFT polymerization of the arm or conjugation of RAFT agent to the arm was not required for this approach.⁸⁵ The PEGMA arm, RAFT agent, and radical initiator were soluble in phosphate buffer pH 7 while the crosslinker and optional comonomer formed oil droplets. As polymerization proceeded a random copolymer of the macromonomer arm, crosslinker, and spacer comonomer (if used) was produced. This is in contrast to the macroRAFT approach where polymerization results in a block copolymer. The resulting amphiphilic copolymers then self-assembled with the hydrophobic portions forming the core while the PEGMA chains radiated outwards. The authors investigated each component of the system, varying the concentration of phosphate buffer and macromonomer, ratio of crosslinker, presence of comonomer, and molecular weight of the macromonomer. Most star polymers formed showed greater than 90% arm incorporation, narrow size distributions and dispersities below 1.2. However, poor star formation was observed when the molecular weight of the arm was increased to 4 kDa.⁸⁵

1.4.2.2.1 Developments in Arm-First Star Synthesis

The synthesis of star polymers *via* the arm-first approach has undergone a number of developments in the last decade that have led to improved polymers. During the early days of RAFT polymerization, the arm-first approach was plagued by low arm incorporation into the star resulting in high polydispersities. Low incorporation of arms was thought to be due to “dead” chain ends that could not participate in further polymerization. However, major developments by the Boyer, Davis, An, and Qiao groups have made the arm-first approach the new method of choice.

In a seminal paper by Boyer and Davis, the synthesis of well-defined star polymers with low molecular weight dispersities and high arm incorporation were reported using the arm-first approach by selecting poorly soluble crosslinkers.⁸⁶ Star polymers with POEGA arms and *N,N'*-bis(acryloyl)cystamine or 1,6-hexanediol diacrylate as crosslinker were synthesized using DMF, acetonitrile, and toluene as solvent to investigate the effect of crosslinker solubility on star formation (Figure 1.13A). 1,6-hexanediol diacrylate is soluble in all three solvents while *N,N'*-bis(acryloyl)cystamine is soluble in DMF and acetonitrile but only partially soluble in toluene. POEGA arms crosslinked with *N,N'*-bis(acryloyl)cystamine in toluene resulted in well-defined star polymers with a dispersity of 1.12 and greater than 90% arm incorporation, a dramatic improvement over previously reported star polymers. All other reactions resulted in dispersities greater than 1.5 demonstrating the importance of crosslinker and solvent choice (Figure 1.13B-E). The authors hypothesized the improved star formation using *N,N'*-bis(acryloyl)cystamine in toluene was a result of nano-phase separation that induced compartmentalization of the crosslinking. Further experiments demonstrated high arm incorporation and low dispersity could be achieved using arms of varying composition and molecular weight as well as other poorly soluble crosslinkers.⁸⁶

Applying a similar concept, the An group has developed and thoroughly investigated the use of heterogeneous RAFT polymerization systems to develop well-defined star polymers by emulsion and dispersion RAFT.^{85,87-94} First reported in 2011, the An group demonstrated the synthesis of CCS polymers in water with a solid content of 10% w/v *via* emulsion and dispersion polymerization and that these star polymers stabilized emulsions during particle synthesis.⁸⁷ The versatility of the method was shown using poly(*N,N'*-dimethylacrylamide) (PDMA), poly(poly(ethylene glycol) methyl ether methacrylate) (PPEGMA), and poly(2-methoxyethyl acrylate-co-poly(ethylene glycol) methyl ether acrylate) (P(MEA-co-PEGA) arms. The emulsion system was conducted with spacing monomer butyl acrylate and crosslinker poly(ethylene glycol) diacrylate (PEGDA) while the dispersion system contained *N*-isopropylacrylamide (NIPAAm)

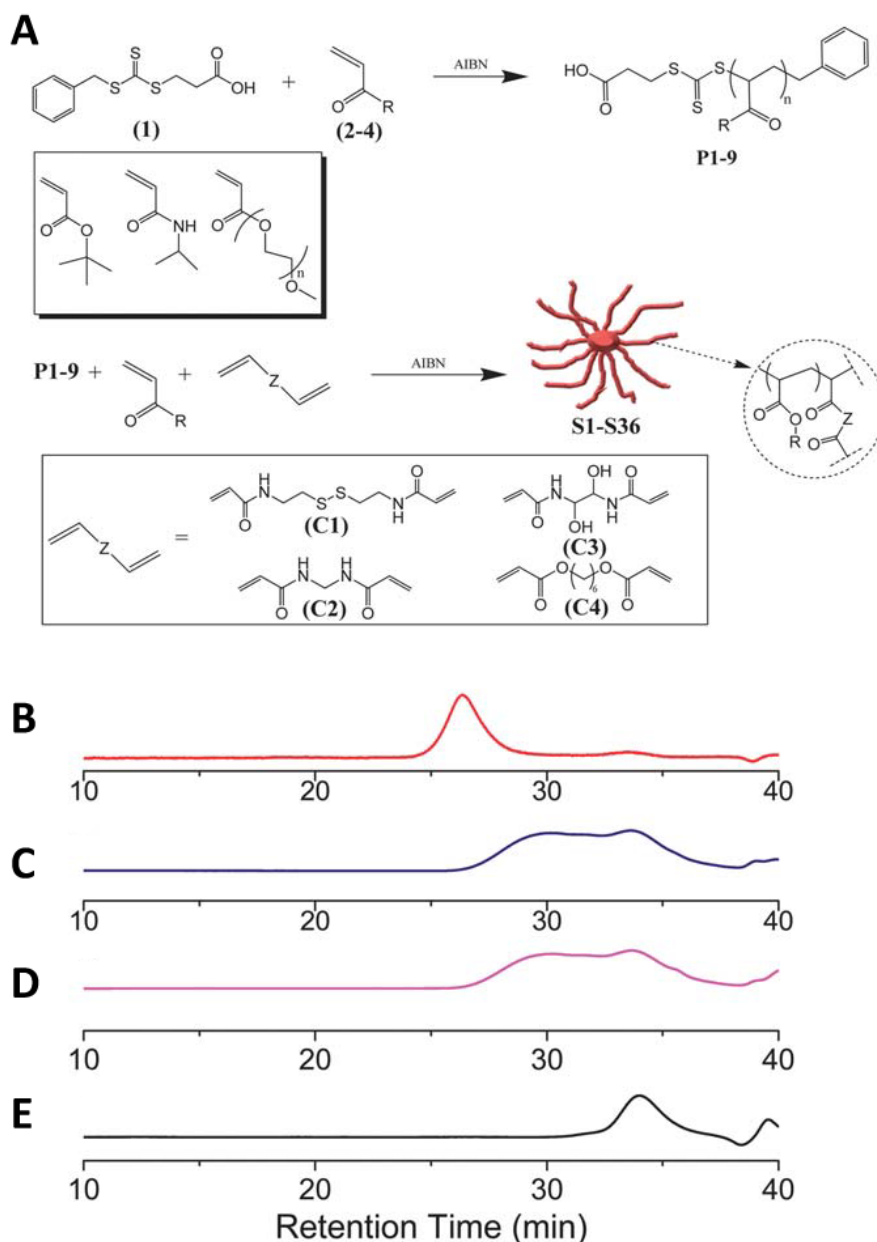


Figure 1.13 (A) Synthesis of star polymers with varying arm compositions and crosslinkers in a range of solvents to investigate the impact of crosslinker solubility on star formation. GPC traces of star polymers obtained by RAFT, using *N,N'*-bis(acryloyl)cystamine (BAC) as the cross-linker, in (B) toluene, (C) DMF, (D) acetonitrile and (E) arm polymer used in this study before crosslinking. BAC is poorly soluble in toluene leading to well-defined stars and is soluble in DMF and acetonitrile where limited star formation was observed.⁸⁶ Adapted from Ref. 86 with permission from the Royal Society of Chemistry.

and methylene bisacrylamide (MBA) as the spacing monomer and the cross-linker, respectively (Figure 1.14A). In all cases a high degree of arm incorporation and dispersities of 1.12 and below were observed from GPC analysis. Interestingly, emulsion polymerization led to greater arm

incorporation, higher molecular weight, and lower dispersity than dispersion polymerization (Figure 1.14B-E).

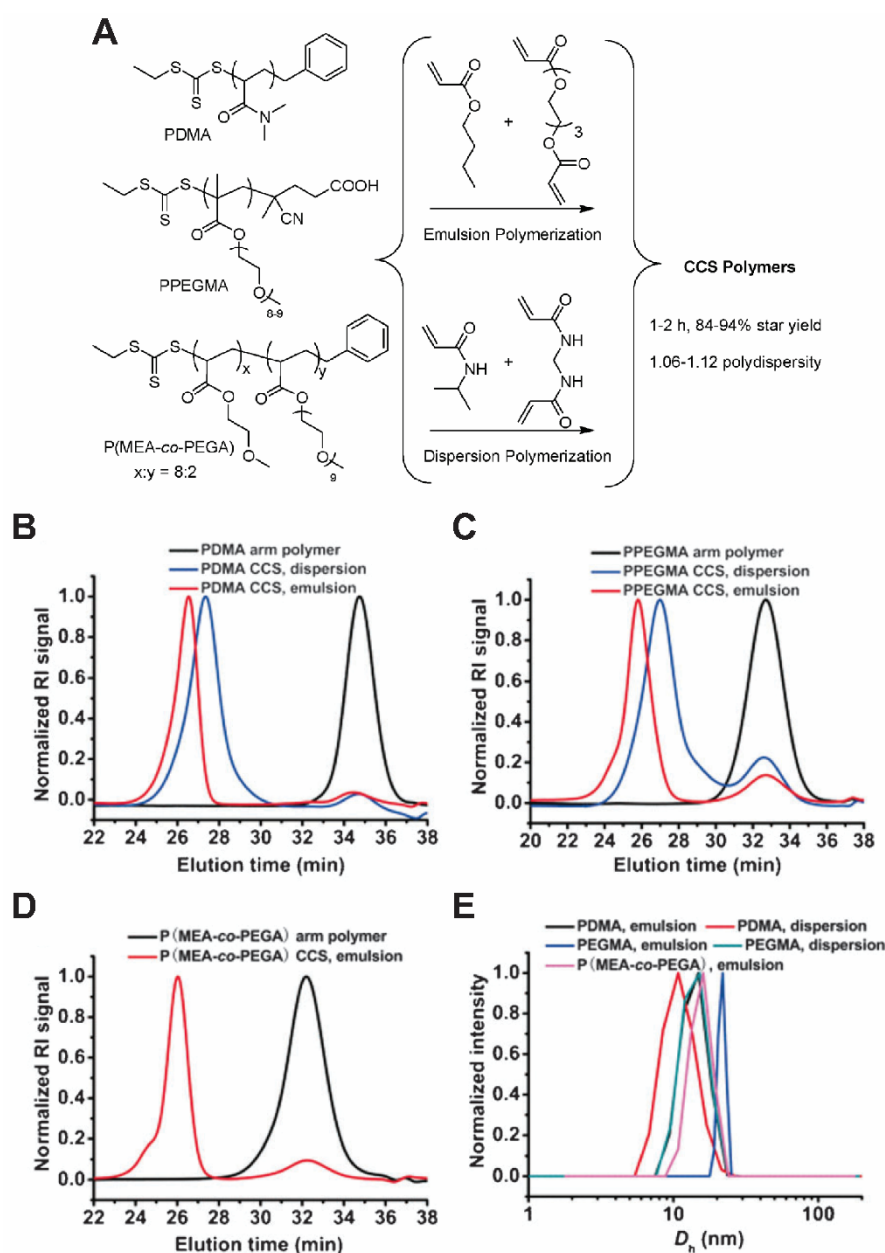


Figure 1.14 Core-crosslinked stars synthesized using emulsion and dispersion RAFT polymerization. (A) Monomers and crosslinkers used for emulsion and dispersion polymerization. GPC chromatograms of (B) PDMA, (C) PPEGMA, (D) P(MEA-co-PEGA) arm and star polymers. (E) Dynamic light scattering chromatogram showing the hydrodynamic radius of star polymers synthesized using emulsion and dispersion polymerization.⁸⁷ Adapted from Ref. 87 with permission from the Royal Society of Chemistry.

The next major development was reported by the Qiao group when they used photoiniferter RAFT polymerization in the synthesis of star polymers *via* the arm-first approach.⁹⁵ The highly living nature of polymers synthesized *via* photoiniferter RAFT made them excellent candidates for the synthesis of star polymers. Using a two-step, one-pot approach, McKenzie et al synthesized linear poly(methyl acrylate) (PMA) which was then crosslinked using ethylene glycol diacrylate (EGDA) (Figure 1.15A). The ratio of crosslinker to arm was investigated with a ratio of 10:1 resulting in the best star formation. A ratio of 5:1 resulted in incomplete star formation while a ratio of 15:1 led to a broader size distribution. The degree of polymerization (DP) of the arm was also important in the synthesis of star polymers. The crosslinker to arm ratio was held at 10:1 while PMA arms with DPs of 60, 80, and 120 were crosslinked. GPC analysis of the stars formed from PMA DP 60 were bimodal, with the higher molecular weight product likely the result of coupling between two star cores due to the small arm length. A DP of 80 resulted in well-defined star polymers with a dispersity of 1.41 and arm incorporation of 94%. The largest arms, with DP 120, also resulting in well-defined stars albeit with a slightly higher dispersity and only 89% arm conversion. The lower conversion of DP 120 arms into the star is thought to be caused by reduced access to the core due to the greater arm length.⁹⁵

Importantly, the RAFT chain ends were shown to still be living following star polymerization. An in/out star was synthesized, with monomer added to the already formed stars. Further irradiation with blue light resulted in the polymerization of new arms from the star core (Figure 1.15B). The number of arms in the initial star was calculated to be 31 and the number of arms following the in/out polymerization was found to be 60. The kinetics of monomer conversion during the in/out chain extension were compared to a control synthesis of linear PMA, with the apparent rate constant indicating the efficiency of polymerization from the core was 95% and that the functionality of most RAFT agents was maintained.⁹⁵

In 2017 the Qiao and Kamigaito groups demonstrated the synthesis of novel star polymers using a mechanistic transformation from cationic RAFT polymerization to radical RAFT polymerization.⁹⁶ Cationic RAFT was first reported by the Kamigaito and co-workers, who demonstrated polymerization of electron-rich vinyl ether monomers that are not readily polymerized *via* radical RAFT polymerization to obtain linear polymers with dispersities below 1.1.⁹⁷ Synthesis of star polymers *via* cationic RAFT was demonstrated in a one-pot approach, with linear poly (isobutyl vinyl ether) (PIBVE) prepared and then crosslinked using a divinyl ether crosslinker. A mechanistic transformation to radical RAFT was also possible with PIBVE arms being crosslinked using a diacrylate crosslinker. Finally, the authors showed that a block copolymer of PIBVE and a heterobifunctional monomer with structure vinyl ether-R-acrylate (eg.

2-(vinylloxy)ethyl acrylate) could be synthesized *via* cationic polymerization of the vinyl ether groups and then crosslinked to form a star polymer through radical RAFT polymerization of the pendant acrylate groups. This mechanistic switch enables the synthesis of star polymers containing monomers not easily synthesized by cationic or radical RAFT polymerization alone and resulted in the synthesis of well-defined star polymers with high arm incorporation and dispersities as low as 1.18.⁹⁷

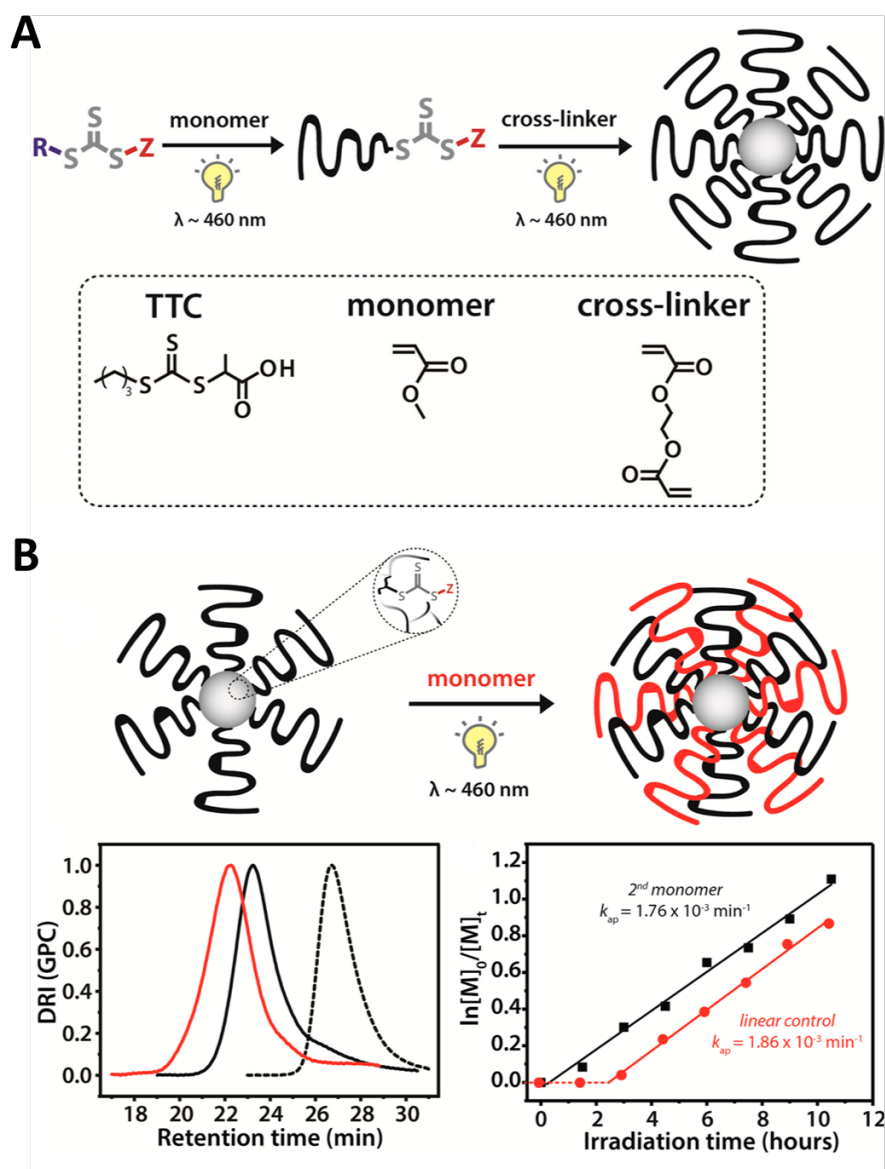


Figure 1.15 Photo-mediated RAFT polymerization for the synthesis of star polymers *via* the (A) arm-first approach and (B) from the resulting star core using the in/out technique. GPC chromatograms show the RI trace of the arm, CCS polymer, and star polymer following the in/out reaction. The apparent rate of polymerization of the second monomer compared to the linear control indicated the efficiency of polymerization from the core was 95%.⁹⁵ Adapted with permission from ACS Macro Lett. 2015, 4, 9, 1012-1016, Copyright 2015 American Chemical Society.

In 2017 the Qiao and Kamigaito groups demonstrated the synthesis of novel star polymers using a mechanistic transformation from cationic RAFT polymerization to radical RAFT polymerization.⁹⁶ Cationic RAFT was first reported by the Kamigaito and co-workers, who demonstrated polymerization of electron-rich vinyl ether monomers that are not readily polymerized *via* radical RAFT polymerization to obtain linear polymers with dispersities below 1.1.⁹⁷ Synthesis of star polymers *via* cationic RAFT was demonstrated in a one-pot approach, with linear poly (isobutyl vinyl ether) (PIBVE) prepared and then crosslinked using a divinyl ether crosslinker. A mechanistic transformation to radical RAFT was also possible with PIBVE arms being crosslinked using a diacrylate crosslinker. Finally, the authors showed that a block copolymer of PIBVE and a heterobifunctional monomer with structure vinyl ether-R-acrylate (eg. 2-(vinylloxy)ethyl acrylate) could be synthesized *via* cationic polymerization of the vinyl ether groups and then crosslinked to form a star polymer through radical RAFT polymerization of the pendant acrylate groups. This mechanistic switch enables the synthesis of star polymers containing monomers not easily synthesized by cationic or radical RAFT polymerization alone and resulted in the synthesis of well-defined star polymers with high arm incorporation and dispersities as low as 1.18.⁹⁷

The development of simple, rapid, high-throughput techniques to synthesize well-defined polymers of all architectures has been an important area in recent years. In 2017 the Cooper-White group reported the development of rapid one-pot sequential aqueous RAFT (rosa-RAFT) for the high-throughput synthesis of star polymers (Figure 1.16).⁹⁸ Polymerizations of acrylate and acrylamide monomers were performed in an water/dioxane mixture (80/20 v/v) using water-soluble thermal radical initiator VA-044. Crucially, these polymerizations did not require degassing. Arm polymerization and crosslinking steps only required 3 minutes each at 100 °C in a thermocycler. The high-throughput technique enabled the rapid synthesis of a library of star polymers using different crosslinkers, crosslinking ratios, and mixing of macroRAFT arms to synthesis miktoarm star polymers, marking an important development in the synthesis of star polymers.

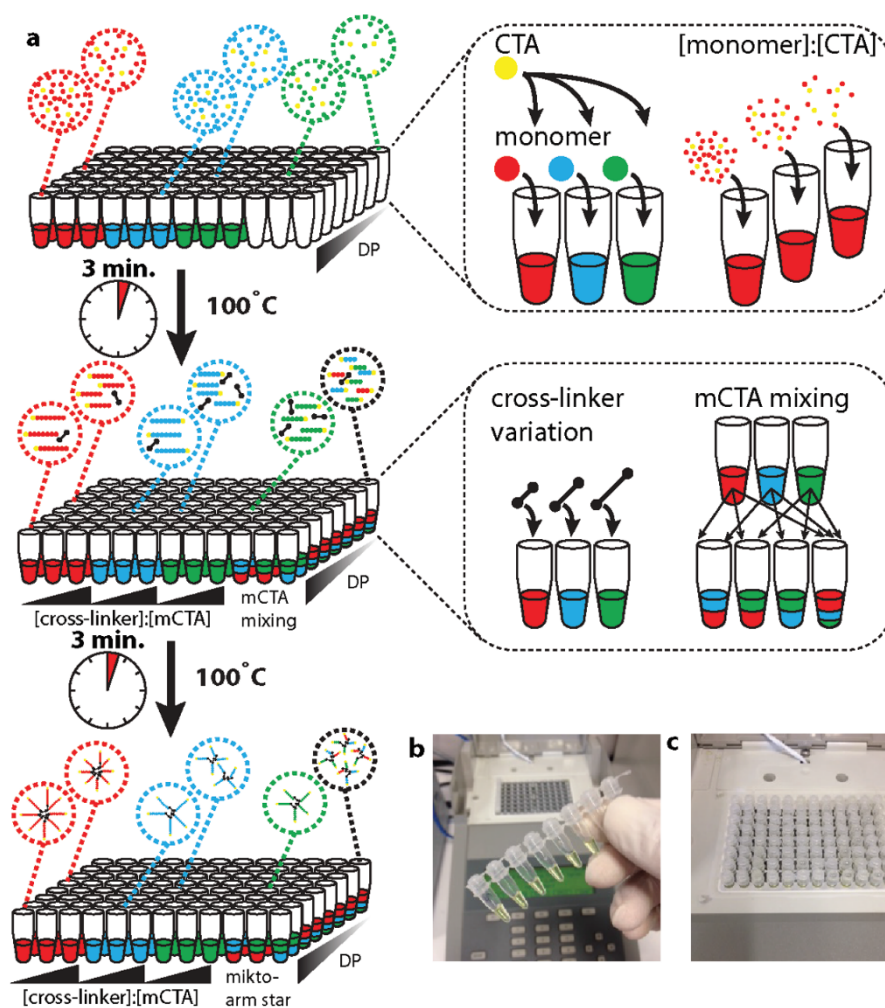


Figure 1.16 Synthesis of star polymers *via* high-throughput ROSA-RAFT. MacroCTA arms were synthesized in 3 min followed by 3 minutes for the crosslinking step. The species and ratio of crosslinker could be varied as well as mixing of the macroCTA arms to rapidly synthesis a large star polymer library.⁹⁸ Reprinted with permission from Ref. 98. Copyright 2017 John Wiley and Sons.

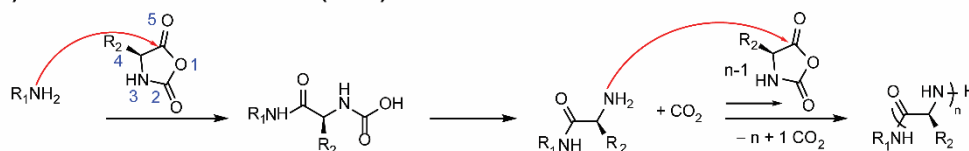
1.4.3 NCA ROP

NCA ROP provides a straightforward method to synthesize polypeptide materials with controlled molecular weights, compositions, and architectures. NCA ROP originated in 1906, when Leuchs reported the synthesis of the first α -amino acid N-carboxyanhydrides (NCA).⁹⁹ Although studies of NCA ROP were performed throughout the 1900s, Deming's seminal work reported in 1997 on the use of organonickel initiators to synthesize well-controlled high molecular weight and block polypeptides was pivotal for generating the widespread interest in NCA ROP that exists today.¹⁰⁰

Developments in NCA ROP have progressed rapidly over the past 20 years as high conversions, molecular weights, and block polymerizations have become more accessible. Although the side chains of proteinogenic amino acids already offer many opportunities to incorporate specific characteristics, functionalization of amino acid side chains offers near unlimited potential for tailoring polypeptide properties, with over 200 NCA monomers described in the literature.¹⁰¹

Despite significant synthetic developments, most application driven NCA ROP still employs the normal amine mechanism (NAM) with a primary amine serving as the initiator, as shown in Figure 1.17A. The nucleophilic amine attacks the CO-5 carbon atom, causing the ring to open.¹⁰² Carbon dioxide is released from the unstable carbamic acid intermediate, exposing the amino group which propagates the polymerization. The activate monomer mechanism (AMM, Figure 1.17B) occurs when the NCA nitrogen is deprotonated and an NCA anion is formed, which then attacks the CO-5 carbon of a monomer, initiating polymerization. The NAM and AMM often occur simultaneously, competing and causing reduced control of molecular weight and dispersity.¹⁰³ NCA ROP is extremely sensitive to contaminants, including water. It is therefore essential to ensure monomers, solvents, initiators, and catalysts are pure and completely anhydrous and to reduce side reactions and termination during polymerization. Readers interested in more advanced NCA ROP mechanisms are directed to several excellent reviews on the developments in NCA ROP,¹⁰⁴⁻¹⁰⁷ including a comprehensive review by Qiao and coworkers focusing on the synthetic advances in NCA ROP from α -amino acids and the applications of these materials.¹⁰³

A) Normal Amine Mechanism (NAM)



B) Activated Monomer Mechanism (AMM)

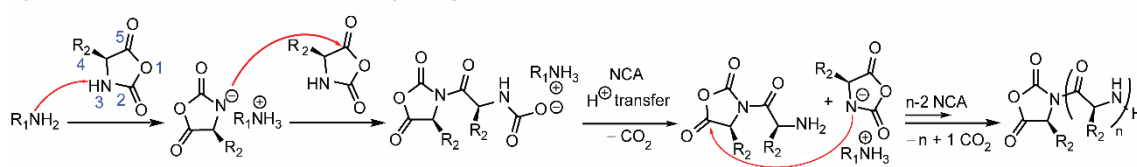


Figure 1.17 The (A) normal amine mechanism (NAM) and (B) activated monomer mechanism of NCA ROP.

1.4.4 Star Polymers by NCA ROP

The strategies described for the initiation of linear polymers via NCA ROP can also be utilized for the synthesis of star polymers, which are most often synthesized with biomedical applications in mind. This section will focus on the synthetic approaches to star polymers via NCA ROP and highlight important works published in recent years. Meanwhile, the uses of polypeptide star polymers will be covered in the applications section.

1.4.4.1 Core-First Approach *via* NCA ROP

The core-first approach has been the most commonly used approach in the synthesis of star polymers *via* NCA ROP. The core-first approach requires a multifunctional initiator from which arms can be polymerized, most commonly a terminal amine for NCA ROP. A selection of reported cores is shown in Figure 1.18. Although the number of arms and core size are limited, the core-first approach gives high yields and the star polymers are easily purified.⁵ Commercially available dendrimers such as poly(amidoamine) (PAMAM),¹⁰⁸⁻¹¹⁰ poly(ethyleneimine) (PEI),¹¹¹ and poly(propyleneimine)^{112,113} have been used as cores due to their widespread availability and the variety of generations available, enabling facile synthesis of star polymers with the desired number of arms. Groups have also synthesized dendrimers and hyperbranched structures, typically *via* click chemistry, to use as cores for star polymer synthesis.^{114,115} The use of less conventional cores has also been investigated.¹¹⁶⁻¹²⁰ The Moretto group used carbon quantum dots (CQD) containing amine groups to initiate the polymerization of PBLG, resulting in a star-shaped CDQ-polypeptide hybrid.¹¹⁶ A CDQ shell was then formed *via* functionalization of the star arm terminal amines with CQD, which induced aggregation of the star polymers. Interestingly, the aggregates maintained the characteristic emission properties of the unmodified CDQs. In a separate report, the Moretto group synthesized photoresponsive linear and star-shaped polymers by combining the photo-induced *cis/trans* conformational changes of azobenzene groups with polypeptides arms.¹¹⁷

Star polymers with block copolymer arms can be synthesized by using multiple NCAs as well as by combining NCA ROP and other polymerization methods. Jia *et al.* synthesized a star with block copolymer arms by combining ROP and NCA ROP, first polymerizing ϵ -caprolactone from a porphyrin core.¹¹⁹ The resulting terminal hydroxyl groups were converted to bromide, then azide, and finally amino groups, which were used to initiate NCA ROP of a poly(ϵ -carbobenzyloxy-L-lysine) (PZLL) block. Recent advancements in NCA ROP from hydroxyl initiators have reduced the need for end group modifications and expanded the pool of possible initiators.^{121,122} Building on work by the Zhang group using 1,1,3,3-trimethylguanidine to promote NCA ROP from hydroxyl groups,¹²¹ Shen *et al.* reported the synthesis of polypeptide stars with

3, 4, and 6 arms from alcohol groups using 1,1,1-tris(hydroxymethyl)propane, pentaerythritol, dipentaerythritol respectively, as cores.¹²³

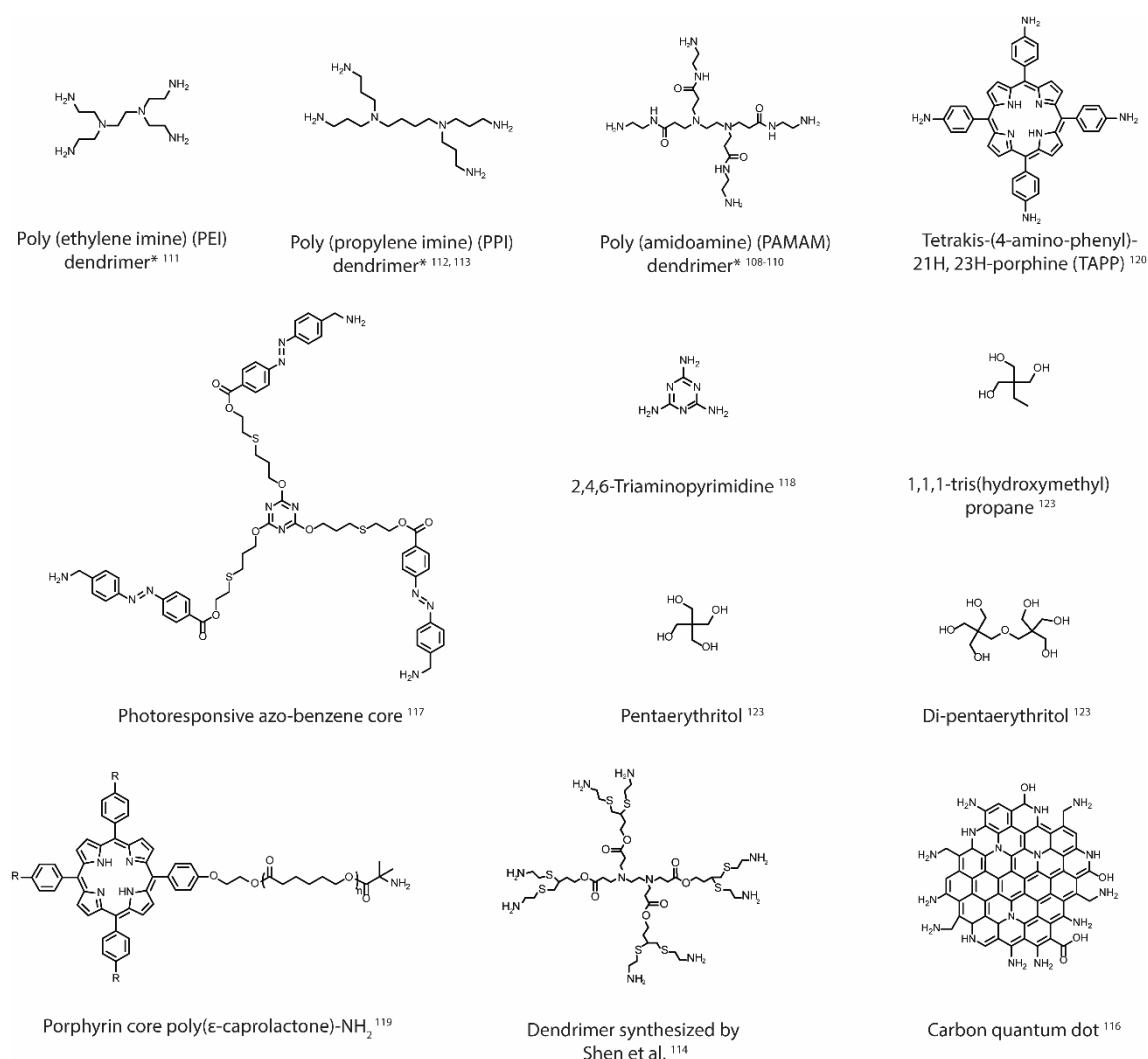


Figure 1.18 Multifunctional initiators used for the synthesis of star polymers *via* the core-first approach using NCA ROP. *Dendrimers of varying generations and with different number of arms have been used. For simplicity, only the 4 arm versions are shown.

One disadvantage of the core-first approach unique to stars synthesized *via* NCA ROP is cross-chain termination, or “backbiting”. Cross-chain termination occurs when the propagating amine attacks the benzylester protecting group of an amino acid instead of the CO-5 of an NCA, and results in chain termination.^{124,125} Stars are particularly susceptible due to the dense nature of star polymers. This phenomenon has most commonly been seen during polymerization of PBLG, and with PBLA (poly(γ -benzyl-L-aspartate) to a lesser extent.^{124,125} Temperature plays a role with higher temperatures leading to more cross-chain termination during the synthesis of linear polymers and reactions at 0°C showing little to no cross chain termination.¹²⁴ The Qiao group was

able to exploit cross-chain termination for the synthesis of capsules, which were crosslinked *via* chain termination reactions.¹²⁵

1.4.4.2 Arm-First Approach *via* NCA ROP

The arm-first approach was used to obtain the first star polymers synthesized entirely *via* NCA ROP.¹²⁶ The Qiao group described the synthesis of linear PZLL arms, which then served as macroinitiators for the crosslinking step. The star synthesis was performed in one pot, with cystine NCA crosslinker added to the reaction mixture following polymerization of the PZLL arms. The use of a cystine crosslinker incorporated biodegradable disulfide groups into the star core. Taking advantage of unreacted NCA remaining in the core, primary amines were used to introduce functional groups into the core. Subsequent reports further investigated exploitation of the unique architecture of star polymers to functionalize distinct regions of the star, including drug loading in the core, functionalization of amino acid side chains, and the functionalization of arm end groups with PEG and targeting ligands.^{127,128}

The synthesis of star polymers *via* NCA ROP exclusively is rare. More commonly, alternate polymerization methods such as controlled radical polymerizations are used for the crosslinking step.^{129,130} Wong *et al.* reported the combination of NCA ROP and RAFT polymerization to synthesize diblock, mikto-arm core cross-linked (CCS) polymers.¹³⁰ The combination of NCA ROP, RAFT polymerization, and coupling chemistries allowed the authors to synthesize complex star polymers with multiple functionalities (Figure 1.19). One arm was synthesized *via* RAFT polymerization while the second arm used a primary amine to initiate polymerization of ZLL NCA. Following synthesis of the polypeptide block, a trithiocarbonate (TTC) RAFT agent was conjugated to the terminal amine and a second block was prepared using RAFT polymerization, resulting in a block copolymer with a terminal TTC group. Star synthesis was achieved through RAFT polymerization of both diblock arm species and di-functional crosslinker *N,N'*-methylenebis(acrylamide) (MBA).

Recently the Heise group investigated the use of both RAFT and free radical polymerization (FRP) for the crosslinking step of polypeptide-based star polymers.¹³¹ ROP of BLG NCA was initiated by 4-vinyl-benzylamine, ensuring a vinyl end group was present on every polymer chain. Crosslinking agent divinylbenzene was used with and without RAFT agent to study star formation *via* RAFT polymerization and FRP, respectively. Star polymers synthesized *via* RAFT polymerization showed narrower molecular weight distributions than those synthesized *via* FRP although both methods suffered from low conversion of linear polymers to star polymers.

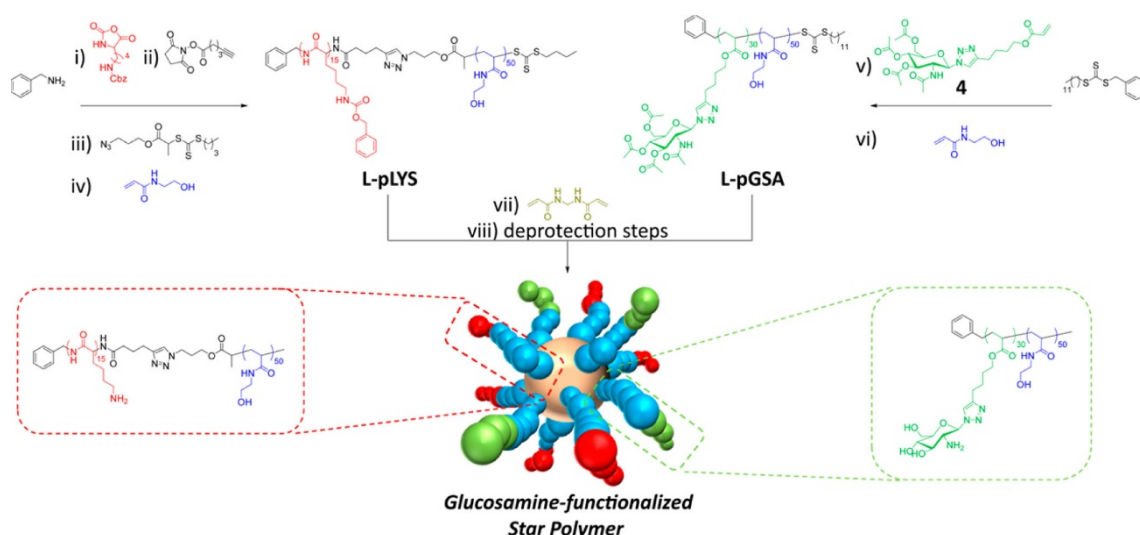


Figure 1.19 Synthesis of diblock miktoarm star polymers using the arm-first approach and RAFT polymerization for the crosslinking step.¹³⁰ Reprinted with permission from *Biomacromolecules* 2016, 17 (3), 1170. Copyright 2016 American Chemical Society.

1.4.4.3 Grafting-Onto Approach with arms synthesized *via* NCA ROP

The grafting-onto approach often capitalizes on the quick and near-quantitative reactions of click chemistry.⁵ Chang *et al.* reported the synthesis of linear PZLL with a disulfide centre from a cystamine initiator.¹³² The two terminal amines were converted to alkyne groups, to each of which two PEG-SH polymers were conjugated using UV-initiated thiol-ene click chemistry. The result was 4 PEG arms attached to a disulfide-centred PZLL core. Pu *et al.* reported the synthesis of a diblock star polymer with a polyhedral oligomeric silsesquioxanes (POSS) core (Figure 1.20).¹³³ POSS are inorganic-organic hybrids composed of alternating silicon and oxygen atoms that form cage-like structures used in a wide variety of applications.¹³⁴ An amine-terminated PEG was used to initiate the ROP of β -benzyl-L-aspartate (BLA) NCA, yielding a diblock polymer. Diblock polymers were then conjugated to the carboxylic acid group of the POSS using the terminal amine of the polypeptide block. Due to their hydrophobic cores and hydrophilic shells, the star polymers self-assembled to form micelles. Pranantyo *et al.* synthesized miktoarm stars using a combination of the core-first and grafting-onto approaches with a modified tetra-azide core.¹³⁵ Two azide groups were converted to propionyl bromide groups which served as initiating sites for ATRP of vinyl monomers containing pendant carbohydrate moieties. Linear PZLL polymers initiated from propargylamine were then conjugated to the two remaining azide groups *via* copper-mediated azide-alkyne cycloaddition.

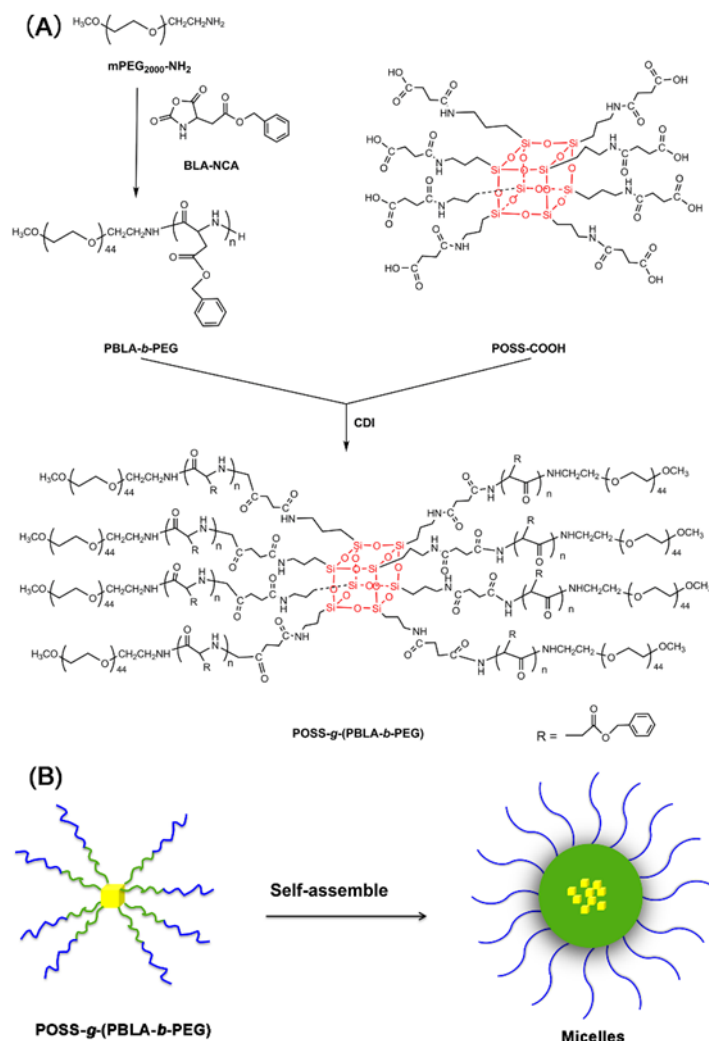


Figure 1.20 Synthesis of star polymers with polyhedral oligomeric silsesquioxane (POSS) core and PEG-*b*-PBLA arms *via* the grafting-onto approach. The resulting stars self-assembled to form micelles with a hydrophobic POSS core and hydrophilic PEG corona. Reproduced with permission from Ref. ¹³³. Copyright 2013 John Wiley and Sons.

The grafting-onto approach has also been used in conjunction with the core-first approach, with linear polymers being “clicked” onto the ends of the star polymer ends. Lin *et al.* described the core-first ATRP of styrene from a tri-bromo-functionalized core.¹³⁶ Post-purification, the bromine end groups were reacted with sodium azide to yield terminal azide groups. PBLG synthesized from a propargylamine initiator was then conjugated to the three-arm star polymer by copper-catalyzed azide-alkyne cycloaddition, yielding di-block star polymers.

1.4.5 Other Methods for Star Polymer Synthesis

As previously mentioned, star polymer synthesis can be performed using numerous polymerization techniques. Here we provide a brief overview of other common polymerization methods.

1.4.5.1 Ring-Opening Polymerization (ROP)

Traditional ROP involves the ring-opening of cyclic ester and carbonate monomers via polycondensation to produce degradable poly(ester) and poly(carbonate) materials. Due to the biodegradable nature of the resulting polymers, ROP techniques are frequently used to synthesize polymers for biomedical applications. Polymerization is typically initiated from hydroxyl groups and requires the use of organometallic complexes or organic catalysts to achieve fast polymerizations and low dispersities. The use of organic catalysts marks an important development for ROP, as organometallic complexes are difficult to remove from the final polymer products due to their oxophilicity.^{137,138} The Qiao group demonstrated methanesulfonic acid (MSA) was an efficient catalyst for the one-pot synthesis of CCS stars by ROP.¹³⁹ The mechanism of MSA-catalyzed ROP is shown in Figure 1.21. In addition, stars synthesized using MSA showed no star-star coupling and reached higher conversions.

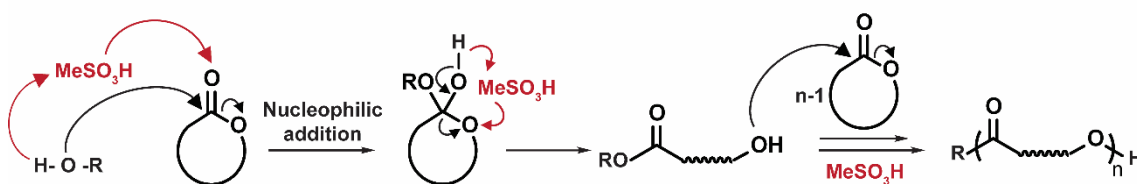


Figure 1.21 Mechanism of methanesulfonic acid-catalyzed ring opening polymerization.

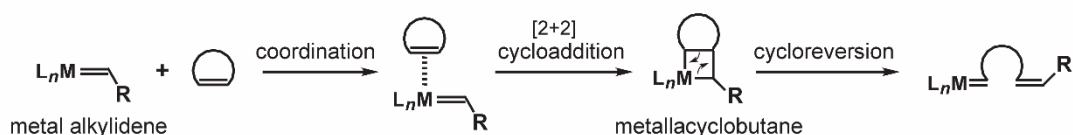
The incorporation of functional side groups has traditionally been limited in polymers synthesized by ROP but of great of interest in recent years. An in-depth review by Beck and Wurm on biodegradable polymers synthesized by ROP of monomers without protecting groups covers many of the recent developments.¹⁴⁰

1.4.5.2 Ring-Opening Metathesis Polymerization (ROMP)

ROMP also proceeds *via* ring-opening of cyclic monomers and results in well-defined polymers with readily modified end groups. The general mechanism of ROMP is illustrated in Figure 1.22.¹⁴¹ The development of novel ruthenium and molybdenum catalysts has led a wider pool of monomers with a greater range of functionalities, however the presence of transition metals is often undesirable.⁵

One particularly interesting development in star polymer synthesis by ROMP was the development of brush-arm star polymers (BASPs) by the Johnson group. “Brush initiators” were prepared by ROMP of norbornene macromonomers, then crosslinked with a bis-norbornene crosslinker in a manner similar to arm-first star polymerization.¹⁴² This approach led to well-defined star polymers with low dispersities. Since their initial development in 2012, BASPs have been investigated for applications drug delivery,¹⁴³ medical imaging,^{144,145} and lithium ion batteries.¹⁴⁶

Initiation:



Propagation:



Termination:



Figure 1.22 General mechanism of ring opening metathesis polymerization (ROMP). Adapted by permission from Springer Nature: Polymer Journal, Recent advances in ring-opening metathesis polymerization, and applications to synthesis of functional materials, Sutthasupa et al, 42, 905–915, Copyright 2010.

1.4.5.3 Nitroxide-Mediated Polymerization (NMP)

NMP was the first type of RDRP developed and therefore one of the early techniques for controlled radical star polymer synthesis. The basic mechanism and activation-deactivation equilibrium of NMP is shown in Figure 1.23.¹⁴⁷ Since the developments of ATRP and RAFT, the use of NMP has declined due to high reaction temperatures, poor control over methacrylate and methacrylamide monomers, and comparatively lower chain-end fidelity.⁵ However, NMP still provides benefits such as compatibility with amine and carboxylic acid groups and the fact no catalysts are needed.

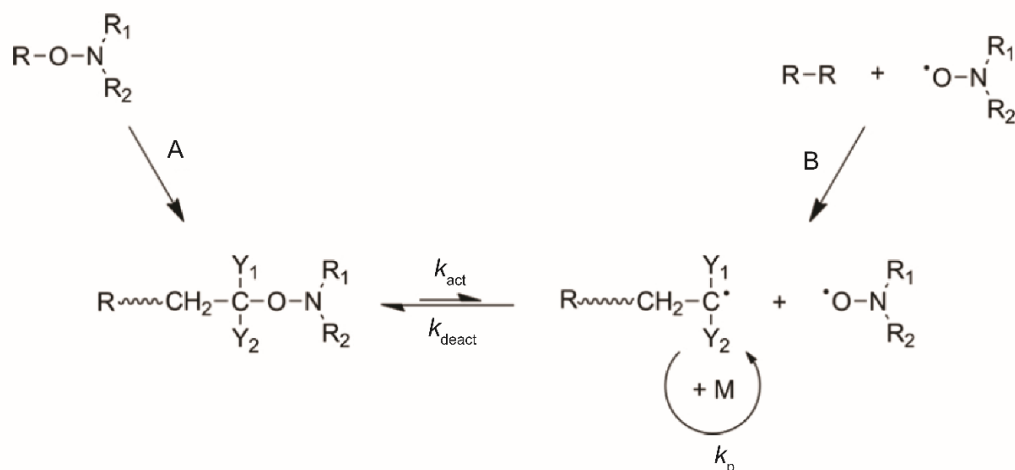


Figure 1.23 Activation-deactivation mechanism of nitroxide-mediated polymerization (NMP). Polymerization can be initiated using an (A) monocomponent or (B) bicomponent initiating system. Modified from Progress in Polymer Science, 38 (1), Nitroxide-mediated polymerization, Nicolas et al, 63-235, Copyright 2013, with permission from Elsevier.

1.4.5.4 Atom Transfer Radical Polymerization (ATRP)

ATRP has been widely reported for the synthesis of star polymers using both the arm-first and core-first approaches.⁵ Like RAFT polymerization, ATRP is compatible of with a large array of vinylic monomers and capable of conversions greater than 95%. While earlier work required the use of transition metal catalysts which was not ideal for certain applications, this obstacle was overcome with the development of metal-free ATRP in 2014.¹⁴⁸ Significant work has been performed in recent years to develop novel mechanisms of initiating and mediating polymerization. Light, ultrasound, enzymes, and electrochemical ATRP have all been reported for the synthesis of exquisitely controlled polymers.¹⁴⁹

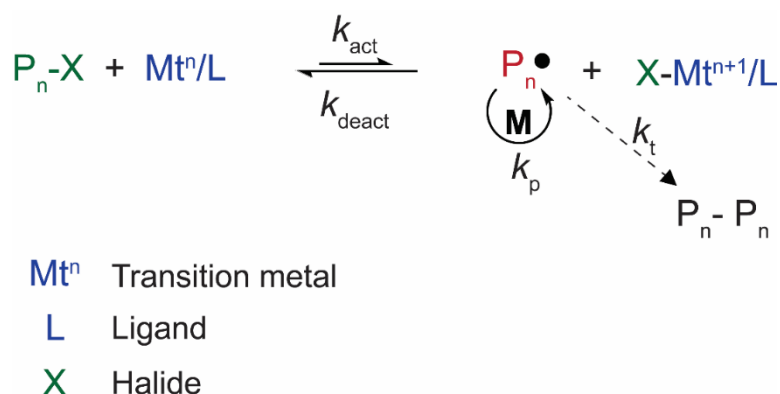


Figure 1.24 Activation-deactivation mechanism of ATRP.

1.5 Applications of Star Polymers

The ease with which star polymers of various sizes, compositions and functionalities can be synthesized has seen the development of a broad array of star polymers for targeted applications. The unimolecular structure of star polymers ensures they maintain their structure and are not susceptible to shear or disassembly under fluctuating or extreme conditions. The availability of numerous polymerization techniques and the ease with which they can be combined promotes incorporation of functional groups while the distinct regions of star polymers facilitate the generation of core-, arm-, or end-functionalized star polymers.^{5,150} Despite these benefits, star polymers do suffer from some disadvantages. The synthesis of multifunctional cores can be labour-intensive while the arm-first approach can require extensive purification. These issues mean large-scale synthesis could be difficult and expensive, and must be considered when synthesizing star polymers for specific applications.

By leveraging the unique properties of star architectures, star polymers have found application in diverse fields; including as drug delivery and imaging contrast agents, viscosity modifiers, emulsion stabilization, and in advanced materials synthesis. This section will provide an overview of the applications of star polymers, and important considerations when designing star polymers for specific applications. Significant developments or examples will be highlighted, with an emphasis on those synthesized via RAFT polymerization or NCA ROP.

1.5.1 Biomedical Applications

The combination of well-defined unimolecular structures with distinct regions for functionalization and versatile synthetic techniques make star polymers promising architectures for biomedical applications. The most common biomedical applications of star polymers include drug delivery, nucleic acid delivery, and use as antibacterial agents. An overview of key considerations in the design of star polymers for these applications and recent examples will be presented.

1.5.1.1 Drug Delivery

Star polymers offer numerous advantages in the field of drug delivery. Unlike self-assembled structures, star polymers are not susceptible to disassembly when diluted or protein adsorption occurs. Nanoparticle drug delivery systems (DDS) can protect therapeutics, improve water solubility, and reduce adverse effects. These benefits are especially advantageous in cancer therapy, where the off-target effects are severe and often limit the dose administered to patients.

Key considerations in the design of nanoparticle drug delivery systems include i) size, ii) structure, iii) targeting, iv) responsiveness, and v) degradability. These considerations and how they can be addressed by star polymers will be presented. Although they will not be specifically covered here, many of the same principles apply to the delivery of imaging agents.

The size of star polymers for drug delivery is of the utmost importance. Nanocarriers such as star polymers are most commonly developed with cancer therapy in mind due to improved efficacy of treatment while reducing toxicity compared to unbound/free chemotherapeutics.¹⁵¹⁻¹⁵⁴ Polymeric nanocarriers also aim to exploit the enhanced permeability and retention (EPR) effect to improve drug delivery to tumours. Tumours are thought to possess a “leaky” vasculature, allowing nanocarriers between 10-800 nm in diameter to escape the vasculature and permeate into the tumour.¹⁵⁵ Star polymers are generally between 10 and 100 nm in diameter, which is often considered the ideal size of nanoparticles for cancer therapy.⁵ Particles greater than 10 nm avoid the removal from circulation by the renal system experienced by smaller particles while nanoparticles smaller than 100 nm are thought to passively accumulate in tumours. It is hypothesized that nanocarriers can accumulate in the tumour while being too large for renal clearance or extravasate at the normal capillary bed.

The core-shell structure of star polymers can shield loaded therapeutics or imaging agents from unwanted degradation and uptake by the body’s immune system. This results in increased delivery efficiency while also facilitating functionalization of the star surface with specific targeting moieties such as peptides, antibodies, and sugars, thus improving delivery to the target site or tissue. In addition, intelligent design of star polymers can result in therapeutic release over a desired timescale or upon exposure to a particular stimulus. As with all materials designed for biomedical applications, it is essential to ensure the star polymers and their possible degradation products do not induce inflammatory response or toxicity before, during, or after delivery.^{5,156-158}

Polypeptides synthesized *via* NCA ROP are excellent materials for biomedical applications such as drug delivery because they are generally regarded as safe. Multiple clinical trials involving polypeptide-containing nanocarriers have been conducted during the past two decades, with the first clinical trial involving polypeptide-containing micelles beginning in 2001.¹⁵⁹ Since then, several formulations have reached clinical trials for cancer therapy and have demonstrated they are well-tolerated by patients.^{160,161}

The unique structure of star polymers facilitates the functionalization of the core, arm, or peripheral regions and can be accomplished by incorporating functional groups into the crosslinkers, monomers, and initiator or RAFT agent used. Common functionalities include

therapeutic molecules, imaging agents, and dyes. N-hydroxysuccinimide (NHS) functionalized RAFT agents have been used to prepare end-functionalized star polymers¹⁶³ while arm-functionalized star polymers were synthesized by incorporating monomers such as pentafluorophenyl acrylate (PFPA)^{162,163} and 4-vinylbenzyl chloride (VBC)¹⁶⁴ into the arms. PFPA^{163,165} and vinyl benzaldehyde (VBA)^{166,167} have also been incorporated into cores as secondary monomers during crosslinking to generate core-functionalized star polymers. It has been shown that two types of functional groups could be incorporated into the star polymer to conjugate both chemotherapeutic drug and MRI contrast agent. Star polymers were synthesized by incorporating aldehyde-functional monomer VBA into the core and activated-ester monomer PFPA in the arm (Figure 1.25). The VBA and PFPA monomers were subsequently modified with chemotherapeutic drug Doxorubicin and an MRI contrast agent based on disparate reactivities of VBA and PFPA.¹⁶²

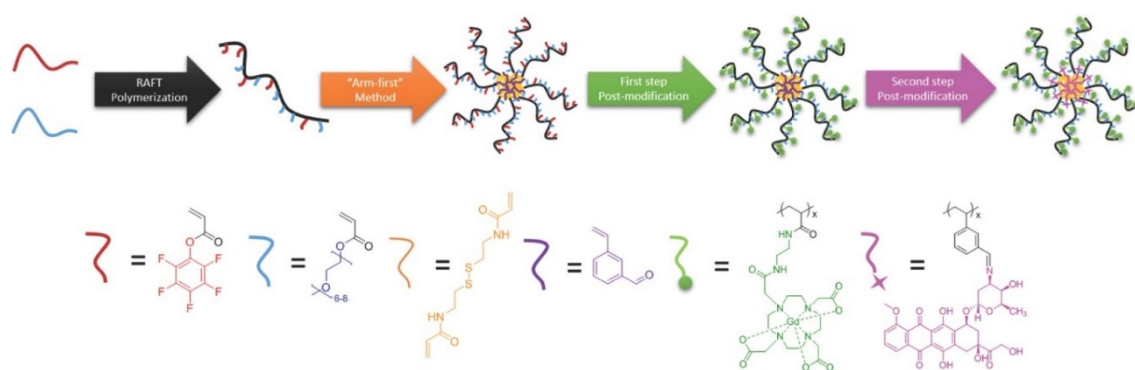


Figure 1.25 Schematic illustration of the synthesis of multifunctional core cross-linked star polymers functionalized with MRI contrast agent and chemotherapeutic drug.¹⁶² Reprinted with permission from Ref. 162. Copyright 2017 John Wiley and Sons.

The Whittaker and Davis groups designed and synthesized star polymers by RAFT polymerization for biomedical applications including the delivery of chemotherapeutic agents, genes, imaging agents, protein inhibitors, and signaling molecules.¹⁵⁸ In order to prepare star polymers which are soluble in water and the biological environment, hydrophilic monomers such as poly(oligo(ethylene glycol) methyl ether acrylate/methacrylate) (POEGA/MA) are commonly used to form the star polymer arms. Star polymers comprising POEGA/MA arms have demonstrated reduced protein adsorption and also provide stability to star polymers during circulation as they are not susceptible to dissociation in the same way as self-assembled structures.¹⁶⁷⁻¹⁶⁹ *In vivo* and *in vitro* studies have shown that the POEGA star polymers with different molecular weights (49, 64, and 94 kDa), and therefore hydrodynamic volumes, exhibit different pharmacokinetic and biodistribution behaviours, with the largest star polymers displaying longer plasma exposure and improved uptake into solid tumours.¹⁶⁸

In recent years researchers have placed greater focus on the active targeting of drug nanocarriers to tumour cells. Cancer cells are known to overexpress certain surface molecules (biomarkers) that can be targeted by displaying a targeting ligand on the nanocarrier surface. The most commonly used targeting ligands are small molecules, antibodies, peptides, sugars, and aptamers.¹⁷⁰ Although the overexpression of biomarkers often varies between different cancers or even between individuals with the same cancer, some receptors such as folic acid and transferrin receptors are overexpressed in many forms of cancer,^{171,172} and conjugation of their ligands can improve targeting and cell internalization.^{173,174}

Glycopolymers offer another opportunity for targeted drug delivery due to their interactions with cell surface proteins.¹⁷⁵ Hyaluronic acid (HA) is able to specifically interact with multiple HA-binding proteins, among which its interaction with cell surface adhesion receptor cluster of differentiation 44 (CD44) in particular has been widely studied due to the overexpression of CD44 in subpopulations of cancer cells. CD44 is also recognized as a molecular marker for cancer stem cells.¹⁷⁶⁻¹⁷⁸

Glyco-amino acids, which have both amino acids and carbohydrates in a single molecule and whose corresponding polymers can be synthesized via NCA ROP, have also been reported in recent years and are being investigated for potential applications in biomaterial and drug delivery applications.¹⁷⁹⁻¹⁸¹ Gupta and co-workers reported the synthesis of miktoarm stars containing one hydrophilic poly(glycol-amino acid) arm and two hydrophobic poly(ϵ -caprolactone) (PCL) arms which could subsequently self-assemble, with the poly(glycol-amino acid) arms forming the exterior of the micelle.¹⁸² The glycol-amino acid consisted of a lysine residue with galactose or mannose conjugated to the lysine side chain amine. Morphologies including nanorods, vesicles, and micelles could be achieved by varying the lengths of the hydrophilic and hydrophobic arms, helicity of the glycopolymer arm, and the crystallinity of the PCL arms, although no difference was observed between mannose- and galactose-functionalized polymers. The authors also demonstrated the targeting of the mannose-functionalized polymersomes *in vitro* using the breast cancer cell line MDA-MB-231, which are known to overexpress certain mannose-specific receptors. Model hydrophobic drug rhodamine B octadecyl ester (RBOE) was encapsulated in galactose- and mannose-functionalized polymersomes and the mannose-functionalized polymersomes were shown to have nearly double the cell internalization of the analogous galactose-functionalized polymersomes.

The synthesis of star polymers via RAFT polymerization also lends itself to functionalization of the star periphery, although some forethought is required. Depending on the synthetic approach

used, the RAFT agent R- or Z-group may be located at the periphery, either of which can be functionalized. Chen and coworkers used a RAFT agent functionalized with an RGD peptide to synthesize linear arms, followed by star polymerization, to give a miktoarm star with RGD targeting ligands.¹⁸³ If post-polymerization functionalization is desired, RAFT agents with clickable R-group handles can be employed. For example, azido-functionalized RAFT agents are commercially available. If using a one-pot arm-first approach, the azido-R-group would be located at the star periphery and could be functionalized with a targeting ligand using azide-alkyne cycloaddition following star polymerization.

Alternatively, core-first star polymerization using the R-group approach results in the thiocarbonylthio moiety at the star periphery. The thiocarbonylthio can easily be modified to a thiol through aminolysis of the RAFT agent, which can then participate in thiol-based click reactions for functionalization.⁷⁷

The use of stimuli to release drug at the intended site is an important area of focus in the development of DDS. The stimuli can be internal (e.g., pH, redox potential and enzymes) or external (e.g., temperature, UV or infrared irradiation and magnetic fields).¹⁸⁴ The use of stimuli-responsive materials promotes on-demand drug release, with payloads released at the desired time or location.

Ding et al. reported the synthesis redox- and pH-responsive core-crosslinked star polymers with a sheddable PEG corona for delivery of camptothecin to cancer cells.¹⁸⁵ Linear PEG-b-P(L-cysteine)(PLCys) was synthesized with an acid-labile linker present between the PEG and PLCys blocks, allowing PEG to be released in the tumour microenvironment. PEG-b-PLCys and camptothecin formed self-assembled nanoparticles and H₂O₂ was added to oxidize the cysteine side chains, forming disulfide bonds between polymer chains. *In vitro* experiments demonstrated the release of the PEG chains at pH 6.5 while the use of reducing agent dithiothreitol (DTT) caused dissociation of the star polymers. Interestingly, shedding of the PEG corona resulted in greater drug release than DTT treatment. As expected, the greatest drug release was observed at pH 6.5 plus DTT treatment.

Star polymers synthesized via RAFT polymerization can incorporate stimuli-responsive functionalities through inclusion of responsive monomers. Examples include thermoresponsive monomer NIPAAm¹⁸⁶ and pH-responsive monomer 2-(dimethylamino)ethyl methacrylate (DMAEMA),^{169,187} which were used in star arms or as secondary monomer in the core. These star polymers change their size when exposed to changing temperature or pH. Because the tumour

microenvironment is slightly warmer and more acidic than healthy tissue, release of the drug is stimulated at the target site.

While not specific to any particular architecture or polymerization techniques, it is important to mention that pH-sensitive linkers have been used to conjugate therapeutics to polymeric nanocarriers, enabling pH-responsive release.¹⁸⁸⁻¹⁹² The use of acid-labile hydrazone linkages in DDS is ubiquitous because they are readily cleaved between pH 5-5.5 allowing the release of therapeutics inside the endosomal pathway. The use of acid-labile hydrazone linkages in DDS has been eloquently reviewed by Sonawane et al.¹⁹³

Stars polymers for biomedical applications should be large enough to avoid removal by the renal system, leading to improved circulation half-lives. However, star degradation is desirable after cell internalization to improve therapeutic delivery or after delivery to prevent bioaccumulation. While polypeptides stars are susceptible to enzymatic degradation, most stars synthesized by RAFT polymerization are not readily degraded. To achieve this end, biodegradable linkers or polymers can be incorporated into the star structure.

Degradable crosslinkers are often responsive to the tumour environment or intracellular stimuli, such as acid-labile^{194,195} and reduction-sensitive disulfide^{6,169,183} crosslinkers. These crosslinkers are readily hydrolyzed in the acidic environment of tumour tissue or reduced by glutathione upon exposure to the glutathione-rich intracellular environment, resulting in degradation of the core and therefore the star polymer. Another mechanism degradation is the incorporation of known enzyme-sensitive sequences.(cite) These peptide sequences can be incredibly specific and are generally synthesized by solid phase peptide synthesis.

Another promising approach still early in development is radical ring opening polymerization, which results in a degradable polyester backbone.¹⁹⁶ Excitingly, recent investigations into the use of xanthates to control radical ROP of these monomers demonstrates that RAFT polymerization may also be used to synthesize degradable polymers and enable facile synthesis of block copolymers with degradable segments.¹⁹⁷⁻²⁰⁰

1.5.1.2 Gene Delivery

Delivery of nucleic acids is a rapidly expanding field with enormous potential to treat numerous pathologies by altering gene expression. Drug and nucleic acid delivery share several requirements, and nanoparticles with core-shell properties are therefore a popular method of nucleic acid delivery. Nucleic acids are sensitive to serum nucleases and requires protection, while non-fouling shells are desired to reduce protein adsorption. High transfection of the genetic

material is essential to achieve a therapeutic result and the development of effective vectors remains a key challenge.

Nucleic acids are negatively charged due to their phosphodiester backbone. Polymeric delivery systems often capitalize on this negative charge by using polycations, which form complexes referred to as “polyplexes” through electrostatic interactions. These polyplexes protect nucleic acids from degradation while in the circulation and compact the nucleic acid material to facilitate loading and delivery.

Polypeptide-based nucleic acid delivery systems often use poly(L-lysine) (PLL) as the polycation. Lam and coworkers synthesized PLL star polymers via NCA ROP from PAMAM cores containing 16 or 32 initiation sites.¹⁰⁸ PEG was conjugated to the terminal amine of the star polymers to reduce fouling and improve the biodistribution of the star polymers. The authors demonstrated successful complexation of nucleic acid and the biocompatibility of the PEGylated poly(lysine) star *in vitro*.

Byrne and coworkers polymerized a library of PLL stars initiated from PPI cores as well as a linear PLL.²⁰¹ The star polymers demonstrated greater compaction of nucleic acid than the linear polymer, as measured by nitrogen to phosphate ratio. *In vitro* transfection studies using a 64 arm PPI-PLL star polymer with an N/P of 5 showed a 300-fold increase in luciferase expression compared to linear PLL, highlighting the advantages of star polymers for nucleic acid delivery.

When synthesizing nucleic acid delivery systems using RDRP techniques, cationic monomers or cationizable monomers such as 2-(dimethylamino)ethyl acrylate/methacrylate (DMAEA/MA) are often employed.²⁰²⁻²⁰⁵ In a recent study, the Themistou group synthesized star polymers containing DMAEMA for nucleic acid delivery using arm-first RAFT polymerization with different crosslinkers to investigate the impact of star degradability on endosomal escape.¹⁹⁵ Early work in nanoparticle delivery systems were focused on the uptake of nanoparticles into cells, but researchers have since learned that release of therapeutics from the endocytic pathway is equally important. This is especially true with enzyme-sensitive materials like nucleic acid which are degraded in the lysosome. With this challenge in mind, the authors compared nucleic acid endosomal escape from star polymers with pH-labile and non-degradable crosslinked cores. Unsurprisingly, the ability of the nucleic acid to escape the endosomal pathway was a key determinant of transfection efficiency, highlighting the importance of endosomal escape when designing and investigating delivery systems.

1.5.1.3 Antibacterial Agents

Antibacterial agents are of intense interest as multi-drug antibiotic resistance is on the rise worldwide. Current predictions estimate more people will die from bacterial infections than cancer by the year 2050 as traditional antibiotics become ineffective.²⁰⁶ Polymeric antibacterial agents are often inspired by naturally occurring antimicrobial peptides (AMPs). AMPs and their mimics are typically amphiphilic, containing cationic and hydrophobic moieties. The positive charge promotes interactions between the polymer and the negatively charged bacterial cell walls, while the hydrophobic components enables penetration of the bacterial membrane.²⁰⁷

Polypeptides synthesized by NCA ROP offer the best mimicry of AMPs that can be produced large-scale. Interestingly, star polymers show exceptional potential as antibacterial agents. A recent review by Rasines Mazo and coworkers examined all antibacterial polypeptides synthesized *via* NCA ROP in the past 5 years and found star polymers displayed superior properties over other architectures.¹⁰³ Star-shaped structurally nanoengineered antimicrobial peptide polypers (SNAPPs) have generated significant interest due to their antibacterial effects and high selectivity for bacterial cells over mammalian cells.^{109,110} First reported by the collaborative team of Qiao and O'Brien-Simpson in 2016, SNAPPs were synthesized by NCA ROP copolymerization of L-lysine and D,L-valine NCA monomers from PAMAM dendrimer cores.¹⁰⁹ SNAPPs containing 16 arms showed excellent antibacterial behaviour at low concentrations against colistin and multidrug resistant *A. baumannii*, a Gram-negative bacteria, both *in vitro* and *in vivo*. Mechanistic studies indicated the SNAPPs efficacy resulted from interactions with the bacterial cell wall and cell membrane, destabilizing of the membrane and leading to cell lysis. Importantly, culturing bacteria with SNAPPs below the minimum bactericidal concentration (MBC) for 600 generations did not result in any acquired resistance. Subsequent studies demonstrated the degree of polymerization and number of star arms play an important role in SNAPP antibacterial activity.¹¹⁰

Wong and coworkers combined NCA ROP and photoiniferter RAFT polymerization to synthesize diblock, mikto-arm star polymers, as mentioned previously in section 1.4.4.2.¹³⁰ The star polymers contained poly(L-lysine) (PLL) to imbue antibacterial activity. Varying amounts of glucosamine were incorporated into the arms using a glucosamine-modified acrylic monomer to investigate the impact on antibacterial activity and selectivity. Incorporation of glucosamine led to selective targeting of Gram-positive bacteria, with little to no effect on Gram-negative bacteria. Star polymers containing 25 mol% glucosamine arms were found to be the most effective for treating Gram-positive bacteria, demonstrating good biocompatibility with mammalian cells and significant antibacterial effects.

Finally, star polymers synthesized entirely by RAFT polymerization have also shown excellent activity against bacteria. Mimicking AMPs, the Boyer group synthesized antibacterial polymers with cationic and hydrophobic acrylic monomers, as well as oligoethylene glycol moieties to reduce fouling. The authors synthesized linear statistical copolymers, linear block copolymers, and hyperbranched copolymers of varying molecular weights. The hyperbranched polymers demonstrated the best overall performance against Gram-negative bacteria; although the minimum inhibitory concentration (MIC) was slightly higher than that of the linear random copolymers, they hyperbranched polymers demonstrated four-fold higher hemocompatibility.

1.5.2 Other Applications

The unique properties of star polymers allow tunable interactions with other species in solution. The compact nature of star polymers leads to significantly different properties than linear polymers of the same molecular weight, while their flexible arms lead to different behaviours than particles of the same size. Such interactions can be leveraged for controlling the reactivity of these other species, leading to altered chemical pathways and affording the preparation of advanced materials. This section will present a selection of applications in which star polymers were essential for improved performance.

1.5.2.1 Emulsion Stabilization

Since reporting the synthesis of core-crosslinked star (CCS) polymers via emulsion and dispersion polymerization in 2011,⁸⁷ An and co-workers have pioneered the use of CCS polymers as emulsion stabilizers.^{91,92,94,208,209} Emulsion systems have wide industrial relevance, including in food, cosmetics, pharmaceuticals, coatings, oil recovery, and chemical processing. Qiu et al demonstrated the use of a fluorescein-tagged PDMA CCS that adsorbed to the interface of a toluene/water emulsion with excess PDMA stars remaining the aqueous phase, shown in Figure 1.26A, and Nile red-stained toluene droplets shown in Figure 1.26B. Stabilized emulsions were used as templates for the synthesis of spherical polystyrene particles inside the oil droplets. Following solvent evaporation, aggregation of PDMA stars around the particles (Figure 1.26C) and Nile red-stained poly(styrene) particles were observed (Figure 1.26D).

Exploiting the versatility of RAFT polymerization, the An group developed reversible emulsification/ demulsification systems readily triggered by external stimuli, including heat/salt/pH through targeted interactions between stars and between star arms and solvents (Figure 1.27).^{91,92,94,208} While previous work had been carried out using amphiphilic star polymers to stabilize emulsions by having them act as surfactants, An and co-workers focused on CCS polymers with entirely hydrophilic arms and the structural properties of star polymers that may cause this stabilizing behaviour. Star polymers are similar in size to particulate emulsifiers but

display greater flexibility, which Chen et al hypothesized would allow the CCS polymers to adopt favourable configurations once adsorbed on to the surface and aid their residence at the surface.⁹¹

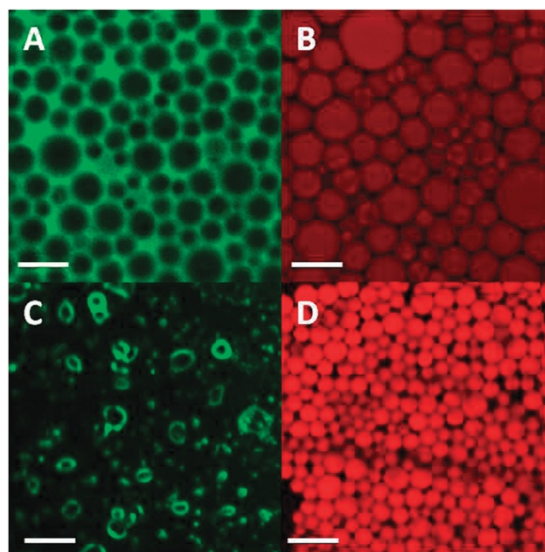


Figure 1.26 Confocal images of water/toluene (60/40 v/v%) emulsions containing (A) 0.2wt% fluorescein-tagged PMDA stars and (B) Nile red stained toluene demonstrating the emulsion stabilizing activity of the PDMA stars. Following solvent evaporation, (C) PDMA stars at particle surface and (D) particles synthesized using emulsion templating. Scale bar is 20 microns.⁸⁷ Adapted from Ref. 87 with permission from the Royal Society of Chemistry.

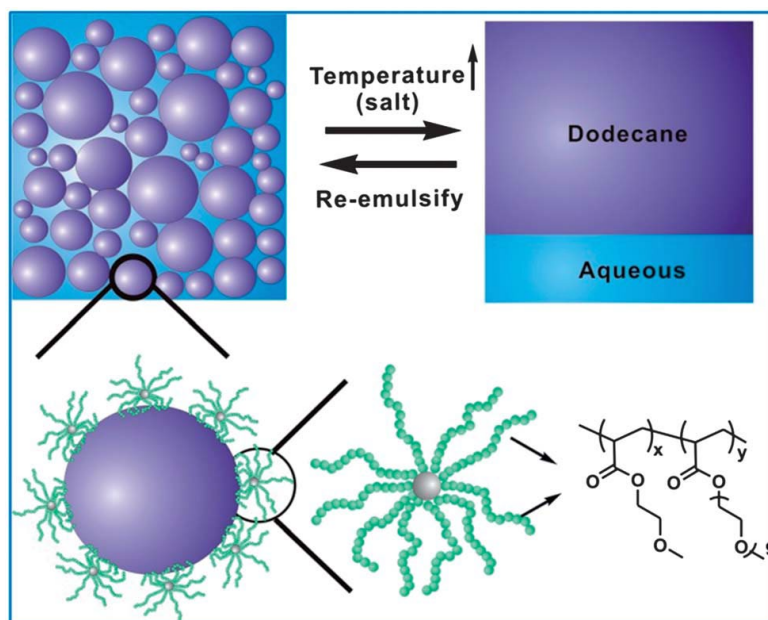


Figure 1.27 High internal phase emulsion (HIPE) stabilized by poly(MEA_x-co-PEGA_y) star co-polymers exhibit on-demand demulsification triggered by salt and temperature.⁹⁴ Adapted from Ref. 94 with permission from the Royal Society of Chemistry.

Star polymers have also been recently used as dispersants of multiwalled carbon nanotubes (MWNTs) in aqueous and acetone solutions.²¹⁰ Gao et al reported the synthesis of an amphiphilic “octopus” star polymer with a RAFT agent-functionalized silsesquioxane cage using the core-first approach. Star polymers were shown to produce more stable MWNT dispersions than linear polymers, which the authors suggested was due to hydrophobic core anchoring to the nanotube surface while the hydrophilic arms extended outwards and reduced the hydrophobic area of the MWNT surface (Figure 1.28).²¹⁰

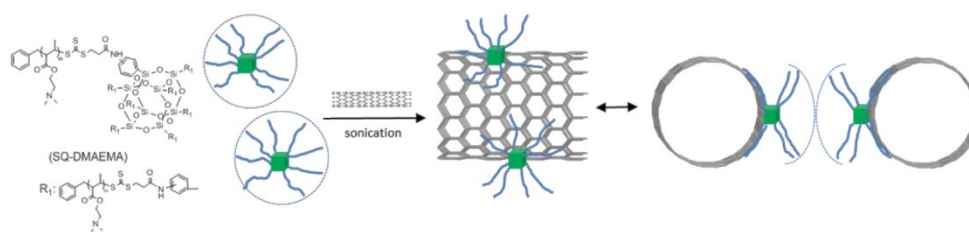


Figure 1.28 Star polymers with POSS core and hydrophilic arms stabilized MWNTs in aqueous solvent.²¹⁰ Adapted from Ref. 210 with permission from the Royal Society of Chemistry.

1.5.2.2 Advanced Materials

Numerous applications have been reported in which use of star polymers resulted in improved properties in comparison to linear polymers. Hendrich and Vana synthesized linear and star-shaped polymers that underwent redistribution of copolymer blocks due to the incorporation of bifunctional RAFT agents.²¹¹ Tensile testing demonstrated that inclusion of as little as 5 mol% star polymer led to higher ultimate strength and material toughness compare to films of pure linear multiblock polymers. Zheng et al reported improved resolution using a novel star polymer photoresist for krypton fluoride (KrF) photolithography.²¹² Star copolymers were synthesized from a tetra-RAFT functionalized pentaerythritol core using *tert*-butyl acrylate and *p*-acetoxystyrene as monomers. Use of the star copolymers resulting in a pattern resolution of 200 nm, demonstrating greater resolution than analogous linear photoresists.

Star polymers have also shown superior properties as single-ion conduction polymer electrolytes (SCPE) which are valued for their potential use in energy storage applications.²¹³ Miktoarm star polymers were composed of a polyhedral oligomeric silsesquioxane (POSS) core and poly (ethylene glycol) (PEG) and lithium poly ([styrene-4-sulfonyltrifluoromethyl-sulfonyl] imide) (PSTF-Li⁺) arms. The lithium-ion conducting stars demonstrated high thermal stability and tunable dc-conductivity. The improved thermal stability of the star polymer in comparison to a

linear analogue was thought to be a result of the restricted motion of the star polymer, which is less sensitive to heat. PEG-*b*-PSTF linear copolymers were found to have greater dc-conductivity than the star polymer because of the star's higher T_g value, however the addition of more PEG arms led to significantly increased conductivity, highlighting the tunability of star polymer composition and performance.²¹³

Star polymers can also be used in situations where the use of linear polymers is not possible. Lin and co-workers synthesized degradable star polymers through a combination of ring opening polymerization (ROP) and RAFT polymerization.²¹⁴ ROP of ϵ -caprolactone was performed from a β -cyclodextrin core and R-group of RAFT agents were conjugated to the resulting terminal alcohol groups. RAFT polymerization of 4-chloromethylstyrene was performed followed by azide-functionalization by reacting sodium azide with the chlorine groups. The star polymers were irradiated with UV under dilute conditions to crosslink the azide groups, resulting in monodisperse nanoparticles with a crosslinked shell (Figure 1.29). The authors were able to tailor the nanoparticle size and shell thickness by altering the size of each block and could control the density of shell crosslinking by varying the duration of UV irradiation. Finally, degradation of the poly(caprolactone) core was possible under acidic conditions.²¹⁴

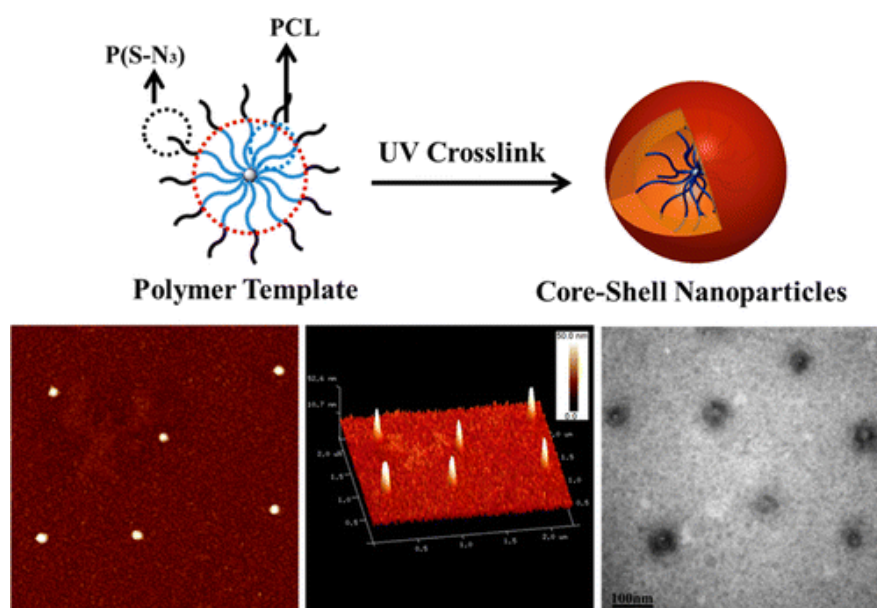


Figure 1.29 Star polymers with degradable inner block and crosslinkable shell were synthesized using ROP and subsequent RAFT polymerization. Crosslinking of outer block and degradation of polyester block resulted in formation of hollow nanoparticles.²¹⁴ Reprinted with permission from *Chemistry of Materials*, 2014, 26, 20, 6058-6067. Copyright 2014 American Chemical Society.

1.6 Objectives

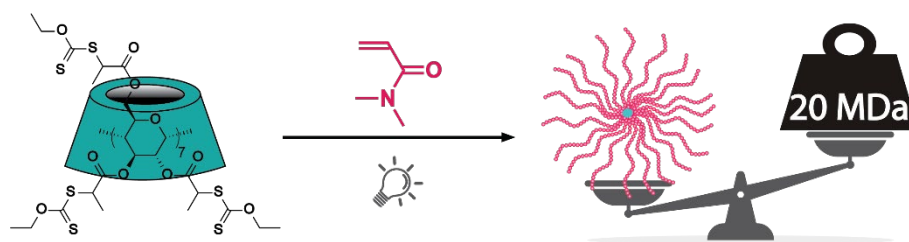
Recent developments in photo-mediated RAFT polymerization have led to polymers with improved dispersities and chain-end fidelity while providing spatiotemporal control. In addition, photo-mediated RAFT polymerization is easily combined with other polymerization techniques to synthesize complex materials. However, studies to date have primarily focused on linear polymers. Thus, the objective of this thesis is to investigate how photo-mediated RAFT polymerization can be applied to the synthesis of star polymers and the potential uses of these star polymers for biomedical applications. To achieve these aims, the following studies were performed:

1. Exploration of photoiniferter RAFT polymerization for the synthesis of star polymers using a core-first approach, leading to the synthesis of UHMW star polymers.
2. Synthesis and study of a novel photocatalyst for PET-RAFT polymerization, which enabled the synthesis of polymers via light irradiation across the visible light spectrum, including linear and star-shaped polymers using NIR irradiation.
3. Synthesis of star polymers *via* core-first NCA ROP and photoiniferter RAFT polymerization to probe the lysosomal escape of dye-loaded star polymers with degradable polypeptide inner block and an anti-fouling PEG corona.

1.7 Thesis Outline

In Chapter 2, the core-first synthesis of star polymers was investigated using photoiniferter RAFT polymerization. Linear polymers synthesized *via* photoiniferter RAFT polymerization have been shown to exhibit exceptional chain-end fidelity, leading to monomer conversions greater than 95% as well as multiblock and ultra-high molecular weight (UHMW) polymers. Herein, we demonstrated the controlled synthesis of poly(dimethylacrylamide) (PDMA) star polymers from tetrafunctional trithiocarbonate (4-TTC) and xanthate (4-XAN) cores under blue and purple light, respectively. Exploiting the highly “living” nature of the xanthate-mediated photoiniferter RAFT polymerization, we were able to synthesize UHMW star polymers. Further experiments with the 4-XAN core resulted in star polymers with molecular weights of 3.8 MDa, equivalent to 0.96 MDa per arm, using both purple and UV light. Finally, a modified β -cyclodextrin core containing 21 RAFT agents was synthesized. A molecular weight of 23 MDa was targeted, with 96% conversion achieved after 18 hours of UV irradiation for a theoretical

molecular weight of 21.9 MDa. This finding was corroborated by static light scattering as well as GPC analysis of the star polymer arms following their cleavage from the core.



- ✓ Ultra-high molecular weight star polymers
- ✓ Photo-mediated - no exogenous initiators or catalysts
- ✓ Highly “living” and well-controlled

Figure 1.30 Chapter 2: Highly living stars *via* core-first photoiniferter RAFT polymerization: exploitation for ultra-high molecular weight star synthesis.

In **Chapter 3**, a novel photocatalyst was reported for PET-RAFT polymerization which enabled photo-mediated polymer synthesis across the light spectrum in aqueous conditions. By employing a PET-RAFT approach, we were able to overcome the wavelength, volume, and degassing limitations of photoiniferter RAFT polymerization. Self-assembly of a carboxylated porphyrin resulted in micrometre scale fibres able to absorb light with wavelengths of 300-950 nm. Using this novel photocatalyst (SA-TCPP), we reported the first broadband- and NIR-mediated CRP in aqueous conditions. Polymers synthesized using white, blue, red, green, and NIR irradiation all showed exquisite control, with excellent agreement between theoretical and experimental molecular weights and dispersities below 1.1. Two sequential chain extension experiments were performed to demonstrate the retention of the RAFT agent, with a complete shift observed during GPC analysis. We also synthesized a star polymer using NIR irradiation, which to our knowledge is the first advanced architecture synthesized using NIR irradiation with any type of CRP, regardless of solvent. The aqueous nature of system allowed us to conduct polymerization in cell culture conditions, where preliminary studies resulted in a PEG-based brush polymer synthesized in the presence of mammalian cells while maintaining 46% cell viability.

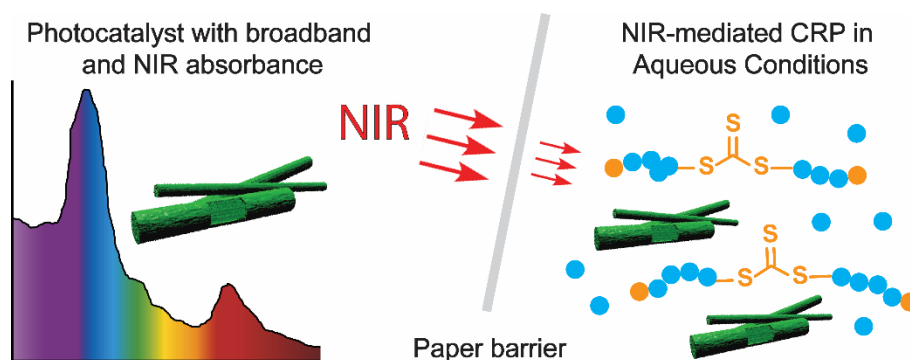


Figure 1.31 Chapter 3: From UV to NIR: a full spectrum metal-free photocatalyst for efficient polymer synthesis in aqueous conditions.

In **Chapter 4**, star polymers were synthesized to investigate a potential mechanism of lysosomal escape by exploiting the protein degradation and amino acid recycling function of lysosomes. Nanoparticles show great promise for cancer therapy, however the release of therapeutics from the endosomal pathway to reach their site of action remains a barrier. We proposed the use of polypeptides can improve lysosomal escape, as degradation of polypeptides and efflux of the resulting amino acids into the cytoplasm is a major function of lysosomes. Photoiniferter RAFT was used in conjunction with NCA ROP to synthesize a degradable, polypeptide-based star, while a nondegradable star was synthesized via photoiniferter RAFT to serve as a control. Both stars were composed of diblock arms, containing an inner polymer block with acid side chains for dye conjugation and an outer poly(PEGA) brush to serve as a protective, anti-fouling corona. Fluorescent microscopy confirmed that dye-loaded nondegradable and degradable star polymers were taken up into the cell. However, only treatment with the polypeptide stars resulting in the fluorescent dye escaping the lysosome. These results suggest the use of polypeptide-based nanoparticles could result in improved drug delivery.

Finally, **Chapter 5** will discuss the future perspectives of this work and offer insight into future applications of these materials and synthetic routes in both academic and industrial settings.

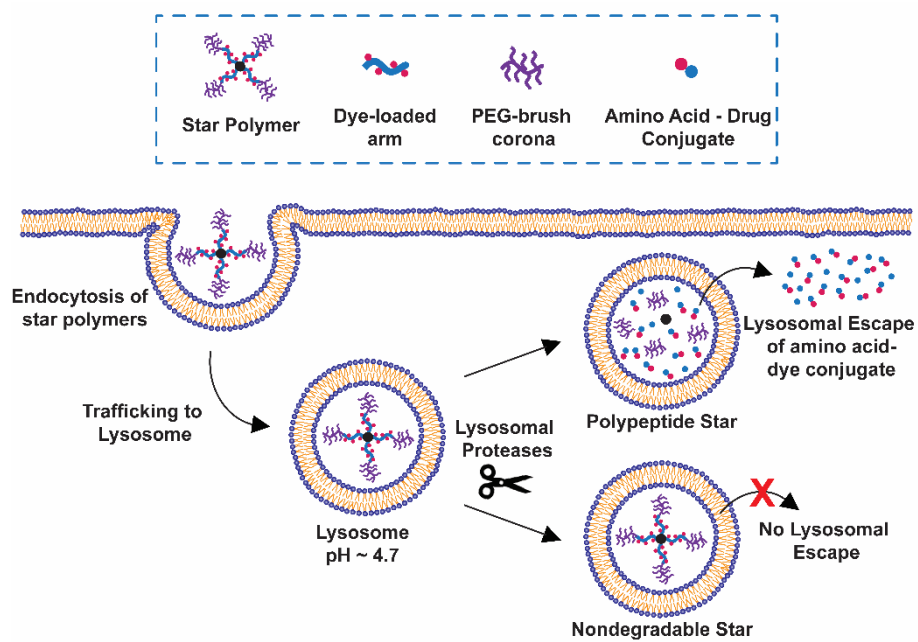


Figure 1.32 Chapter 4: Polypeptide stars for efficient lysosomal escape.

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Chapter 2

Highly Living Stars via Core-First Photo-RAFT Polymerization: Exploitation for Ultra-High Molecular Weight Star Synthesis

2.1 Chapter Perspective

Star polymers are highly functional materials that display unique properties in comparison to linear polymers, making them valuable in a wide range of applications. Currently, ultra-high molecular weight (UHMW) star polymers synthesized using controlled radical polymerization are prone to termination reactions that have undesirable effects, such as star–star coupling. In recent years, photo-mediated reversible addition fragmentation chain transfer (RAFT) polymerization has shown improved chain end fidelity and dispersity when compared to more traditional thermal- and redox-initiated systems. However, the study of photo-mediated RAFT polymerization for advanced architectures, including star polymers, has been limited.

In this chapter, we report the synthesis of the largest star polymers to date using controlled radical techniques via xanthate-mediated photo-RAFT polymerization and a core-first approach. Xanthate-mediated photoiniferter RAFT polymerization enabled the synthesis of well-defined, highly “living” star polymers, even at monomer conversions greater than 95%. The highly living nature of these stars allowed us to synthesize UHMW stars of extremely long arm lengths, with star polymer molecular weights in excess of 20 MDa. The facile synthesis of well-defined UHMW star polymers presented here may provide access to unique star-based polymeric materials that have so far been unattainable.

2.2 Introduction

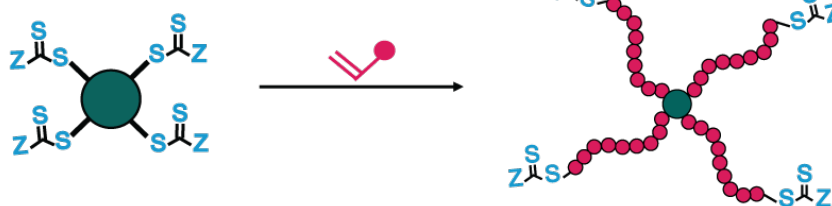
Star polymers are highly functional materials due to their compact, spatially defined, three-dimensional structure that leads to unique physical properties and increased opportunity for functionalization. They are currently used industrially as viscosity modifiers and lubricants, while potential applications in areas as varied as drug nanocarriers and interfacial stabilizers are also being investigated.¹⁻⁵ Star polymers consist of linear arms that radiate from a central core and can be synthesized using a variety of controlled radical polymerization methods. Synthesis via reversible addition–fragmentation chain transfer (RAFT) polymerization has received significant attention and can be performed using either an arm-first or core-first approach.⁶ Arm-first star polymers are synthesized either by conjugation of the linear arms to a multifunctional species or through sequential polymerization with a cross-linker that then forms a gel-like core of the star polymer. The core-first approach requires the use of a multifunctional core from which the arms can be polymerized. Although both arm-first and core-first approaches can lead to well-defined star polymers, only the core-first approach allows for an accurate and predetermined number of arms and facile purification.⁷

Star polymers synthesized via the core-first approach have been synthesized using many different polymerization techniques including atom transfer radical polymerization (ATRP), nitroxide-mediated polymerization (NMP), RAFT polymerization, and ring-opening polymerization (ROP).⁸⁻¹³ The core-first approach via RAFT polymerization is complicated by the asymmetric nature of RAFT agents, which have both a fragmenting (R) and a non-fragmenting (Z) group. The particular synthetic approach is determined by which of these is conjugated to the core (Figure 2.1). The Z-group approach (when the Z-group is covalently conjugated to the core) prevents star–star coupling, a common side reaction when using the R-group approach.^{6,11,14} However, synthesis of high molecular weight star polymers is difficult due to steric hindrance and congestion around the core, as the chain length of the growing arms and/or the number of arms is increased.^{1,6}

Star–star coupling is often observed in reversible-deactivation radical polymerization (RDRP) when a core-first synthetic approach is used, including for RAFT polymerization via the R-group approach. This is due to the location of the propagating radical species at the end of each arm.¹⁵ The R-group approach does have advantages, including the presence of the thiocarbonylthio group at the star periphery, offering additional opportunities for postpolymerization functionalization. Moreover, if star–star coupling could be avoided, then this approach would offer the potential to achieve high molecular weight star polymers with precise arm numbers, which would be challenging for the Z-group or coupling-to techniques.

In recent years, photo-mediated RAFT polymerization (photo-RAFT) has been shown to result in highly “living” polymers.¹⁶⁻¹⁸ As is convention, we use the term “living” here to specifically refer to a high retention of ω -chain end functionality—in our case the thiocarbonylthio moiety—that allows the polymer chain to be reinitiated and to continue chain growth. However, the use of photo-mediated RAFT polymerization has not yet been investigated for the synthesis of star polymers using the core-first approach. We hypothesized that photo-RAFT polymerization would help to minimize termination reactions, enabling us to reach high molecular weights. Additionally, using a photoiniferter-based initiation approach allows us to eliminate exogenous radical initiators which may initiate chains that are not attached to the star core.¹⁹

i) R-Group Approach



ii) Z-Group Approach

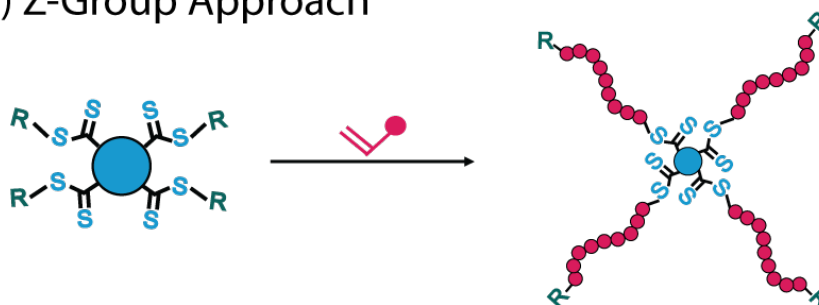


Figure 2.1 Core-first star polymers can be synthesized via RAFT polymerization using the R-group or Z-group approach depending on orientation of the asymmetrical RAFT agent.

2.3 Results and Discussion

2.3.1 Star synthesis via photoiniferter RAFT polymerization

In order to study photoiniferter RAFT star polymerization using a core-first approach, tetrafunctional xanthate (4-XAN) and trithiocarbonate (4-TTC) cores were synthesized. The absorbance spectra of 4-XAN and 4-TTC were measured in DMSO to determine the most appropriate wavelengths of visible light for photoiniferter RAFT polymerization. As shown in Figure 2.2, 4-XAN absorbance was limited to wavelengths of 420 nm and lower, while the absorbance spectrum of 4-TTC stretched to over 500 nm. Based on these results, polymerization with xanthate and TTC cores were carried out using purple and blue light, respectively.

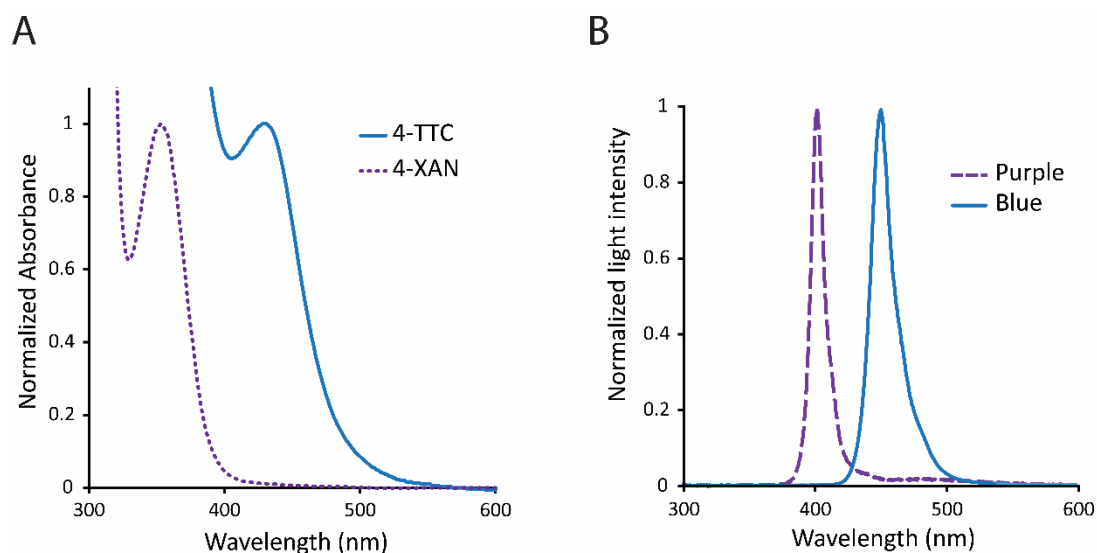
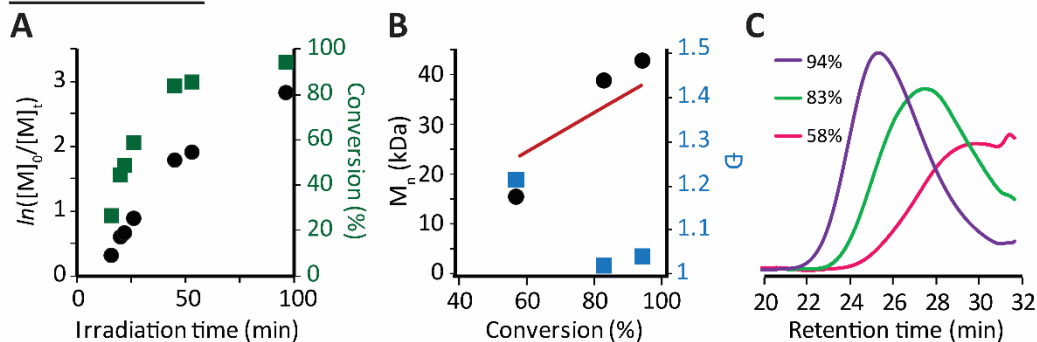


Figure 2.2 (A) Absorbance spectra of 4-TTC and 4-XAN from UV/Vis spectroscopy. (B) Emission spectra of the purple and blue LED sources and used for xanthate- and trithiocarbonate-functionalized cores respectively. The maximum emission was detected at 401 nm for the purple LED and 450 nm for the blue LED.

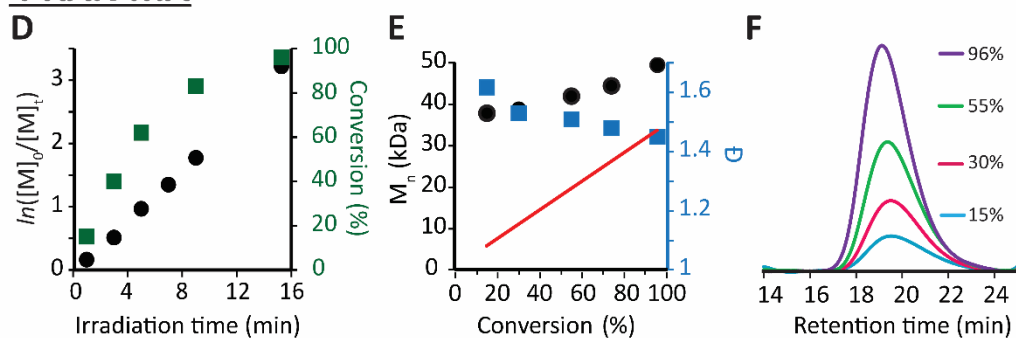
The polymerization of dimethylacrylamide (DMA) was investigated using the 4-XAN core under purple light (Figure 2.3A). A target degree of polymerization (DP) of 100 monomer units per arm was used, with polymerization kinetics shown in Figures 2.3B and 2.3C. The rate of polymerization was rapid, with 95% conversion obtained in less than 3 h of irradiation. Near-linear pseudo-first-order kinetics were observed until 95% conversion, indicating a constant radical concentration and suggesting high chain-end fidelity. The dispersity remained low throughout the polymerization, and the theoretical molecular weight (determined from NMR conversion) (Figure 2.3C red line) was similar to the molecular weight determined using GPC paired with multi-angle light scattering.

opportunity for termination. The 4-TTC core led to PMA stars with improved dispersities and molecular weights that matched theoretical molecular weights; however, a high molecular weight shoulder was observed, indicating star–star coupling had occurred (Figure 2.4I).

4-TTC DMA



4-XAN MA



4-TTC MA

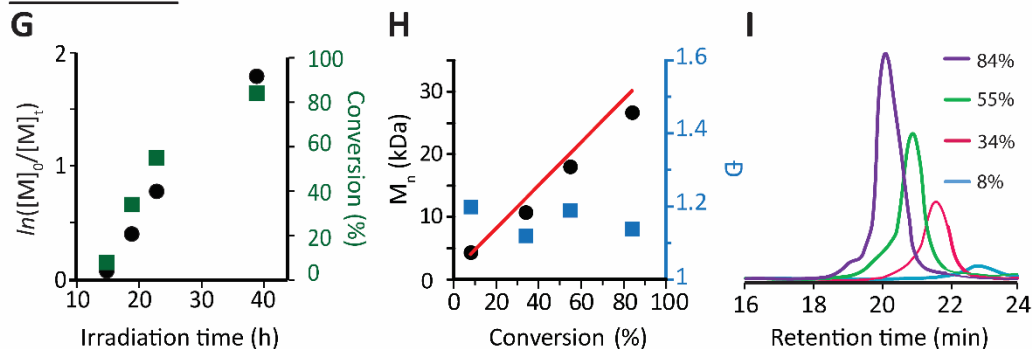


Figure 2.4 Polymerization of DMA from 4-TTCp and MA from 4-XAN and 4-TTCb. Reactions mixtures were irradiated with blue and purple light when using 4-TTC and 4-XAN cores, respectively. Kinetic studies were completed and conversion was determined using ^1H NMR spectroscopy in deuterated chloroform (A, D, G). The M_n (●) and dispersity (■) versus conversion following visible light irradiation (B, E, H). Theoretical molecular weights ($M_{n,theo}$) were determined from monomer conversion from ^1H NMR spectroscopy. The theoretical M_n ($M_{n,theo}$) versus conversion is shown as a solid red line. Molecular weight from GPC (M_n) and dispersity were measured using poly(styrene) standards. GPC traces of the star polymers with increasing conversion (C, F, I).

Using the described photo-RAFT polymerization approach with the tetrafunctional xanthate 4-XAN, we were able to synthesize a range of well-defined star polymers with differing chain lengths per arm ($DP_{n,arm} = 100, 200, 500, \text{ and } 1000$) (Figure 2.5A, Tables 2.4 and 2.5). We further investigated the livingness of the star polymers synthesized from 4-XAN through *in situ* chain extension experiments, with a targeted degree of polymerization (DP) of 100 for the first block and chain extension of DP 400 (Figure 2.5B, Table 2.5). The GPC trace remained monomodal after chain extension, and the near-complete shift to lower retention times demonstrates the highly living property of the xanthate initiator.

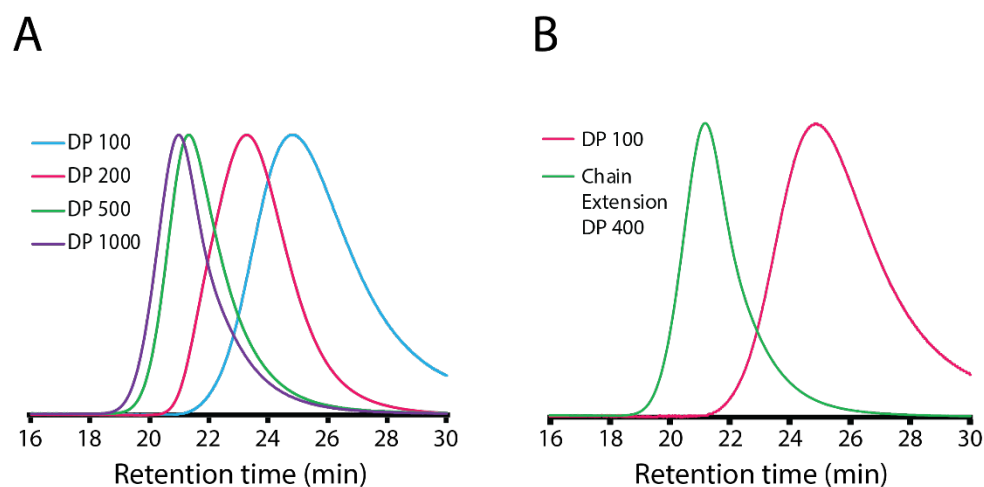


Figure 2.5. PDMA star polymers synthesized from the 4-XAN core via photo-RAFT polymerization under visible light are highly living. (A) GPC refractive index (RI) trace of star polymers synthesized with 4-XAN under purple light with target DPs of 200, 500, and 1000 per arm. (B) GPC trace of diblock polymer synthesized with 4-XAN under purple light with DMA DP 100 per arm for the first block and DMA DP 400 for the second block.

2.3.2 Synthesis of UHMW star polymers

Due to previously mentioned issues with core-first star polymer synthesis, star polymers with high MW arms and precise arm numbers have long been considered out of reach. However, inspired by the livingness observed in our experiments and recent work from the Sumerlin group that used a similar xanthate photo-RAFT polymerization method to synthesize ultra-high molecular weight (UHMW) linear polymers, we decided to attempt the synthesis of UHMW star polymers.²⁰

The 4-XAN core was used to polymerize DMA with a target DP of 10 100 per arm, the equivalent of 1 MDa per arm, using both purple and UV light irradiation. The use of UV was

employed to increase the rate of polymerization, enabling reactions to reach 96% conversion in 6 h, while irradiation with purple light required 102 h to reach the same conversion. The corresponding theoretical molecular weights were 0.96 MDa per arm with a total molecular weight of 3.8 MDa (Figure 2.6A and Table 2.1). GPC traces of 4-XAN DP 10 100 stars initiated with purple light are shown in Figure 2.6B at 25, 43, and 96% conversion, while the trace of the UV-initiated star is shown in black at 96% conversion. The UV-initiated star trace appears slightly broader than the purple-light-initiated star. This increased dispersity may result from direct activation of monomer by UV irradiation resulting in a less controlled polymerization than observed with purple light irradiation, where direct activation of the monomer does not occur.

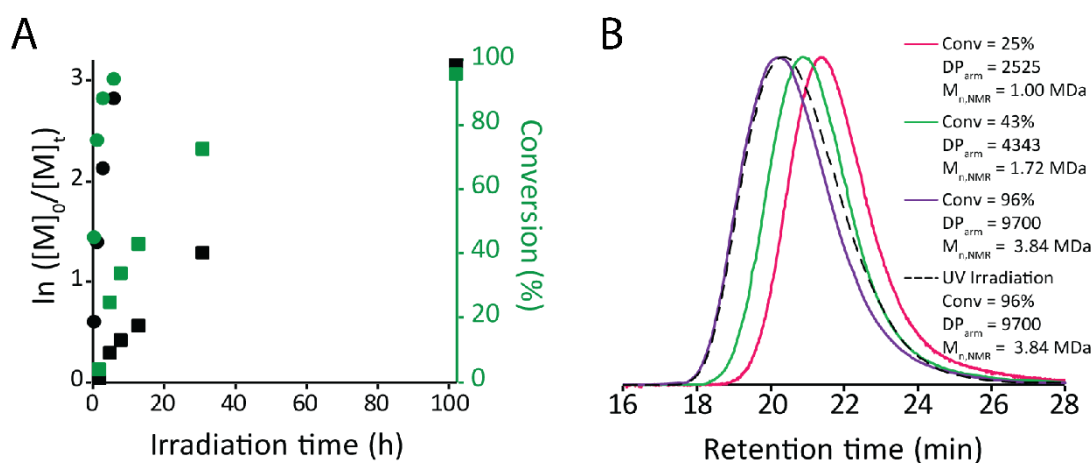


Figure 2.6. Synthesis of 4-arm UHMW star polymers. (A) kinetic study of star polymerization using purple (squares) and UV (circles) light. (B) GPC traces of 4-XAN DP 10 100 irradiated with purple light at 25, 43, and 96% conversion and UV at 96% conversion.

Due to the ultra-high molecular weights of the polymers synthesized—beyond the limits of our GPC-coupled light-scattering detector—additional methods of characterization were investigated. Static light scattering (SLS) requires the use of a laser whose light is scattered upon interaction with a sample and has been previously employed to determine the absolute molecular weight of star polymers.²³ A range of accurately determined concentrations are required to successfully carry out SLS measurements, with the intensity of the scattered light measured at multiple angles. A Zimm plot is generated that can provide the weight average MW and the radius of gyration (although not MW dispersity) (Figures 2.7C and 2.14). Therefore, using the assumption that our polymers were monomodally distributed, SLS indicated that the molecular weights of the 4-XAN DP 10 100 stars were a close match to their theoretical values, with observed MWs of 2.5 and 3.8 MDa for the UV and purple light, respectively (Figure 2.7 and Table 2.1).

To further study the limits of this photo-mediated star polymerization, β -cyclodextrin was modified so that each of its 21 hydroxyl groups was functionalized with a xanthate, resulting in a 21-xanthate core (21-XAN) (Figure 2.13). Star polymerization of DMA from the 21-XAN core with DPs 50, 200, and 500 per arm resulted in well-defined stars with monomodal, symmetrical GPC traces (Figure 2.7A). A star polymer with a per arm target DP of 1900 (target molecular weight of 4 MDa) was synthesized, and conversion of 95% was observed following 3.5 h of UV irradiation (Figure 2.7B). Once again, the theoretical M_n of 3.8 MDa and the experimental MW of 5.3 MDa from SLS were in relatively close agreement.

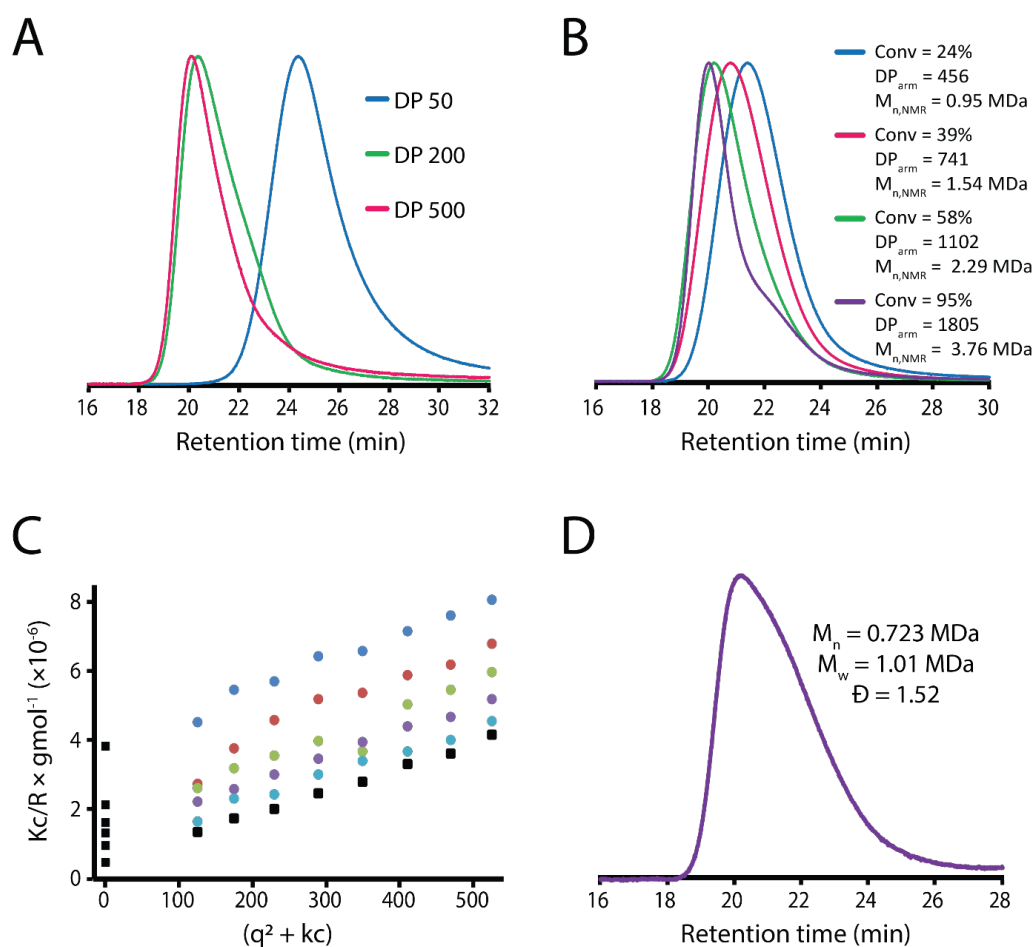


Figure 2.7. PDMA star polymers with 21 arms. GPC traces for star polymers with (A) DP 50, 200, and 500 per arm and (B) DP 1900 per arm at 24, 39, 58, and 95% conversion. (C) Zimm plot of 21-XAN DP 11 000 generated from SLS ($M_{w,(SLS)}$ 20.4 MDa, $M_{n,(theo)}$ 21.9 MDa). (D) GPC trace of PDMA arms cleaved from 21-XAN DP 11 000 after 16 h of stirring in 5 M HCl.

The largest molecular weight star polymer was synthesized using the 21-XAN core and a DP of 11 000 per arm. NMR indicated 96% conversion after 18 h of UV irradiation, corresponding to a total molecular weight of 21.9 MDa. NMR and SLS results were in agreement with

the $M_{w,(SLS)}$ equal to 20.4 MDa. Additionally, the arms were cleaved from the core via acid-catalyzed hydrolysis and resulted in polymers with a M_n of 723 kDa (expected arm M_n was ca. 1043 kDa) and dispersity of 1.5 (Figure 2.5D), indicating a relatively high initiation efficiency from the core. To our knowledge, 20.4 MDa is the largest molecular weight reported for a star polymer synthesized via any RDRP.

There are few rheological studies of star polymers synthesized using RDRP techniques and due to the unprecedented size of our star polymers, the rheological properties of stars with arm molecular weights in the range of 1 MDa have not been reported. In general, star polymers are known to have lower solution viscosities than analogous linear polymers of the same molecular weight.¹ A previous study by the Qiao group has shown the scaling rate of intrinsic viscosity is much lower for star polymers than linear polymers.²⁴ In addition, arm molecular weight plays an important role; star polymers with longer arms have higher shear and relative viscosities than star polymers with the same total molecular weight but lower arm molecular weight. It is important to note that the long arms in this study still only had a molecular weight of 46 kDa.²⁴ Therefore, we plan to pursue rheological studies on our UHMW star polymers. Despite their high total and arm molecular weights, all stars reported in this study were easily dissolved in water at concentrations of 2 mg mL⁻¹ for SLS analysis.

2.3.3 Limits of UHMW star polymerization

A range of polymers were synthesized to determine the limits of polymerization, in terms of both degree of polymerization and molecular weight (Table 2.1). The DP per arm was increased for both the 4-XAN and 21-XAN cores until theoretical and experiment molecular weights disagreed by more than 50%. The maximum successful degree of polymerization was obtained using the 4-XAN core with a target DP of 20 200, resulting in a star polymer with a $M_{n,(theo)}$ of 7.5 MDa and a $M_{w,(SLS)}$ of 6.5 MDa. Polymerizations from 4-XAN with a DP of 50 000 per arm achieved high conversion and theoretical molecular weights of nearly 18 MDa. However, SLS demonstrated the resulting polymers had molecular weights of 2.9 and 2.5 MDa when using purple and UV light, respectively. We hypothesize this could be due to the shear stress of stirring, causing star arms to be broken from the core. Control experiments without a RAFT agent showed no conversion following purple light irradiation, supporting that initiation was only occurring from the star core. The Matyjaszewski group has previously reported the cleavage of arms from a brush-arm star polymer core, which they hypothesized was a result of congestion and the highly strained architecture of the star polymer core.²⁵

Table 2.1. UHMW PDMA star polymers synthesized with the largest known star polymer synthesized to date via RDRP (21 arms, DP 11 000 per arm) highlighted in dark purple.

Number of arms	DP per arm	Target MW (MDa)	$[M]_0$ (M)	Time (h)	Conversion (%) ^a	$M_{n(\text{theo})}$ (MDa) ^b	$M_{w(\text{SLS})}$ (MDa) ^c	R_G (nm) ^c
4	10 100	4	1	6	96	3.8	2.5	71.1
4 ^c	10 100	4	1	102	96	3.8	3.8	74.7
4	20 200	8	3	6	94	7.5	6.3	124.1
21	1 900	4	1	3.5	95	3.8	4.6	105.7
21	11 000	23	0.5	18	96	21.9	20.4	205.1
21	22 000	46	0.5	18	94	43.3	3.2	79.6

^a Conversion was determined using ¹H NMR spectroscopy.

^b Theoretical molecular weights ($M_{n(\text{theo})}$) were determined via monomer conversion values.

^c $M_{w(\text{SLS})}$ and R_G were determined by static light scattering.

^d This polymerization was performed using purple light irradiation, while all others were irradiated with ultraviolet light. Polymers where experimental and theoretical results were in agreement are highlighted in purple. The largest reported RDRP star polymer, with $M_{w(\text{SLS})}$ of 20.4 MDa, is highlighted in dark purple and bold text.

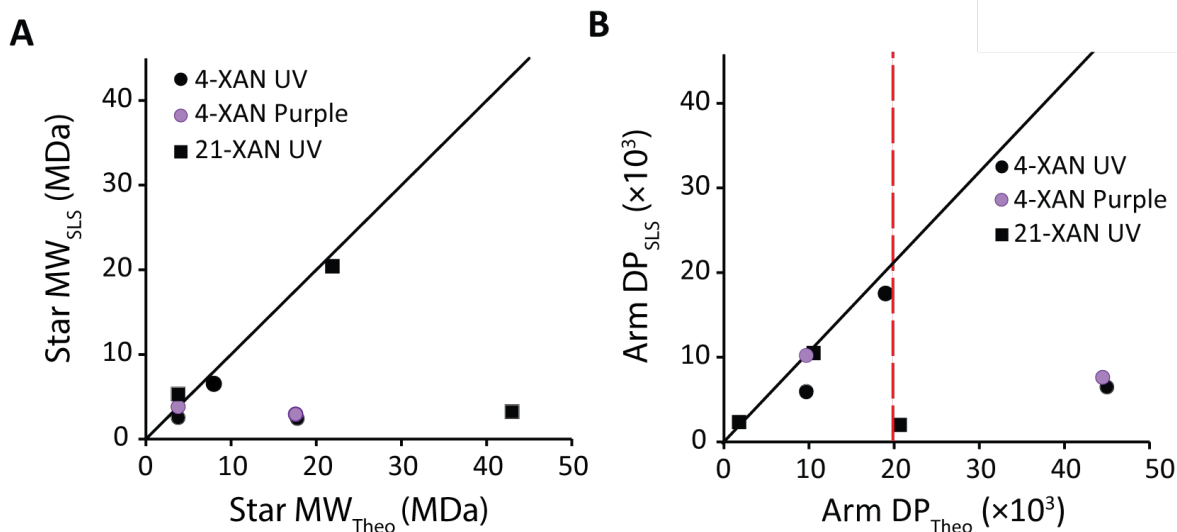


Figure 2.8 Investigation of the limit of core-first star polymer synthesis in terms of (A) total molecular weight and (B) degree of polymerization per arm. The solid black line shows where theoretical and observed values are equivalent.

To determine the limit in terms of total molecular weight, we attempted to synthesize a star polymer from the 21-XAN core with a target DP of 22 000 per arm. Following 18 h of UV irradiation, monomer conversion was 94% for a theoretical MW of 43.3 MDa, yet SLS analysis revealed an apparent experimental molecular weight of 3.2 MDa. We propose that this discrepancy is due to the extreme dilution of the photoactive (xanthate) species, leading to background polymerization. Control experiments demonstrated free radical polymerization of DMA in DMSO with UV irradiation, explaining how high conversion could be achieved, while a much lower than expected MW was observed. These observations indicate that it is more likely an effect of the arm chain length rather than the overall molecular weight that results in these synthetic limitations, as can be seen in Figure 2.8.

2.4 Conclusions

In this chapter, we report the synthesis of well-defined, monomodal UHMW star polymers via photo-mediated RAFT polymerization to conversions of over 95%. The highly “living” nature of these star polymers enabled the synthesis of UHMW stars of extremely long arm lengths, including the largest star polymer synthesized to date using RDRP techniques. The facile synthesis of well-defined UHMW star polymers may provide access to unique star-based polymeric materials such as gels and films that were previously unattainable. Future works will also include rheological studies of these unprecedented star polymers.

2.5 Experimental Section

2.5.1 Materials

Monomers methyl acrylate (MA, 99%) and *N,N'*-dimethylacrylamide (DMA, 99%) were purchased from Sigma and passed through a column of basic alumina to remove inhibitors. Potassium ethyl xanthate, β -cyclodextrin, 1,2,4,5-tetrakis(bromomethyl)benzene, and 2-bromopropionyl bromide were purchased from Sigma and used as received. Dimethyl sulfoxide (DMSO, 99%, Chem-Supply) and deuterated chloroform (CDCl_3 , 99.5%, Cambridge Isotope Laboratories) were used as received. A Beaufly Nail Lamp was used as the UV light source (36 W, $\lambda_{\text{max}} = 365 \text{ nm}$). The purple photoreactor was made using commercial strip lighting (60 LEDs/m 12V (DC) 5 m total strip length, working distance of approximately 5 cm) wrapped around the inside of a cardboard cylinder. The LED light emission spectrum ($\lambda_{\text{max}} = 401 \text{ nm}$) was measured using a Maya 2000Pro spectrometer fitted with optical fibre (OceanOptics 100UV).

2.5.2 Characterization

2.5.2.1 Nuclear Magnetic Resonance (NMR) Spectroscopy

^1H spectroscopic analysis was conducted using a Varian Unity Plus 400 MHz spectrometer operating at 400 MHz, with deuterated chloroform used as a reference. All samples were analysed at a concentration of approximately 10 mg mL^{-1} .

2.5.2.2 Gel Permeation Chromatography (GPC)

GPC characterization of poly(MA) (PMA) star polymers was carried out using tetrahydrofuran as eluent. GPC analysis was performed at 50°C using a modular Shimadzu system comprised of a Shimadzu RID-10A interferometric refractometer ($\lambda = 633 \text{ nm}$) using three Agilent MIXED-C columns ($5 \mu\text{m}$ bead size) operating at 45°C .

GPC characterisation of poly(DMA) (PDMA) star polymers was carried out using Milli-Q water containing 0.1% (v/v) trifluoroacetic acid as eluent at a flow rate of 1 mL/min . GPC analysis was performed at 25°C using a modular Shimadzu system comprised of a Shimadzu RID-10A interferometric refractometer ($\lambda = 633 \text{ nm}$), Wyatt 3-angle light scattering detector, and three Waters Ultrahydrogel columns in series ((i) $250 \text{ \AA}$ porosity, $6 \mu\text{m}$ bead size; (ii) and (iii) linear, $10 \mu\text{m}$ bead size). All samples were filtered through a $0.45 \mu\text{m}$ nylon filter prior to injection. The dn/dc value of PDMA star polymers were measured using batch mode.

2.5.2.3 Static Light Scattering (SLS)

SLS analysis was used to determine the weight average molecular weight ($M_{w, SLS}$) of UHMW star polymers. Analysis was carried out using an ALV-5022F light scattering spectrometer. Samples were illuminated with a HeNe laser ($\lambda = 622$ nm), and measurements were made at up to five concentrations as a function of scattering angle. Scattering data was analyzed at 30-150° in 10° increments with star polymer concentrations ranging from 0.1-2 mg/mL. Measurements were the average of 3 repeats for 30s at each angle. Zimm plots were generated using ALV Zimm Analysis software. Samples were dissolved in DI water filtered through a 0.45 μ m nylon filter and mixed for a minimum of 72h prior to SLS analysis. Samples were filtered immediately before analysis to ensure aggregates were not present. The previously determined dn/dc values determined by GPC was used for the PDMA star polymers.

2.5.3 Methods

2.5.3.1 4-arm trithiocarbonate initiator (benzene core) (4-TTCb)

Sodium hydroxide (0.474 g, 11.85 mmol) was dissolved in Milli-Q water (35 mL) and the solution was added to 1-dodecanethiol (2 g, 9.88 mmol). Carbon disulfide (1.19 mL, 19.76 mmol) was added dropwise to the mixture and stirred at room temperature for 3 h until the solution colour turned to orange. Then, 1,2,4,5-tetrakis(bromomethyl)benzene (0.88 g, 1.96 mmol) dissolved in acetone (30 mL) and was added dropwise to the reaction mixture and the solution stirred at room temperature for 24 h. By the end of the reaction, the solution colour became yellow. The organic solvent was removed under reduced pressure and the product was extracted with DCM (50 mL). The organic phase was washed with 0.5 M HCl solution (40 mL $\times$ 1), deionized water (40 mL $\times$ 1), brine (40 mL $\times$ 1), and dried over $MgSO_4$. DCM was removed via rotary evaporation. The product was recrystallized in methanol:ethanol:DCM (4.5:4.5:1, 50 mL) overnight and filtered to give yellow crystals (2.126 g, 86.72 % yield). NMR 400 MHz, 1H NMR ($CDCl_3$); 7.34 (2H, s, 2 $\times$ CH), 4.58 (8H, s, 4 $\times$ CH₂), 3.34 (8H, t, $J=7.4$ Hz, 4 $\times$ CH₂), 1.73-1.63 (8H, m, 4 $\times$ CH₃), 1.42-1.34 (8H, m, 4 $\times$ CH₂), 1.31-1.18 (64H, m, 32 $\times$ CH₂), 0.86 (12H, t, $J=6.8$ Hz, 4 $\times$ CH₃) (Figure S1). Calculated mass: 1238.53, observed mass: 1239.53 (M+H). 1H NMR Spectrum is shown in Figure 2.9.

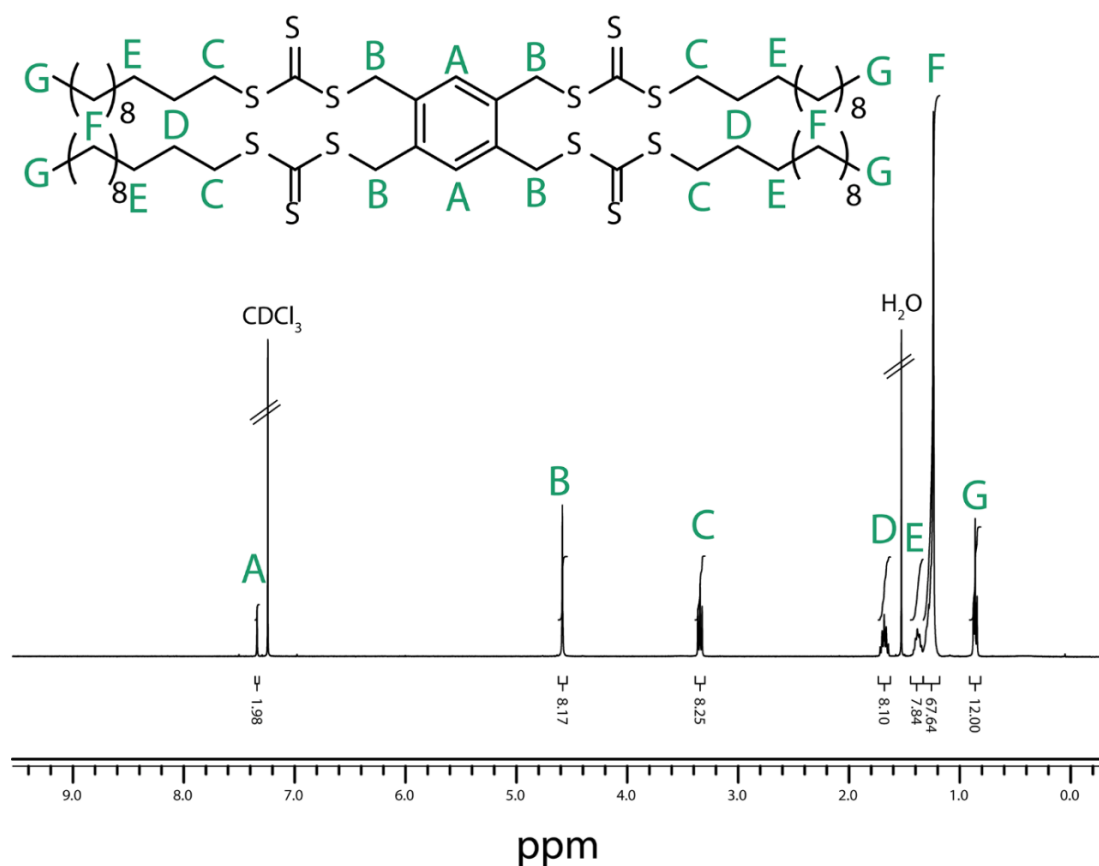


Figure 2.9. ^1H NMR spectrum of 4-arm trithiocarbonate initiator with benzene core in deuterated chloroform.

2.5.3.2 4-arm trithiocarbonate initiator (pentaerythritol core) (4-TTCp)

2-(((butylthio)carbonothioyl)thio)-propanoic acid (4 g, 16.8 mmol), pentaerythritol (0.48 g, 3.5 mmol), and 4-dimethylaminopyridine (DMAP, 0.085 g, 0.7 mmol) were dissolved in 100 mL anhydrous DCM. The reaction mixture was purged with nitrogen and cooled to 0 °C for 20 min. N, N'-dicyclohexylcarbodiimide (DCC, 3.17 g, 15.4 mmol) was dissolved in 50 mL anhydrous DCM and was added dropwise to the reaction mixture over 15 min. The reaction mixture was mixed under nitrogen at room temperature for 48 h. The precipitate was filtered off and the organic solvent was removed under reduced pressure. The product was purified using column chromatography on silica gel (hexane: ethyl acetate 9.5:0.5) to give a yellow oil (0.5 g, 14.1 % yield). ^1H NMR Spectrum is shown in Figure 2.10.

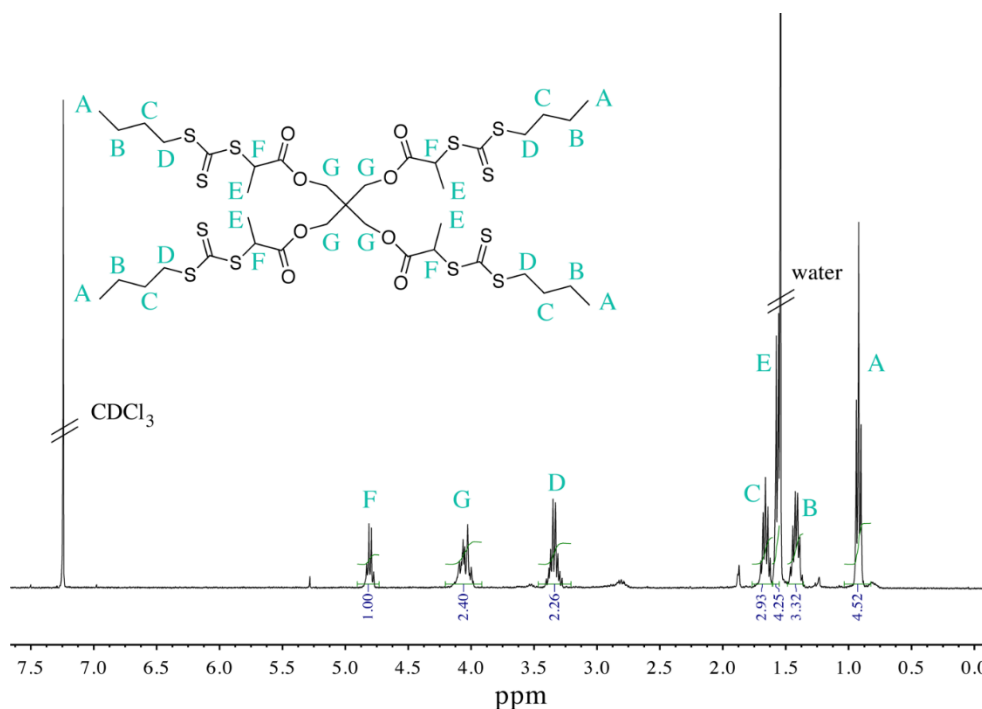


Figure 2.10 ^1H NMR spectrum of 4-arm trithiocarbonate initiator with pentaerythritol core in deuterated chloroform.

2.5.3.3 4-arm xanthate initiator (4-XAN)

Potassium ethyl xanthogenate (2.68 g, 16.7 mmol) was added to a solution of 1,2,4,5-tetrakis(bromomethyl)benzene (1.5 g, 3.33 mmol) in acetone (30 mL) and stirred at room temperature for 20 h. The precipitate was filtered off and the organic solvent was removed under reduced pressure. The crude was dissolved in dichloromethane (DCM, 50 mL) and washed with water (20 mL $\times$ 3), brine (20 mL $\times$ 1), and dried over MgSO_4 . After solvent removal, the product was purified using column chromatography on silica gel (hexane: ethyl acetate 9:1) to give a light yellow solid (1.27 g, 62.13 % yield). NMR 400 MHz, ^1H NMR (CDCl_3); 7.37 (2H, s, $2\times\text{CH}$), 4.65 (8H, q, $J=7.1$ Hz, $4\times\text{CH}_2$), 4.39 (8H, s, $4\times\text{CH}_2$), 1.41 (12H, t, $J=7.1$ Hz, $4\times\text{CH}_3$) (Figure 2.11).

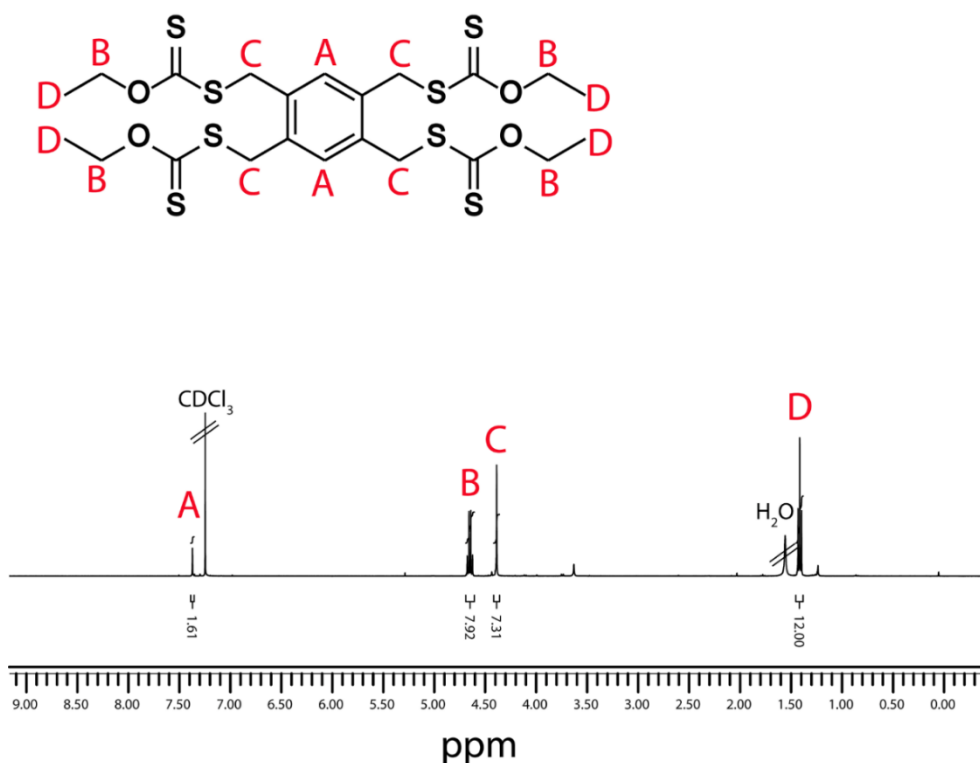


Figure 2.11 ^1H NMR spectrum of 4-arm xanthate initiator in deuterated chloroform.

2.5.3.4 21-arm xanthate initiator (21-XAN)

β -Cyclodextrin (β -CD, 2.2 g, 1.94 mmol, vacuum dried at 50 °C for 48 h) was dissolved in anhydrous 1-methyl-2-pyrrolidone (NMP, 20 mL) and was cooled to 0 °C. 2-Bromopropionyl bromide (26.4 g, 122.2 mmol) was dissolved in anhydrous NMP (10 mL) at 0 °C and added dropwise to the β -CD solution. The reaction was allowed to react at 0 °C for 2 h and at room temperature for 18 h. The reaction mixture was concentrated by removing the solvent under reduced pressure and then diluted with DCM (60 mL) and washed with saturated NaHCO_3 (60 mL $\times$ 2), deionized water (60 mL $\times$ 2), and dried over MgSO_4 . DCM was removed via rotary evaporation. Then, the mixture was purified by column chromatography on silica gel (hexane: ethyl acetate 6.5:3.5) to obtain a white solid (3.4 g, 34.05 % yield). ^1H NMR spectrum is shown in Figure 2.12.

Potassium ethyl xanthogenate (0.56 g, 3.49 mmol) was added to the product of previous step (0.6 g, 0.1511 mmol) in acetone (12 mL) and stirred at room temperature for 21 h. The precipitate was filtered off and the organic solvent was removed under reduced pressure. The reaction mixture was dissolved in ethyl acetate (50 mL) and washed with water (50 mL $\times$ 3) and dried over MgSO_4 . The solvent was evaporated and the product was purified using column chromatography

on silica gel (hexane: ethyl acetate 6.5:3.5) to give a light yellow solid (0.5 g, 69.58 % yield). ^1H NMR Spectrum is shown in Figure 2.13.

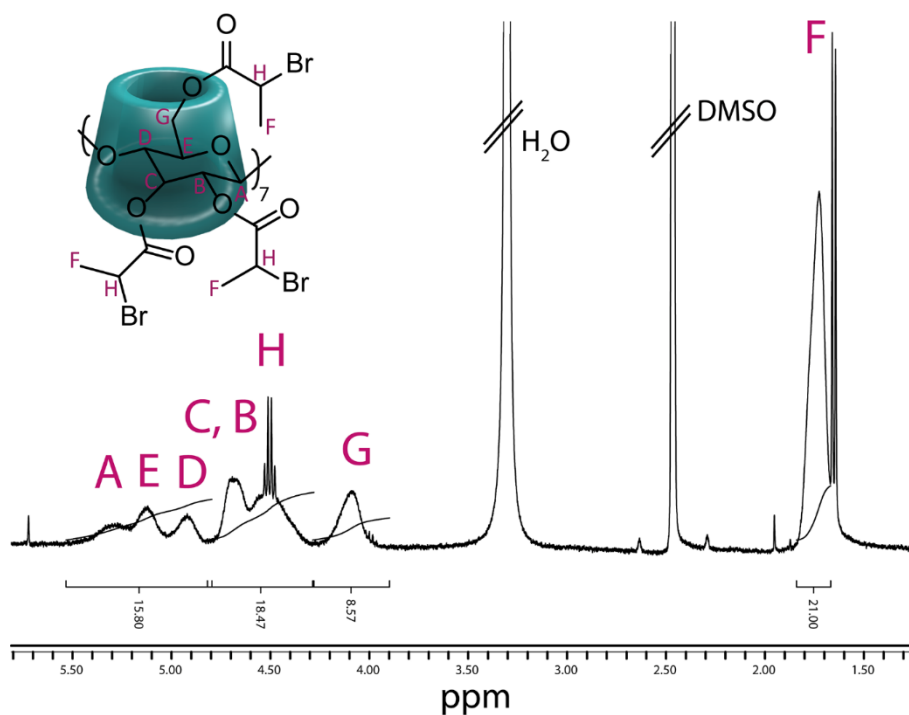


Figure 2.12 ^1H NMR spectrum of 21-arm bromo-functionalized β -cyclodextrin in deuterated DMSO.

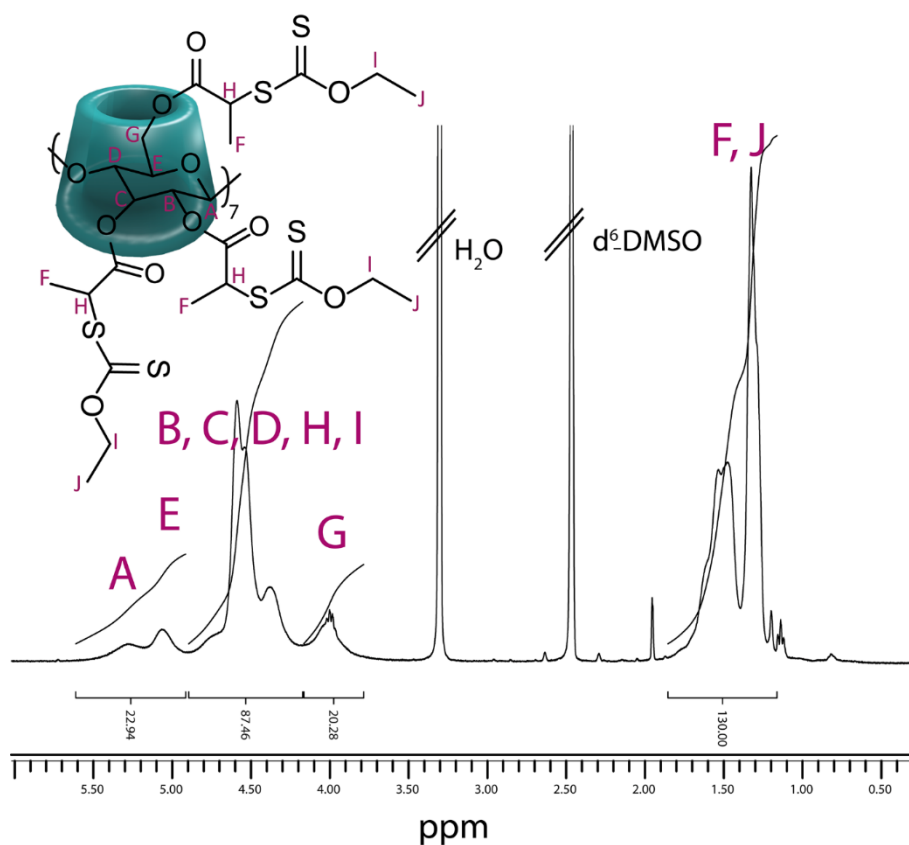


Figure 2.13 ^1H NMR spectrum of 21-arm xanthate (21-XAN) initiator in deuterated DMSO.

2.5.3.5 Synthesis of UHMW star polymer

In a representative experiment, DMA (0.33g, 3.3 mmol), multifunctional core 4-XAN (0.05 mg, 8.14×10^{-5} mmol), and dimethylsulfoxide (DMSO) (5.8 mL) were added to a Schlenk flask and degassed by 3 freeze-pump-thaw cycles, then back-filled with ultra-high purity argon. The flask was placed in a photoreactor and left to stir under light irradiation. At pre-determined times, samples were taken via degassed syringe and dissolved in CDCl_3 and water for NMR and GPC analysis, respectively.

2.6 Additional Information

Table 2.2 Polymerization kinetics of DMA DP 100 from 4-TTCp in DMSO initiated by purple LED irradiation. ^aConversion was determined using ¹H NMR spectroscopy in deuterated chloroform. ^bTheoretical molecular weights ($M_{n, \text{theo}}$) were determined from monomer conversion from ¹H NMR spectroscopy. ^cMolecular weight from GPC ($M_{n, \text{GPC}}$) and dispersity were measured using light scattering with $dn/dc = 0.190$.

Time (h)	Conversion (%)^a	$M_{n, \text{theo}}$ (kDa)^b	$M_{n, \text{GPC}}$ (kDa)^c	$\bar{D}$
26	58	24.02	15.7	1.21
45	83	33.93	39.45	1.02
96	94	38.3	43.56	1.04

Table 2.3 Polymerization kinetics of DMA DP 100 from 4-XAN in DMSO initiated by purple LED irradiation. ^aConversion was determined using ¹H NMR spectroscopy in deuterated chloroform. ^bTheoretical molecular weights ($M_{n, \text{theo}}$) were determined from monomer conversion from ¹H NMR spectroscopy. ^cMolecular weight from GPC ($M_{n, \text{GPC}}$) and dispersity were measured using light scattering with $dn/dc = 0.190$.

Time (min)	Conversion (%)^a	$M_{n, \text{theo}}$ (kDa)^b	$M_{n, \text{GPC}}$ (kDa)^c	$\bar{D}$
15	25	10.53	14.08	1.06
25	45	18.46	23.1	1.08
70	63	25.59	27.9	1.14
100	86	34.71	29.33	1.12
160	94	37.89	28.06	1.14

Table 2.4 4-XAN star polymerization of DMA in DMSO initiated by purple LED irradiation.

^aConversion was determined using ¹H NMR spectroscopy in deuterated solvent. ^bTheoretical molecular weights ($M_{n, \text{theo}}$) were determined from monomer conversion from ¹H NMR spectroscopy. ^cMolecular weight from GPC ($M_{n, \text{GPC}}$) and dispersity were measured using light scattering with $dn/dc = 0.190$.

DP	Conversion (%)^a	$M_{n, \text{theo}}$ (kDa)^b	$M_{n, \text{GPC}}$ (kDa)^c	Đ
200	96	76.9	76.0	1.12
500	95	188.8	215.8	1.14
1000	93	367.8	276.6	1.19

Table 2.5 4-XAN star polymerization of DMA in DMSO initiated by purple LED irradiation.

^aConversion was determined using ¹H NMR spectroscopy in deuterated solvent. ^bTheoretical molecular weights ($M_{n, \text{theo}}$) were determined from monomer conversion from ¹H NMR spectroscopy. ^cMolecular weight from GPC ($M_{n, \text{GPC}}$) and dispersity were measured using light scattering with $dn/dc = 0.190$.

Sample	DP	Conversion (%)^a	$M_{n, \text{theo}}$ (kDa)^b	$M_{n, \text{GPC}}$ (kDa)^c	Đ
Block 1	100	97	39.20	46	1.11
Chain extension	400	94	188.77	267.7	1.18

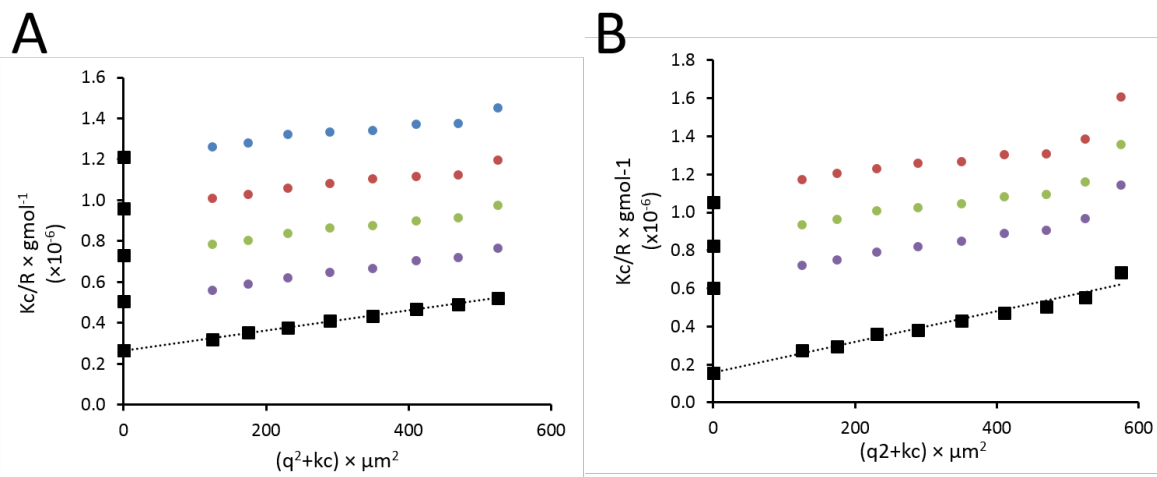


Figure 2.14. Representative Zimm plots generated from SLS analysis. (A) 4-XAN DP 100, irradiated with purple light for 102 h. $M_{n,\text{theo}}$ 3.8 MDa, $M_{W,\text{SLS}}$ 3.8 MDa. (B) 4-XAN DP 20 200, irradiated with UV light for 6 h. $M_{n,\text{theo}}$ 7.5 MDa, $M_{W,\text{SLS}}$ 6.3 MDa.

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Chapter 3

From UV to NIR: A full spectrum metal-free photocatalyst for efficient polymer synthesis in aqueous conditions

3.1 Chapter Perspective

Photo-mediation offers unparalleled spatiotemporal control over controlled radical polymerizations (CRP), with polymerizations mediated by broadband and near-infrared (NIR) light of particular interest due to their potential use in solar-driven and *in vivo* applications. One such technique, photo-induced electron/energy transfer reversible addition-fragmentation chain transfer (PET-RAFT) polymerization, is particularly versatile due to its oxygen tolerance and wide range of compatible photocatalysts. However, NIR-mediated CRP is currently limited to organic solvents as well as linear architectures.

In this work, we report for the first time an aqueous broadband- and NIR-mediated CRP. A novel metal-free, porphyrin-based, organic photocatalyst (SA-TCPP) with an absorbance spectrum covering 300-950 nm enabled well-controlled broadband- and NIR-mediated PET-RAFT polymerization in water. SA-TCPP was compared to three other photocatalysts previously reported for hydrogen evolution and was found to have the highest rate of polymerization when irradiated with white, blue, green, and red light. Using SA-TCPP in conjunction with the 21-XAN core from Chapter 3, we were able to synthesize the first reported star polymer under NIR irradiation. Preliminary investigations with SA-TCPP resulted in the successful microlitre-scale polymerization of poly(ethylene glycol) methyl ether methacrylate in the presence of mammalian cells in complete cell culture medium. These findings have broad implications for “green” polymerizations, where solar-driven reactions in environmentally friendly solvents such as water are highly desirable. Because NIR can penetrate barriers including human tissue, this work also marks a crucial advance towards *in vivo* polymerization for biomedical applications.

3.2 Introduction

Photo-mediation enables spatiotemporal control of controlled/living radical polymerizations (CRP) while maintaining excellent dispersity and chain-end fidelity.^{1,2} For this reason, visible light-mediated reversible addition-fragmentation chain transfer (RAFT) polymerization has been studied extensively since its discovery in 2014.³ Such RAFT polymerization encompasses two distinct mechanisms known as photoiniferter and photo-induced electron/energy transfer (PET)-RAFT approaches. Photoiniferter RAFT polymerization requires direct activation of the RAFT agent via light irradiation and is therefore limited to wavelengths absorbed by the RAFT agent, generally UV, blue, or green light.⁴⁻⁶ The PET-RAFT mechanism depends on the excitation of a photoredox catalyst or a photosensitizer by light irradiation. The excited catalyst/photosensitizer interacts with the RAFT agent via energy or electron transfer to generate the initiating radical for polymerization.^{3,7} Numerous compounds have been employed as photoredox catalysts or photosensitizers for PET-RAFT polymerization including organic dyes,⁸⁻¹¹ metalloporphyrins such as zinc tetraphenylporphyrin (ZnTPP),¹²⁻¹⁵ naturally-occurring photosynthetic pigments,¹⁶⁻¹⁸ enabling PET-RAFT polymerization across the visible light spectrum. Interestingly, a photoenzymatic RAFT mechanism using flavin-dependent enzymes was recently reported, with visible light causing photoexcitation of FADH- to facilitate the PET process and mediate polymerization.¹⁹ In addition, PET-RAFT polymerization is typically less sensitive to oxygen and can therefore be performed under less stringent conditions.²⁰⁻²²

As PET-RAFT polymerization has gained popularity due to its versatility and less stringent reaction conditions, there has been a push to develop photocatalysts with broadband and near-infrared (NIR) absorption, due to potential “green” chemistry and biomedical applications. The solar spectrum covers wavelengths from 250-2500 nm, with IR energy alone making up nearly 50% of solar energy, while photons from visible light and NIR constitute 95% of the solar flux at sea level.^{23,24} Therefore, broadband and NIR absorbance are necessary to achieve efficient solar energy utilisation. NIR can also penetrate opaque materials, including depths greater than 1 cm through human tissues, and therefore has potential use in biomedical applications and even *in vivo* polymerization.²⁵ Boyer and coworkers reported the first NIR-mediated CRP, using bacteriochlorophyll a as a photocatalyst.²⁶ More recently, they reported the use of aluminum naphthalocyanine as a photosensitizer for peroxides to initiate RAFT polymerization under NIR irradiation.²⁷ The authors demonstrated temporal control and achieved high conversions even with paper, chicken skin, and pig skin barriers. These reports are significant developments in NIR-mediated photopolymerization and potential *in vivo* polymerization but to date have been limited to organic solvent.

Focusing on broadband photopolymerization, Matyjaszewski and coworkers exploited the localized surface plasmon resonance (LSPR) effect of nanostructured silver orthophosphate (Ag_3PO_4) photocatalysts.²⁸ PET-RAFT polymerization of methyl acrylate demonstrated high conversions following blue, green, and red light irradiation as well as when exposed to natural sunlight. In addition, polymerization of benzyl acrylate was possible using NIR irradiation. However, polymerization was again only limited to organic solvent.

To date, all NIR-mediated CRP polymerization systems reported have been employed in organic solvents.²⁶⁻³⁰ However, the development of aqueous systems is essential for any potential *in vivo* applications and could also offer less expensive and more environmentally friendly options for industrial use. To aid with the development of aqueous broadband- and NIR-responsive systems, we looked to recent advances in the field of photocatalysis for solar-driven hydrogen evolution. Our group has previously reported efficient PET-RAFT polymerization with organic semiconductor graphitic carbon nitride ($\text{g-C}_3\text{N}_4$),³¹ an organic photocatalyst previously used in hydrogen evolution.³² With the use of $\text{g-C}_3\text{N}_4$, RAFT polymerization can be carried out with no degassing or monomer purification.³¹ Due to the limited absorbance spectrum and relatively hydrophobic surface functional groups of $\text{g-C}_3\text{N}_4$, its activity for polymerizations was only studied under UV irradiation in DMSO. More recently, novel photocatalysts such as covalent triazine-based framework-1 (CTF-1)³³ and formic acid-treated carbon nitride (FAT)³⁴ have shown narrower band gaps and red-shifted absorbance spectra in comparison to $\text{g-C}_3\text{N}_4$. Due to the improved function of these new catalysts, our previous successful PET-RAFT polymerizations using $\text{g-C}_3\text{N}_4$, and the need of aqueous PET-RAFT systems under NIR, we decided to investigate the use of these photocatalysts for PET-RAFT polymerization under various wavelengths in an aqueous system.

In addition, we synthesized a novel self-assembled carboxylated porphyrin (SA-TCPP) photocatalyst. Although it has been previously shown that nanometric SA-TCPP particles had a high solar spectrum efficiency and enhanced hydrogen and oxygen evolution from water under visible light irradiation,³⁵ our prepared micro-size SA-TCPP photocatalyst displayed a broader absorbance spectrum from 300 to 950 nm. Due to the micro-fibre like morphology, the lifetime of photoexcited electron-hole pairs is further extended, which in turn can lead to an improved polymerization efficiency compared to other recently reported photocatalysts for hydrogen evolution. As our PET-RAFT polymerization with this catalyst is in aqueous conditions under broad wavelengths, we further tested its polymerization ability in biological cell culture media, investigating its potential for *in situ* biomedical applications as well as highly efficient “green” controlled polymerizations.

3.3 Results and Discussion

3.3.1 Synthesis and characterization of SA-TCPP photocatalyst

We prepared the SA-TCPP photocatalyst through a modified approach (Figure 3.1). Commercially available 5,10,15,20-tetra (4-carboxy-phenol) porphyrin (TCPP) was deprotonated in an aqueous 1M KOH solution and heated to 80 °C, yielding a purple solution. 0.1M HCl was added dropwise until the pH became neutral, following which the precipitate was purified via dialysis and freeze-dried.

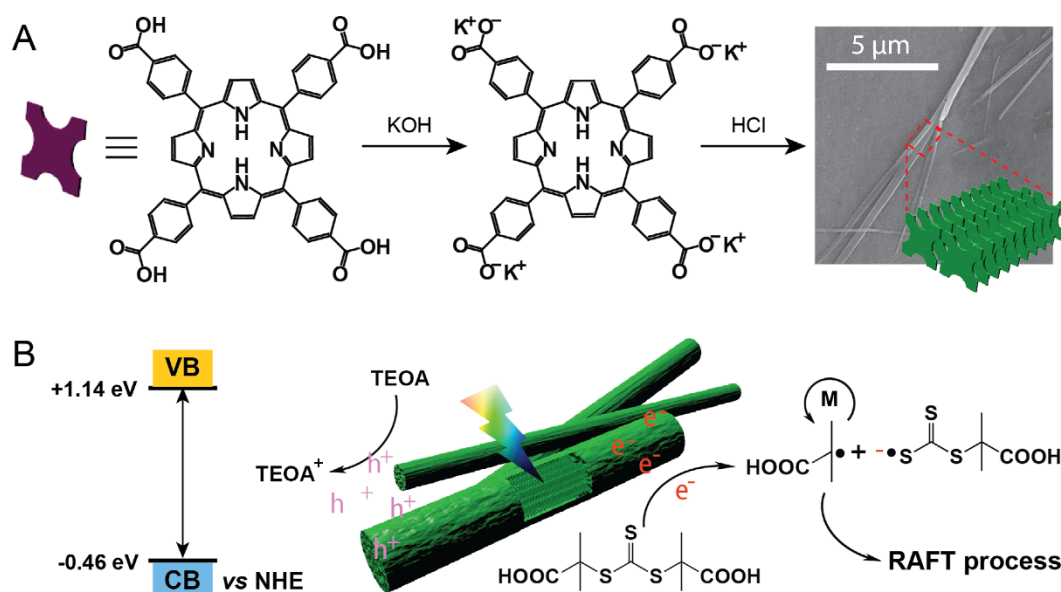


Figure 3.1 (A) The synthetic strategy for rod-like SA-TCPP and (B) schematic mechanism of the photocatalytic PET-RAFT polymerizations by SA-TCPP.

The resultant self-assembled TCPP (SA-TCPP) showed a dark green colour and formed long fibres/rods as seen using SEM (Figure 3.1). The obtained rods displayed a non-crystalline X-ray diffraction (XRD) pattern and this result was further confirmed by TEM diffraction measurement (Figure 3.2B). Two obvious peaks at 7° and 22° represent the 1.26 nm width of an aggregated TCPP molecule and the 0.4 nm face-to-face distance between TCPP molecules. X-ray photoelectron spectroscopy (XPS) peaks assigned to carbon 1s, nitrogen 1s, and oxygen 1s were located at 285, 400, and 533 eV, respectively, as shown in Figure 3.2C). High resolution of XPS spectra of carbon 1s, oxygen 1s, and nitrogen 1s regions are shown in Figures 3.2D-F. Shakeup, COO, C=O, C-N, C-C, and C=C peaks were found at 292, 289, 288, 286, 285, and 284 eV, respectively. The C-C peak represented 71.7% of the area under the curve, with the remaining peaks representing 3.2-9.4% of the carbon 1s region. OH and C=O peaks were found at 532 and 534 eV and made up 47 and 52% of the area under the curve,

consistent with the molecular structure of SA-TCPP. The nitrogen 1s region revealed peaks for C-N and N-H, binding energies of 400 and 398 eV, at a ratio of 3:1. Furthermore, the electrochemical experiment further revealed that the prepared SA-TCPP rods exhibit a band gap of -0.46 to +1.14 eV (Figure 3.11).

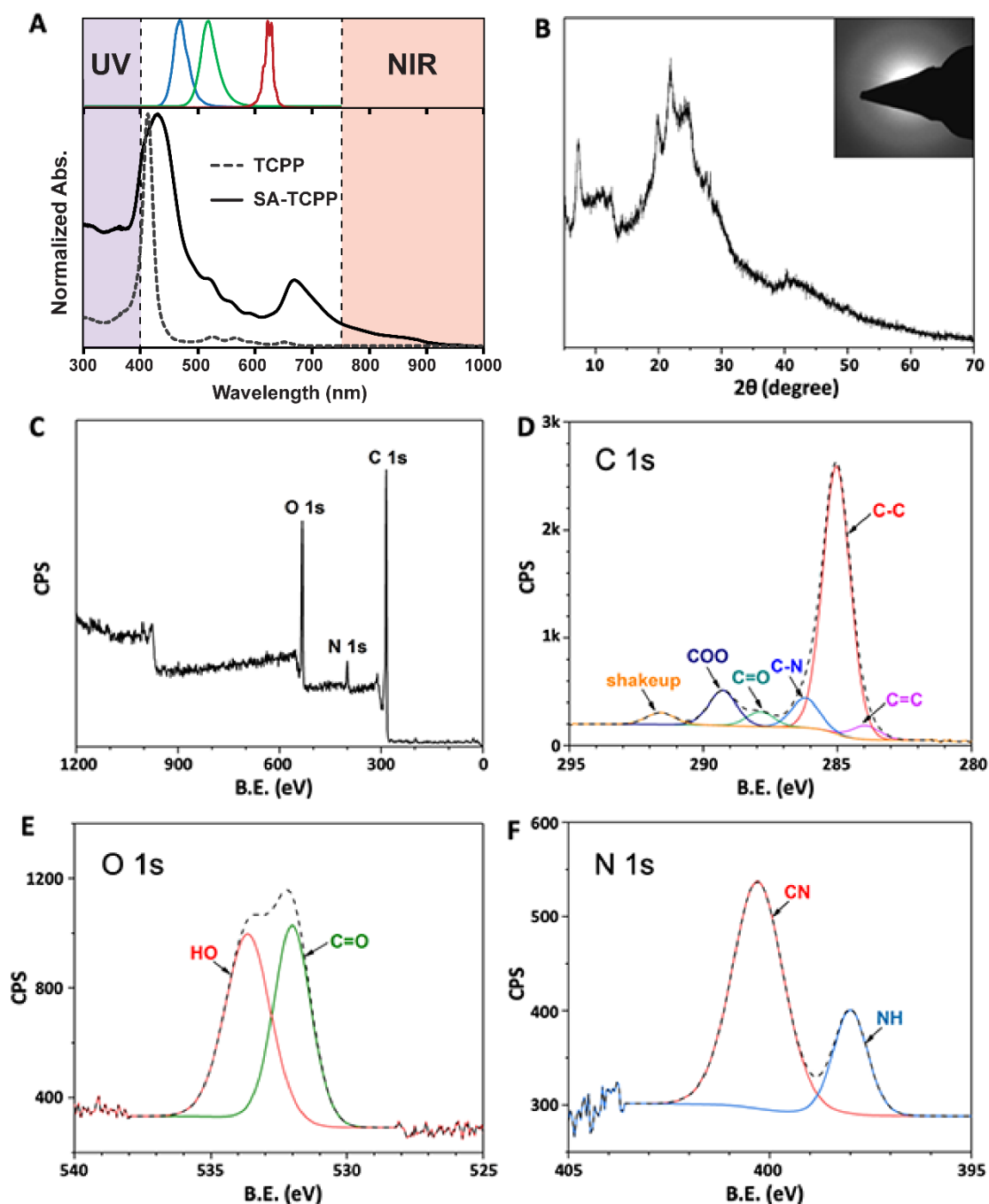


Figure 3.2 (A) UV-vis normalized absorption spectrum of TCPP and SA-TCPP (bottom) and LED light source emission spectra (top). (B) XRD and electron diffraction patterns of SA-TCPP. (C) Full XPS spectrum of SA-TCPP and high resolution XPS of the (D) C 1s, (E) O 1s, and (F) N 1s regions.

3.3.2 PET-RAFT polymerization with SA-TCPP

The broad absorption spectrum of SA-TCPP allowed us to investigate its efficiency in comparison to previously reported H₂ evolution photocatalysts g-C₃N₄, CTF-1, and FAT. In a typical reaction, DMA (100 equivalents), trithiocarbonate (TTC) RAFT agent (1 equivalent), triethanolamine (TEOA, 13.4 equivalents) and photocatalyst (5 mg) were dissolved in DI water (50 % v/v). Reaction mixtures were bubbled with argon for 20 minutes prior to light irradiation to afford faster polymerization and reduced induction period, although degassing and addition of TEOA were not essential, as previously reported.³¹ Polymerizations were performed under blue, green, and red LED irradiation.

In all cases, polymerizations performed with SA-TCPP resulted in the highest conversion (Figure 3.3). All photocatalysts enabled PET-RAFT polymerization of DMA to reach high conversions under blue light irradiation, with SA-TCPP reaching 91% conversion in 3 hours. While all polymerizations achieved high conversions under blue light irradiation, SA-TCPP increasingly outperformed the other catalysts as the irradiation wavelength increased and was the only catalyst that led to polymer formation under red light irradiation. Under green light, >99% monomer conversion was observed in 5 hours using SA-TCPP, while the CTF-1, FAT, and g-C₃N₄ reached 88, 83, and 77% conversion, respectively. Only SA-TCPP resulted in polymer formation after 5 hours of irradiation. All GPC traces were monomodal and symmetrical, with dispersities of 1.1 or below. In all cases where polymers were formed, kinetics show linear semi-logarithmic plots of conversion versus irradiation time, indicating a constant radical concentration and controlled polymerization.

The oxygen tolerance of the SA-TCPP PET-RAFT system was also demonstrated (Figure 3.4). An induction period of nearly 6 hours was observed, with 67% conversion reached in 14 hours. GPC analysis showed a symmetrical, monomodal peak with a dispersity of 1.04, demonstrating that control is maintained in the presence of air.

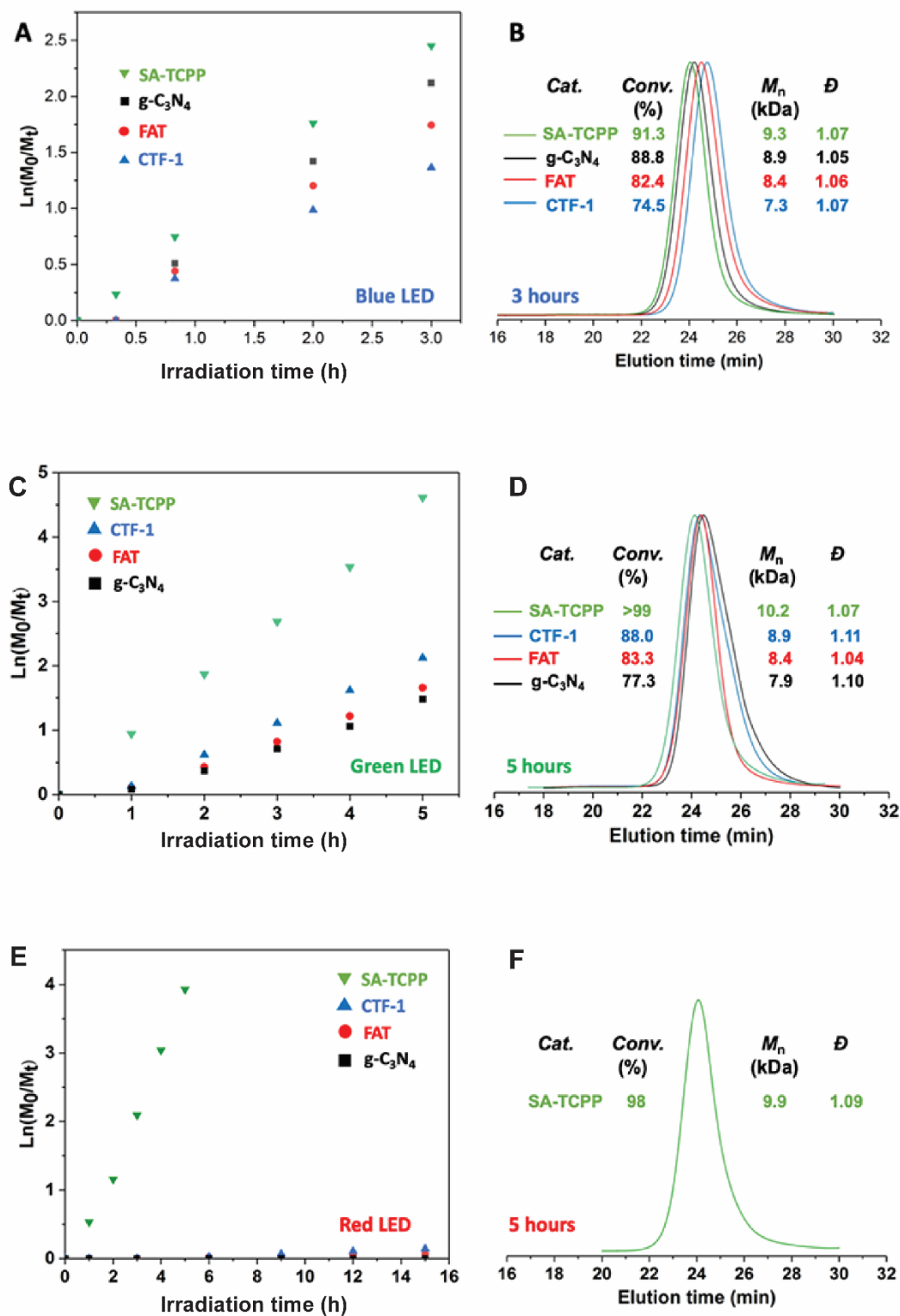


Figure 3.3 Polymerization kinetics and GPC traces from PET-RAFT polymerization of DMA in water with SA-TCPP, g-C₃N₄, FAT, and CTF-1 photocatalysts under blue (A and B), green (C and D), red (E and F) LED light irradiation.

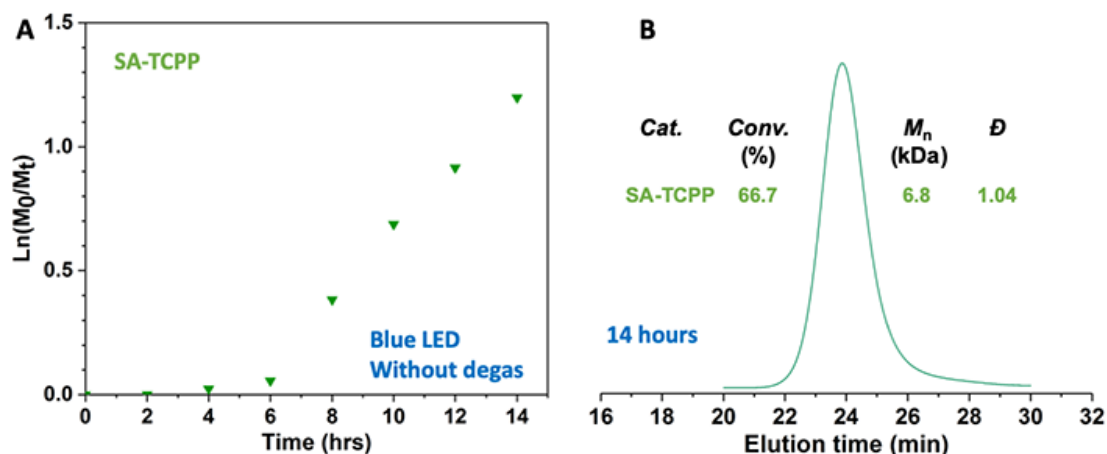


Figure 3.4 (A) Polymerization kinetics and (B) GPC trace of PET-RAFT polymerization of DMA in water under blue light without addition of TEOA or degassing. An induction period of nearly 6 hours was observed, with 67% conversion reached in 14 hours.

The polymerization kinetics of DMA using SA-TCPP under blue, green, red, white, and NIR irradiation are shown in Figure 3.5. The use of CTF-1, FAT, and $g\text{-C}_3\text{N}_4$ was not investigated under NIR irradiation due to their limited absorbance spectra and poor performance under red light. The temporal control of the system was then demonstrated using an “on/off” experiment, where the light source was turned on and off at 30 minute intervals (Figure 3.5B). Several cycles were performed with a different wavelength of light used during each “on” period, highlighting the broadband absorption of the photocatalyst. No polymerization was observed during the off period, demonstrating instantaneous temporal control. After 2 hours of irradiation, 70% monomer conversion was achieved. Pleasingly, the rates of polymerization during the “on” periods were in agreement with those observed in Figure 3.5A, with white light showing the fastest rate of polymerization, followed by blue, green, and red.

Crucially, retention of the TTC RAFT agent and its living nature were demonstrated via two chain extension reactions with greater than 95% monomer conversion attained for each block (Figures 3.5C). GPC traces showed symmetrical, monomodal peaks after each chain extension. Due to the symmetrical nature of the RAFT agent employed (S,S'-Bis(α , α' -dimethyl- α'' -acetic acid)trithiocarbonate), the TTC moiety remained at the centre of the polymer and the two chain extensions experiments resulted in a well-defined *pseudo*-pentablock polymer.

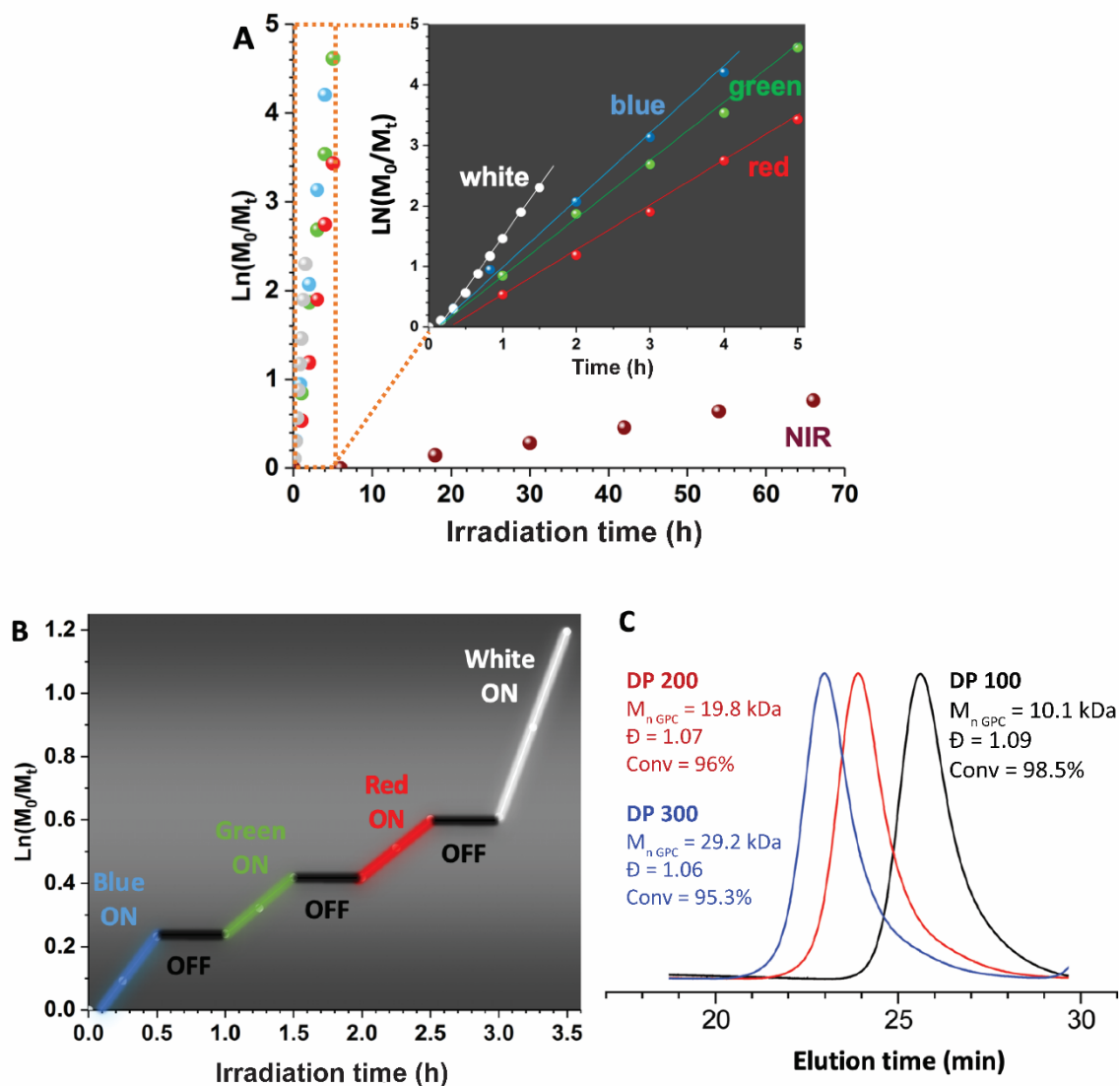


Figure 3.5 Kinetics of SA-TCPP-catalyzed RAFT polymerizations of DMA under (A) various LED irradiation (white to NIR, 4 mW/cm²) and (B) in an “ON/OFF” experiment. (C) GPC evolution of a *pseudo*-pentablock PDMA by chain extension.

While interest in NIR-mediated RDRP polymerizations has been increasing in recent years, focus has remained on the synthesis of linear polymers. Using the modified β -cyclodextrin core reported in Chapter 2 (21-XAN), we synthesized a PDMA star polymer under NIR irradiation. A target DP of 270 repeat units per arm was used. NMR analysis indicated 8.5% monomer conversion was achieved after 25 hours, which is comparable the linear polymer, and corresponds to molecular weight of 53.2 kDa. GPC analysis showed a symmetrical, monomodal peak, although a high dispersity of 1.73 was observed (Figure 3.6).

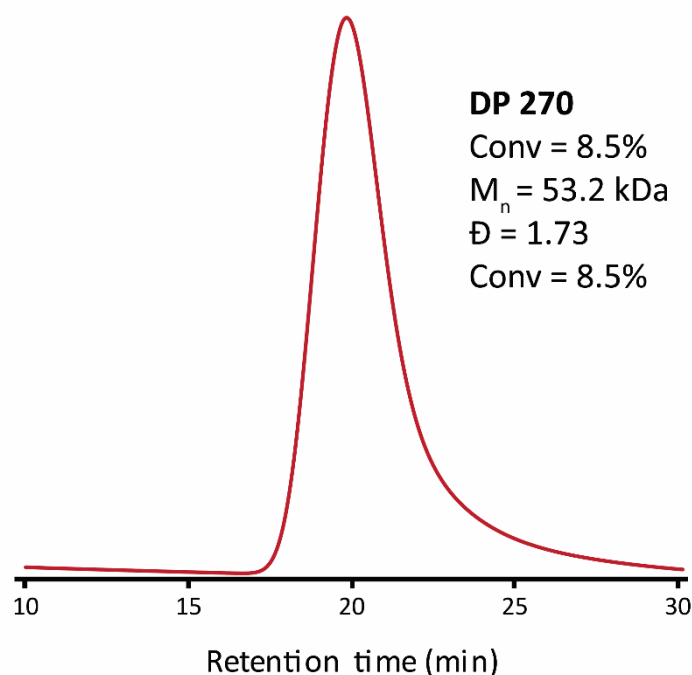


Figure 3.6 GPC chromatogram of 21-XAN PDMA star polymer synthesized under NIR irradiation. A target DP of 270 repeat units per arm was used and 8.5% conversion was achieved after 25 h NIR irradiation.

3.3.3 NIR-mediated polymerization through barriers

Successful NIR-mediated polymerization with SA-TCPP suggested PET-RAFT polymerization could be performed through barriers. NIR is known to penetrate barriers, including mammalian tissues, and the Boyer and Matyjaszewski groups have previously reported successful NIR-mediated PET-RAFT polymerization through materials such as paper and animal skin.^{27,28} Interestingly, Boyer and coworkers observed minimal change in the apparent rate propagation coefficient when polymerizing methyl acrylate through 0.1 mm paper, 1 mm chicken skin, and 2.5 mm pig skin barriers, mediated by NIR (850 nm).²⁷

Therefore, we set out to determine if we could perform our polymerization through a barrier using NIR (850 nm) and SA-TCPP in an aqueous environment. We carried out our investigation using a piece of opaque paper wrapped around the reaction vessel as a barrier (Figure 3.7). An induction period of 6 hours was observed before achieving 30% conversion after 66 h (Figure 3.7B). The GPC trace of the resulting polymer was monomodal and symmetrical, with a low dispersity of 1.07, as shown in Figure 3.7C. To our knowledge, this is the first time NIR irradiation has been reported for RDRP in water. These results signify an essential step towards biocompatible NIR-mediated polymerization, where an aqueous system would be essential.

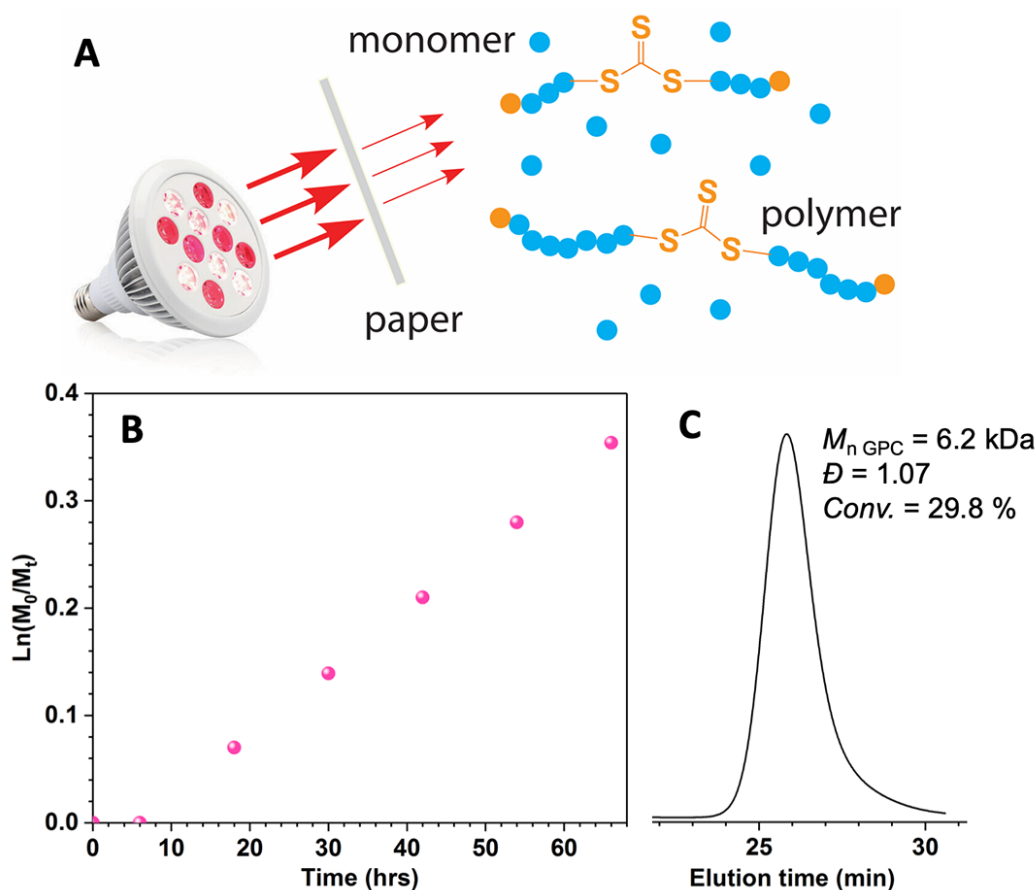


Figure 3.7 (A) Schematic illustration of an opaque paper barrier employed for polymerizations under NIR LED light ($\lambda_{\text{max}} = 850 \text{ nm}$). (B) Kinetic study of SA-TCPP catalyzed RAFT polymerization of DMA under NIR LED irradiation (4 mW/cm^2). (C) GPC curve of the resultant PDMA.

3.3.4 PET-RAFT polymerization in cell culture medium

Any *in vivo* polymerizations would also need to be biocompatible and performed with a low volume. To this end, we attempted a preliminary study of the PET-RAFT polymerization using PEG methacrylate (PEGMA, 480 Da) in the presence of mammalian fibroblast cells in a 96-well plate (Figure 3.8A). Six wells each contained 0.13 mg SA-TCPP, 22 mg PEGMA, 0.7 mg TTC, and 0.5 mg TEOA in complete DMEM cell culture medium to a total volume of 100 μL . The 96-well plate was irradiated with red light for 45 minutes at 37 $^{\circ}\text{C}$. The conversion of PEGMA was 11% and GPC characterization showed a monomodal peak with a small tail (Figure 3.8B). NMR analysis confirmed that no chain transfer to any biomolecules had occurred. Significantly, this work demonstrates our system can conduct polymerization without degassing at microlitre scale, offering the future potential for machine programable multi-block polymerization via computer-controlled microlitre injection systems. We also measured cell viability immediately following

light irradiation and obtained 46% cell viability (Figure 3.8C). The assay used measures viability based on metabolic activity; therefore it is possible to measure reduced viability resulting from cell death or due to decreased metabolic activity. Using a light microscope, it was possible to look at the cells and confirm that many cells were floating, indicating they had died. While optimization of reaction conditions and reagents is needed to reduce the toxicity of the system, these preliminary results suggest a biocompatible, NIR-mediated PET-RAFT polymerization may soon be possible.

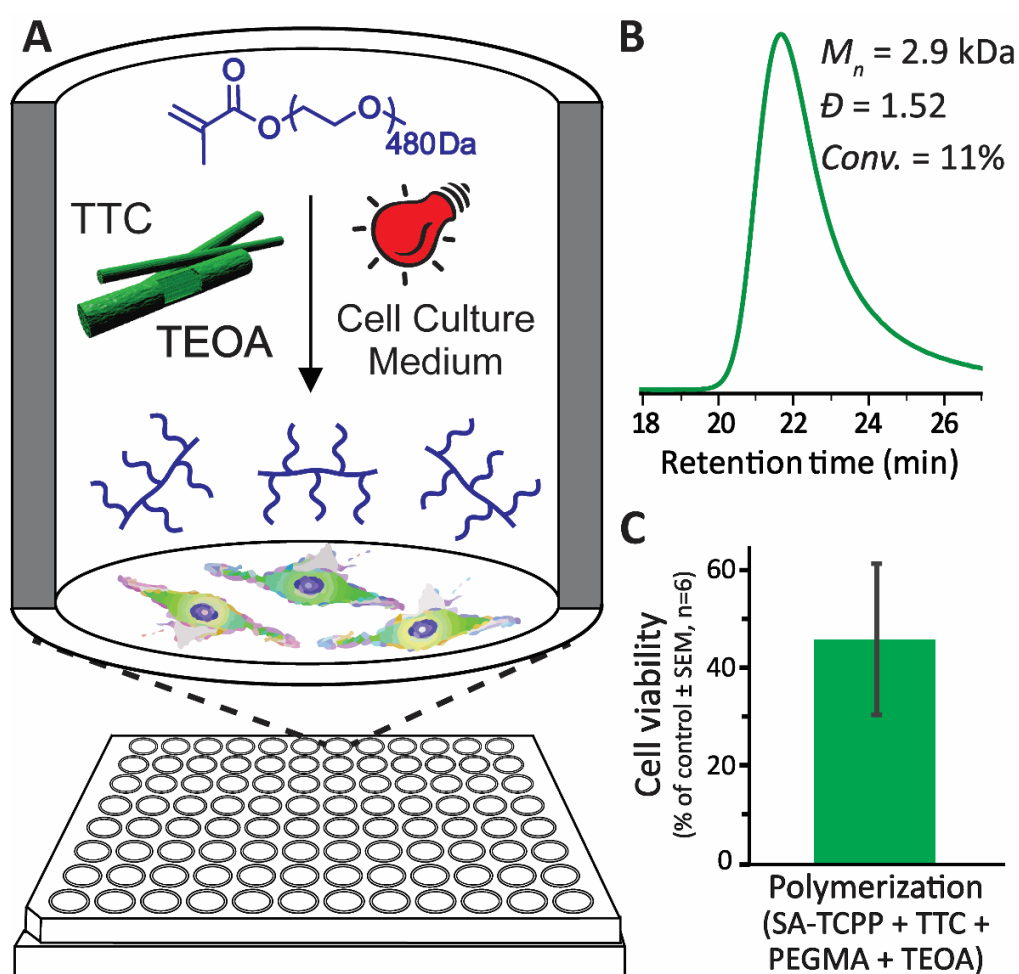


Figure 3.8 (A) Microlitre-scale PET-RAFT polymerization of PEGMA₄₈₀ in a 96-well plate containing fibroblast cells, 45 minutes red light irradiation. (B) GPC trace of poly (PEGMA), and (C) cell viability following PET-RAFT polymerization.

3.4 Conclusions

The recent reports by the Boyer,^{26,27} Matyjaszewski,²⁸ and Yagci²⁹ groups demonstrate exciting developments in broadband- and NIR-mediated CRP. Building on these critical advances, we report for the first time an aqueous NIR-mediated CRP. The use of a metal-free, porphyrin-based organic photocatalyst with an absorbance spectrum covering 300-950 nm enabled broadband- and NIR-mediated PET-RAFT polymerization in water. SA-TCPP was compared to three other photocatalysts previously reported for hydrogen evolution and was found to have the highest rate of polymerization when irradiated with white, blue, green, and red light. Preliminary investigations resulted in the successful microlitre-scale polymerization of PEGMA in the presence of mammalian cells in complete cell culture medium, with future works to focus on improving cell viability. These findings have broad implications for potential *in vivo* polymerizations for biomedical applications as well as “green” polymerizations.

3.5 Experimental Section

3.5.1 Materials

Tetra(4-carboxyphenyl)porphyrin (TCPP), N,N-dimethylacrylamide (DMA, 99%) and triethanolamine (TEOA, 98%) were purchased from Sigma-Aldrich and used as received. Hydrochloric acid, potassium hydroxide and dimethyl sulfoxide (DMSO) were purchased from Chem-Supply Pty Ltd and used as received. Snakeskin dialysis tubing (3.5k, MWCO) was purchased from Thermo Fisher Scientific. The White-Red-Green-Blue RGB waterproof LED strip lighting 5050 was used for PET-RAFT experiments (DC12V, 4.0 mW cm⁻²). The LED light emission spectra (blue: $\lambda_{\text{max}} \sim 465$ nm, green: $\lambda_{\text{max}} \sim 520$ nm, and red: 630 nm, respectively) were measured using a Maya 2000Pro spectrometer fitted with optical fiber 139 (OceanOptics 100UV). Chain transfer agent S,S'-bis(α , α' -dimethyl- α'' -acetic acid)trithiocarbonate (CTA) was synthesized as previously reported.¹ Photocatalysts graphitic carbon nitride (g-C₃N₄), covalent triazine-based framework-1 (CTF-1), and formic acid-treated carbon nitride (FAT) were synthesized as previously reported.²⁻⁵ Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), GlutaMAX, penicillin, streptomycin, and trypsin-EDTA (1 $\times$) were purchased from Gibco, Invitrogen. Cell Counting Kit-8 was purchased from Sigma-Aldrich and used as instructed.

3.5.2 Characterization

3.5.2.1 Scanning Electron Microscopy (SEM)

SEM measurements conducted on a Quanta FEG 200 ESEM. Samples were coated with gold using a Dynavac Mini Sputter Coater prior to imaging.

3.5.2.2 Transmission Electron Microscopy (TEM)

TEM measurements were performed on a FEI Tecnai F20 microscope equipped with an EDAX TEAMTM EDS System.

3.5.2.3 Powder X-Ray Diffraction (PXRD)

XRD pattern of the samples was recorded on a Bruker D8 Advance instrument with Cu K α radiation (40 kV, 40 mA) and a nickel filter, and the samples were exposed at a scanning rate of $2\theta = 0.020$ s⁻¹ in the range of 5-70 $^\circ$.

3.5.2.4 X-ray Photoelectron Spectroscopy (XPS)

XPS was carried out on a VG ESCALAB 220i-XL spectrometer under ultra-high vacuum conditions (6×10^{-9} mbar) with fixed photon energy (Al K α 1486.6 eV). A survey scan was

performed between 0 and 1200 eV with a resolution of 1.0 eV and pass energy of 100 eV. High resolution scans for C 1s (281 to 293 eV), O 1s (528 to 536 eV) N 1s (396 to 405 eV) and Fe 2p (705 to 733 eV) were also conducted with a resolution of 0.05 eV and a pass energy of 20 eV.

3.5.2.5 Nuclear Magnetic Resonance (NMR) Spectroscopy

¹H NMR spectroscopy was conducted on a Varian Unity 400 MHz spectrometer operating at 400 MHz, using the deuterated solvent (CDCl₃) as the reference and a sample concentration of approximately 10 mg·mL⁻¹.

3.5.2.6 Gel-Permeation Chromatography (GPC)

The aqueous GPC system consisted of three Waters Ultrahydrogel columns in series ((i) 250 Å porosity, 6 µm bead size; (ii) and (iii) linear, 10 µm bead size). A Shimadzu RID-10 refractometer and Wyatt 3-angle MiniDawn light scattering detector were connected in series. Milli-Q water with 0.1 vol% TFA was used as eluent at a flow rate of 1 mL min⁻¹ and the system operated at ambient temperature. For all PDMA polymers, dn/dc values were determined via a method of 100% mass recovery. Molecular weight and dispersity values were calculated using the Wyatt ASTRA software package from MALS data using a Debye model. Poly(PEGMA) dispersity values were calculated from PEG standards.

3.5.2.7 Absorbance of photocatalysts

The UV-Vis absorbance of SA-TCPP was recorded by measuring a dilute suspension of SA-TCPP in deionized water using a Shimadzu UV-2101 PC spectrophotometer. The absorption spectra of g-C₃N₄, CTF-1, and FAT were obtained in the solid state using a Shimadzu UV-Vis 2550 spectrophotometer fitted with an integrating sphere. Diffuse reflectance spectra were obtained using powdered samples, with standard barium sulphate powder as a reference. The reflection measurements were converted to absorption spectra via the Kubelka-Mulk transformation.

3.5.3 Methods

3.5.3.1 Synthesis of self-assembled Tetra(4-carboxyphenyl)porphyrin (SA-TCPP)

TCPP (500 mg) was dissolved in 20 mL of 1mol·L⁻¹ KOH aqueous solution in a 500 mL round bottom flask. The mixture was then heated to 80 °C to afford a purple solution. Thereafter, 200 mL of 0.1 mol·L⁻¹ HCl solution was added dropwise until the pH became neutral and no further precipitation was obtained. The prepared SA-TCPP was purified by

dialysis against DI water using a regenerated cellulose dialysis tubing with a molecular weight cut-off of 3.5 kDa, followed by freeze-drying, yielding green powder.

3.5.3.2 Electrochemical measurements

A standard three-electrode cell was employed, with a working electrode (SA-TCPP), a saturated calomel electrode (SCE) as the reference electrode and a platinum wire as the counter electrode. Na₂SO₄ was taken as the electrolyte solution. All the potentials were given with reference to the SCE.

The working electrodes were prepared as follows: 5 mg SA-TCPP was suspended in 1 mL DI water under ultrasonication. A dark green slurry was obtained and drop-coated onto an indium tin oxide (ITO) glass electrode. Electrodes were dried in a vacuum oven at 60 °C.

3.5.3.3 General procedure for photoinduced electron transfer RAFT polymerization

A vial glass (7 mL) charged with photocatalysts (5 mg), DMA (1.03 mL, 10 mmol), CTA (28.2 mg, 0.1 mmol), TEOA (100 mg, 0.67 mmol), and DI water (50 vol %) ([DMA]/[CTA] = 100/1) was sealed with a rubber septa. The reaction mixture was degassed by bubbling Ar for 20 minutes. The LED light source (blue, green, red, or NIR; 4.0 mW cm⁻²) was then switched “on”, and the reaction mixture was stirred. Samples were taken at timed intervals *via* degassed syringe and immediately diluted with either DMSO-*d*₆ or DI water, for NMR and GPC analysis, respectively.

3.5.3.4 “On/Off” reaction

The “on/off” reactions were set up in the same fashion; however, at a given reaction time the LED light source was turned off and the vial covered completely in aluminium foil and placed into a homemade “dark box” for 30 minutes. The polymerization was reactivated by irradiating the reaction mixture with LED light again. Samples were taken at timed intervals *via* degassed syringe and immediately diluted with either DMSO-*d*₆ or DI water for NMR and GPC analysis, respectively.

3.5.3.5 In situ chain extension

A 7 mL vial glass charged with SA-TCPP (5 mg), DMA (0.515 mL, 5 mmol), CTA (14.1 mg, 0.05 mmol), TEOA (100 mg, 6.7 mmol), and DI water (50 vol %) ([DMA]/[CTA] = 100/1) was sealed with a rubber septa. The vial was sparged with Ar for 20 min. The LED light source (white, 4.0 mW cm⁻²) was then switched “on”, and the reaction mixture was stirred. After 2 h the conversion of DMA achieves >95% by ¹H NMR analysis. The second portion of DMA monomer (0.515 mL, 5 mmol) and DI water was

sparged with Ar for 20 min. The deoxygenated mixture was then injected into the reactor. After 2 hours, the monomer conversion achieved over 95% by ^1H NMR analysis. Finally, the last portion of the deoxygenated mixture containing 5 mmol DMA and DI water was injected into the reactor. After 4 hours, the monomer conversion achieved over 95% by ^1H NMR analysis. The sample was also taken and diluted with DI water for GPC analysis.

3.5.3.6 Cell Culture

NIH 3T3 mouse fibroblast cells were maintained in DMEM supplemented with 10% FBS, GlutaMAX (2mM), streptomycin (100 $\mu\text{g}/\text{mL}$) and penicillin (100 units/mL). Cells were passaged every 3-4 days using 0.25% trypsin-EDTA at subconfluence and incubated in a humidified incubator (37 $^{\circ}\text{C}$, 5% CO_2 , and 90% humidity). Cell passages 10-11 were used for experiments.

3.5.3.7 PET-RAFT polymerization in the presence of cells

NIH 3T3 cells seeded in a transparent, poly(styrene), flat-bottom 96-well plate (Corning) at 5×10^3 cells per well in supplemented DMEM cell culture medium. After 20 h the cell culture medium was replaced with 100 μL fresh medium (control wells) or medium containing PEGMA, TTC, TEOA, and/or SA-TCPP and irradiated with red light for 45 min at 37 $^{\circ}\text{C}$, 5% CO_2 , 90% humidity. Each treatment was performed in three or six separate wells as shown below:

Treatment/control (final concentration, in culture medium)	Number of wells
Blank (culture medium, no cells)	6
Control (culture medium)	6
SA-TCCP in culture medium (1.25 mg/mL)	3
PEGMA (20 μL), TEOA (50 mg/mL), TTC (7 mg/mL) in culture medium	3
SA-TCPP (1.25 mg/mL), PEGMA (20 μL), TEOA (50 mg/mL), and TTC (7 mg/mL), in culture medium	6

3.5.3.8 Cell viability assay

Cell viability was assessed using a standard Cell Counting Kit-8 (CCK-8) reagent. Following PET-RAFT polymerization, the treatment or cell culture medium was removed and each well was washed twice with 100 μL cell culture medium, followed by the addition of 100 μL cell culture

medium and 10 μ L CCK-8 reagent. After 2 h incubation, the absorbance was measured using a Tecan M200 Infinite Pro microplate reader at a wavelength of 460 nm, reference wavelength 650 nm. Error bars represent the standard error of the mean, n=3 or n=6 as described above. The cell viability was calculated as a percent of the control using the following formula, where [A] is the absorbance:

$$\text{Cell viability (\% of control)} = \frac{[A]_{460 \text{ (sample)}} - [A]_{460 \text{ (blank)}}}{[A]_{460 \text{ (control)}} - [A]_{460 \text{ (blank)}}} \times 100\%$$

3.6 Additional information

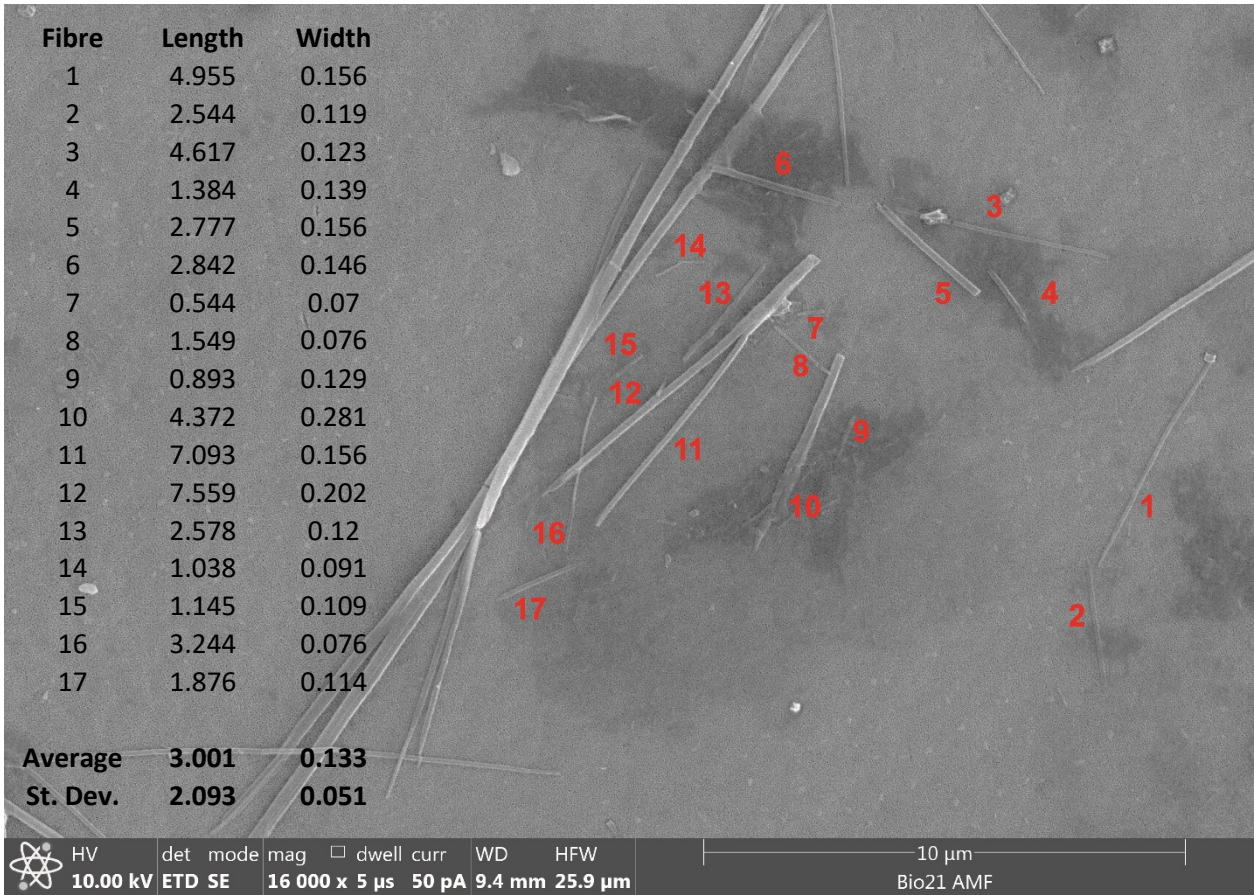


Figure 3.9 A representative SEM image was selected and the fibres in the image were numbered and measured for length and width using ImageJ. The average length and width $\pm$ standard deviation were $3.00 \pm 2.09 \mu\text{m}$ and $0.13 \pm 0.05 \mu\text{m}$, respectively. Measurement was only performed when the entire fibre was visible, therefore a small number of very large fibres were not included in the average and standard deviation calculations.

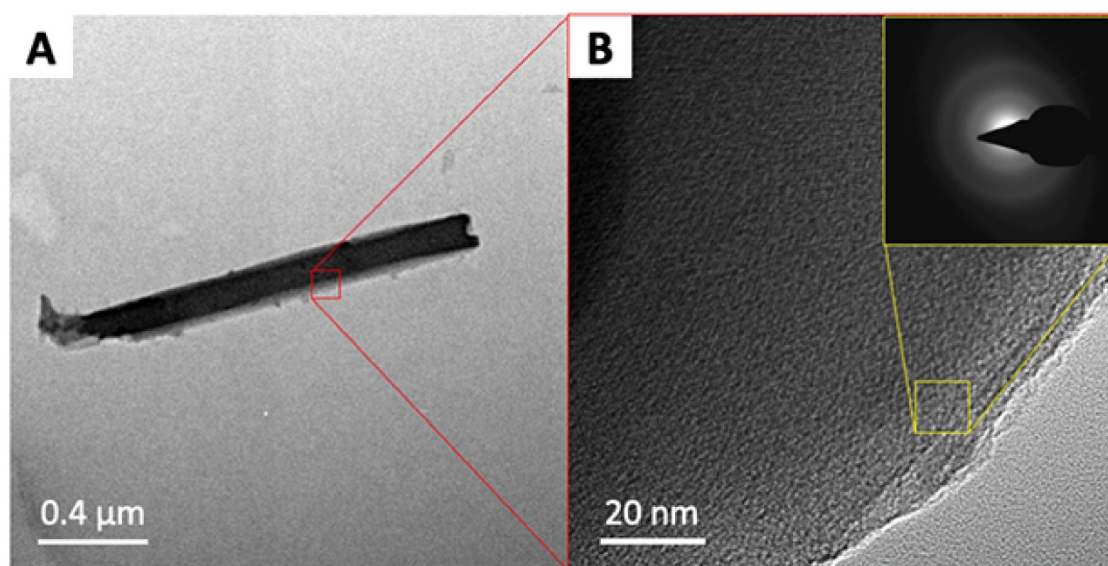


Figure 3.10 (A and B) High resolution TEM images of SA-TCPP. (B inset) Selected area electron diffraction (SAED) of the yellow box.

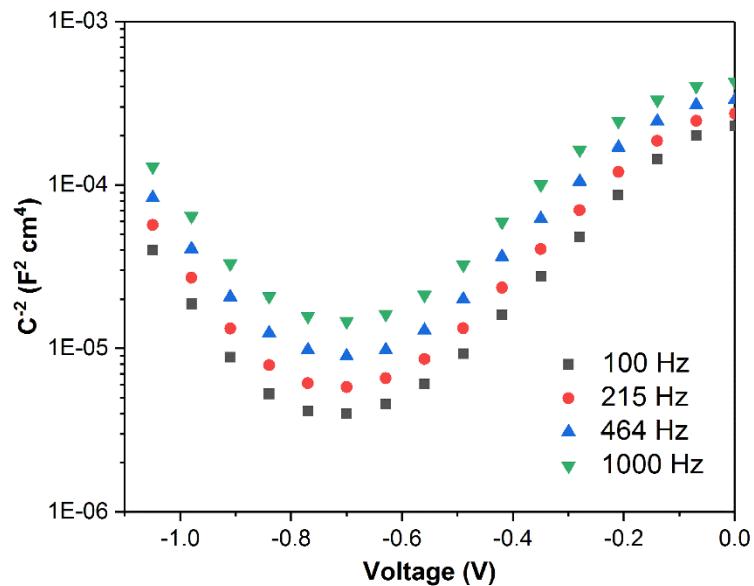


Figure 3.11 The Mott-Schottky plots of SA-TCPP. Four frequencies have been used to detect the flat band position of SA-TCPP semiconductor (-0.7 eV vs. SCE).

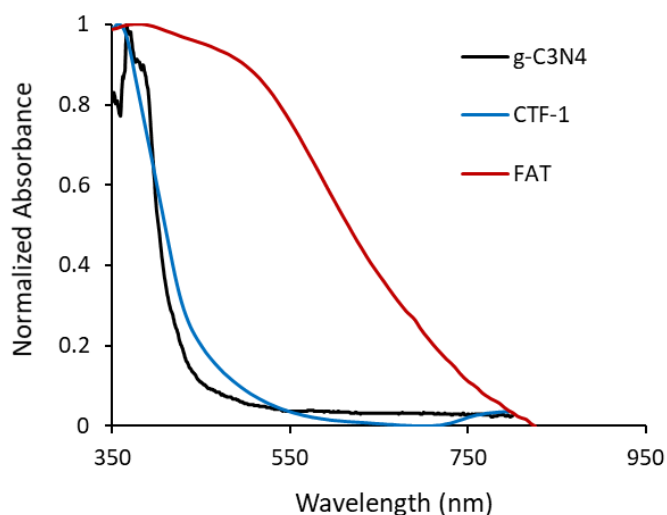


Figure 3.12 Normalized absorbance spectra of CTF-1, FAT, and g-C₃N₄ from 350 to 950 nm. UV-Vis absorbance spectra were calculated from diffuse reflectance measurements taken in the solid state, and therefore should not be directly used as evidence of photocatalytic ability under irradiation at a particular wavelength.

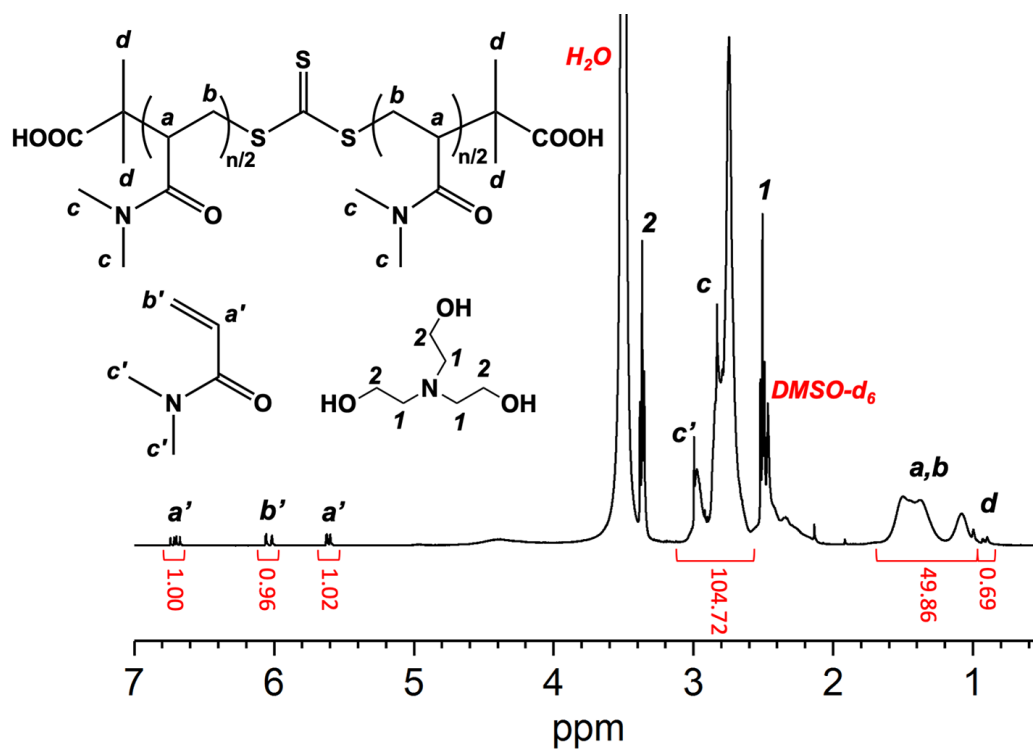


Figure 3.13 NMR spectrum of the final chain extension resulting in a pseudo-pentablock PDMA, with target DP 300. Integration of the RAFT agent methyl groups and polymer backbone indicate 289 DMA repeat units, corresponding to an M_n of 28.9 kDa. This result is in close agreement with the $M_{n, GPC}$ of 29.2 kDa, highlighting the “livingness” of the polymerization.

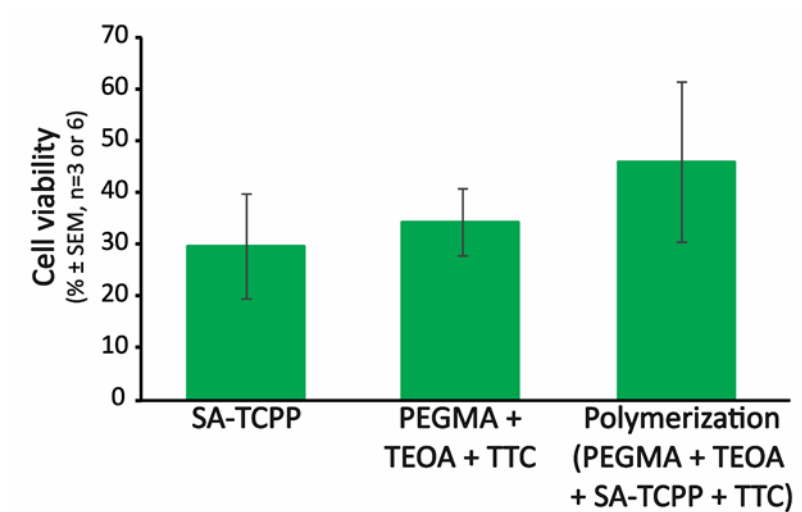


Figure 3.14 Cell viability assay following red light irradiation for 45 minutes. Cells treated with SA-TCCP, PEGMA + TEOA + RAFT agent, or polymerization (SA-TCCP, PEGMA + TEOA + RAFT agent) in supplemented DMEM showed 30 ± 10 (n= 3), 34 ± 6.5 (n = 3), and $46 \pm 15\%$ (n = 6) cell viability $\pm$ standard error of the mean.

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Chapter 4

Polypeptide Stars for Efficient Lysosomal Escape

4.1 Chapter Perspective

Nanoparticles offer great promise in the field of drug delivery, yet delivery of therapeutics to their site of action remains a challenge. Most nanoparticles are taken into the cell via endocytosis and become trapped in endosomes or lysosomes unless they have a mechanism of escape. We proposed that we could exploit the lysosome's role in protein degradation and amino acid recycling to achieve lysosomal escape using polypeptide-based nanoparticles. This chapter investigates the synthesis of a polypeptide-based star polymer and initial *in vitro* cell assays to probe our proposed novel mechanism of lysosomal escape. We synthesized a degradable polypeptide star and a nondegradable control star, to both of which fluorescent dye and chemotherapeutic agent doxorubicin (DOX) was conjugated. Fluorescent microscopy was used to demonstrate degradation of the polypeptide star and the subsequent lysosomal escape of DOX, while the DOX-loaded nondegradable star appeared trapped in the lysosome. In addition, cytotoxicity studies showed the polypeptide star is non-toxic.

The synthetic approach employed in this chapter has expanded on our synthetic developments in photoiniferter RAFT star polymer synthesis demonstrated in Chapter 2. Herein, we synthesize a polypeptide star via NCA ROP, and subsequently use photoiniferter RAFT polymerization to produce a PEG-brush corona. The nondegradable star polymer was synthesized using a one-pot, core-first approach from the 4-XAN core presented in Chapter 2.

4.2 Introduction

Nanoparticles show incredible promise in the delivery of therapeutics to treat a wide array of illnesses. Benefits include increased circulation time, improved water solubility, reduced adverse effects, and the ability to protect therapeutics when compared to free or unconjugated therapeutics.^{1,2} Cancer therapy in particular shows tremendous potential due to the enhanced permeation and retention (EPR) effect, where the “leaky” tumour vasculature allows particles of a particular size to escape blood vessels and enter the tumour.^{3,4} The ideal particle size is thought to be 10 – 100 nm, although larger nanoparticles have been shown to accumulate in the tumour.⁵ Particles smaller than 10 nm are cleared through the renal system, dramatically reducing their circulation time and probability of successful delivery to the tumour, while larger particles are less able to penetrate deep into the tumour.

While there has been significant focus on the delivery of nanoparticles to tumour sites, delivery of therapeutics to the intracellular site of action remains a challenge. Most nanoparticles are taken into the cell via endocytosis and transported via the endosomal pathway. Nanoparticles are transported from early endosomes (pH ~ 6.3), to late endosomes (pH ~ 5.5), and finally to lysosomes (pH ~ 4.7), the recycling centres of the cell.⁶ Nanoparticles can become trapped in these compartments, preventing the release and action of the delivered therapeutics. Escape from the endosome or lysosome, collectively referred to as endo-lysosomal escape, is therefore of the utmost importance. Although the mechanisms of endo-lysosomal escape are still subject to debate, four mechanisms are more or less accepted: i) membrane fusion, ii) osmotic rupture, iii) particle swelling, and iv) membrane destabilization.⁷ In this chapter, we propose a fifth mechanism, exploiting the natural recycling function of the lysosome by conjugating drug to the amino acid side chain of a synthetic polypeptide.

Lysosomes are known as the terminal degradation stations for both exogenous and intracellular cargo.^{8,9} Lysosomes contain numerous enzymes to metabolize macromolecules down to their constituent monomers, which are then exported to the cytoplasm. Proteins are broken down to amino acids, which are released into the cytosol via lysosomal amino acid transporters for protein synthesis.¹⁰⁻¹⁴ We hypothesized that we could capitalize on the recycling function of lysosomes as a novel mechanism of endo-lysosomal escape for improved drug delivery by using a polypeptide-based nanocarrier delivery system which can be metabolized by lysosomal enzymes (Figure 4.1). To test this hypothesis, we need a particle with the following properties:

- 1) Nanoparticle between 10 and 100 nm to maximize the EPR effect;
- 2) Polypeptide segment susceptible to lysosomal degradation;

3) Fluorescent dye with stable conjugation to an amino acid side chain to monitor intracellular location; and

4) Anti-fouling corona to reduce biointeractions, thereby increasing circulation time.

In addition, we would also need an analogous yet nondegradable nanoparticle to serve as a control.

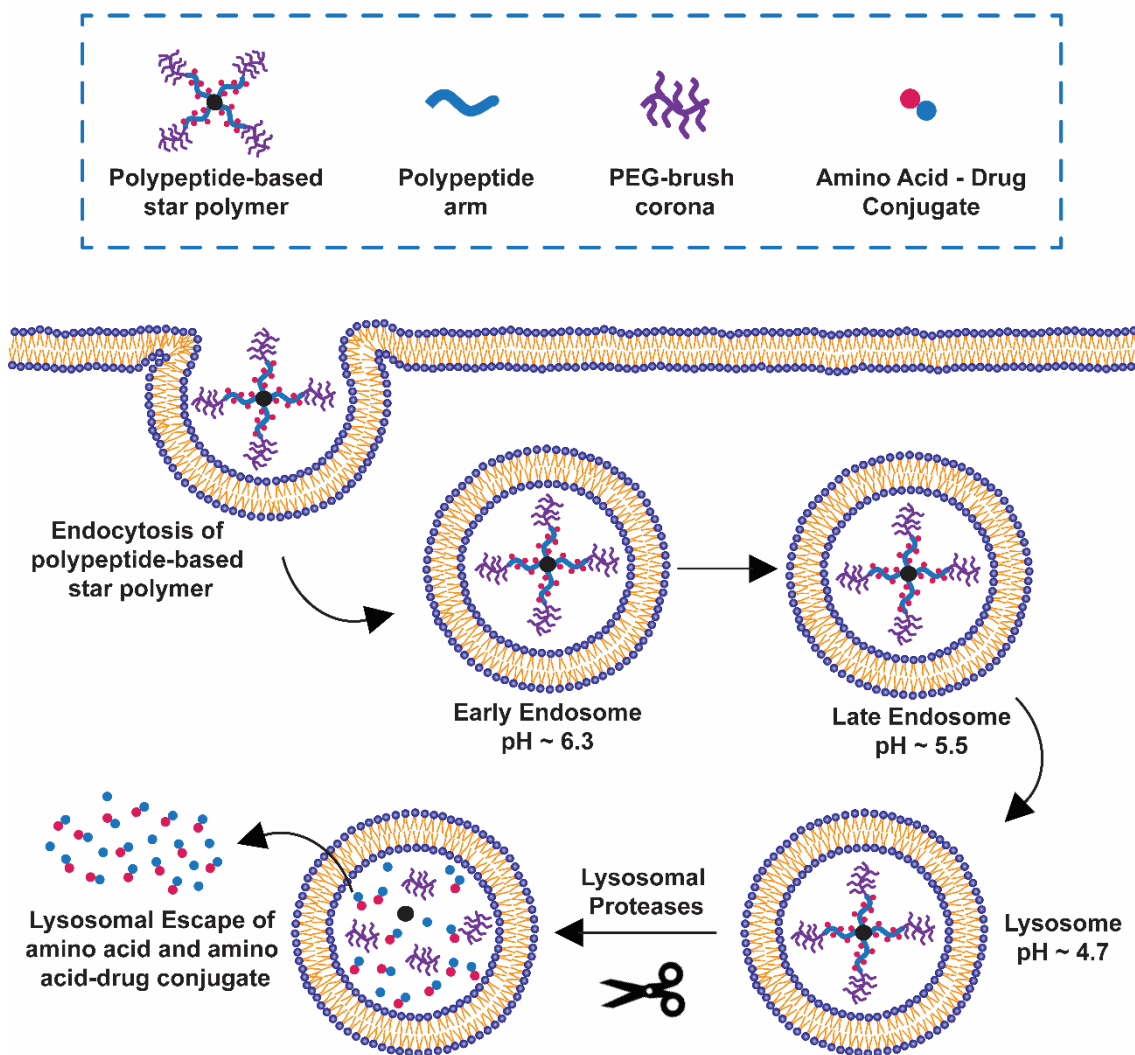


Figure 4.1 Proposed mechanism of lysosomal escape using a polypeptide-based star polymer.

With these criteria in mind, we chose to synthesize a star polymer with polypeptide arms and a PEG-brush corona. Star polymers consist of a central core and 3 or more arms, and are excellent candidates for drug delivery owing to their unimolecular structure and nanometre size.¹⁵ The polypeptide segment was synthesized via N-carboxyanhydride (NCA) ring-opening polymerization (ROP), a well-established polymerization method commonly used for biomedical applications, including drug delivery.¹⁶ The use of poly (L-glutamic acid) (PLG) is particularly widespread, due to its acid side chain which can serve as a site for drug conjugation or endow

pH-responsiveness, with multiple PLG-containing self-assembled structures having reached clinical trials.¹⁷⁻¹⁹ In addition, PLG can be degraded by lysosomal proteases.^{20,21} PLG stars have shown longer circulation times and improved pharmacokinetics in animal studies when compared to their linear counterparts, while still being susceptible degradation by lysosomal proteases.²²

Poly (ethylene glycol) (PEG) is widely used in biomedical applications for its anti-fouling properties, which result from its high hydration capacity.²³ PEG can also serve to improve water solubility due to its hydrophilicity. Herein, we synthesized a poly (PEG acrylate) (PPEGA) brush as a protective corona for our star whilst improving water solubility. The PEG-brush corona and nondegradable star polymers were synthesized *via* photoiniferter RAFT polymerization, using a core-first approach. Fluorescent dye and anticancer therapeutic doxorubicin (DOX) was conjugated to amino acid side chains to demonstrate the drug delivery potential of the stars and to track the location of star polymers and amino acids once inside cells.

In this work, we report the synthesis of a degradable polypeptide-based star polymer to investigate a novel mechanism of lysosomal escape using by conjugating drug to an amino acid side chain. Fluorescent microscopy demonstrated cell internalization of the degradable polypeptides star and the nondegradable control star. Cells treated with the polypeptide stars showed DOX escape from the lysosome, while DOX-loaded nondegradable stars were trapped within the lysosome, supporting our proposed mechanism of lysosomal escape. Cell viability studies clearly demonstrated the biocompatibility of our polypeptide stars.

4.3 Results

To examine our proposed mechanism of lysosomal escape, a star polymer containing degradable polypeptide arms with fluorescent dye and chemotherapeutic drug DOX conjugated to the amino acid side chain was synthesized. An analogous nondegradable star polymer was synthesized as a control (Figure 4.2).

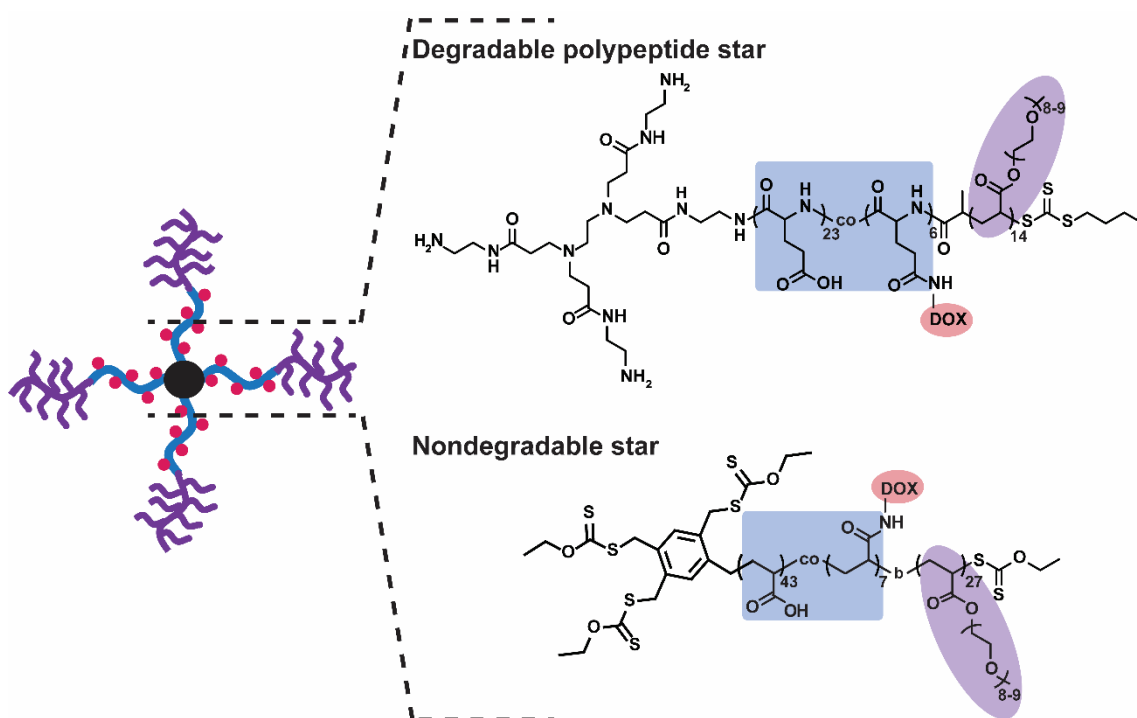


Figure 4.2 Chemical structure of the polypeptide star and the analogous, nondegradable control star.

4.3.1 Degradable polypeptide star

A polypeptide-containing star polymer was synthesized using a combination of NCA ROP and photoiniferter RAFT polymerization. Synthesis of the star was a 5-step process, as outlined in Figure 4.3.

The PAMAM-PBLG star was synthesized via ring-opening polymerization of BLG-NCA from a G₀ PAMAM dendrimer, which contains 4 primary amines as initiating sites. Successful NCA ROP was verified by NMR and GPC analysis. GPC analysis demonstrated a Mn of 27.6 kDa, Mw 41.7 kDa, and a dispersity of 1.51, determined using poly(styrene) standards (Figure 4.4).

PEGylation of the star polymer was necessary to reduce fouling and to improve water solubility of the star. Initially, PEGylation was attempted via conjugation of linear PEG to the terminal amine of the polypeptide block. However, quantitative conjugation could not be achieved, likely due to steric hindrance, and following conjugation of DOX to the acid side chains, the star polymers were no longer water soluble. To overcome these obstacles and ensure adequate PEGylation, a photo-mediated core-first RAFT approach was employed. Photoiniferter RAFT was chosen to avoid the addition of exogenous catalysts.

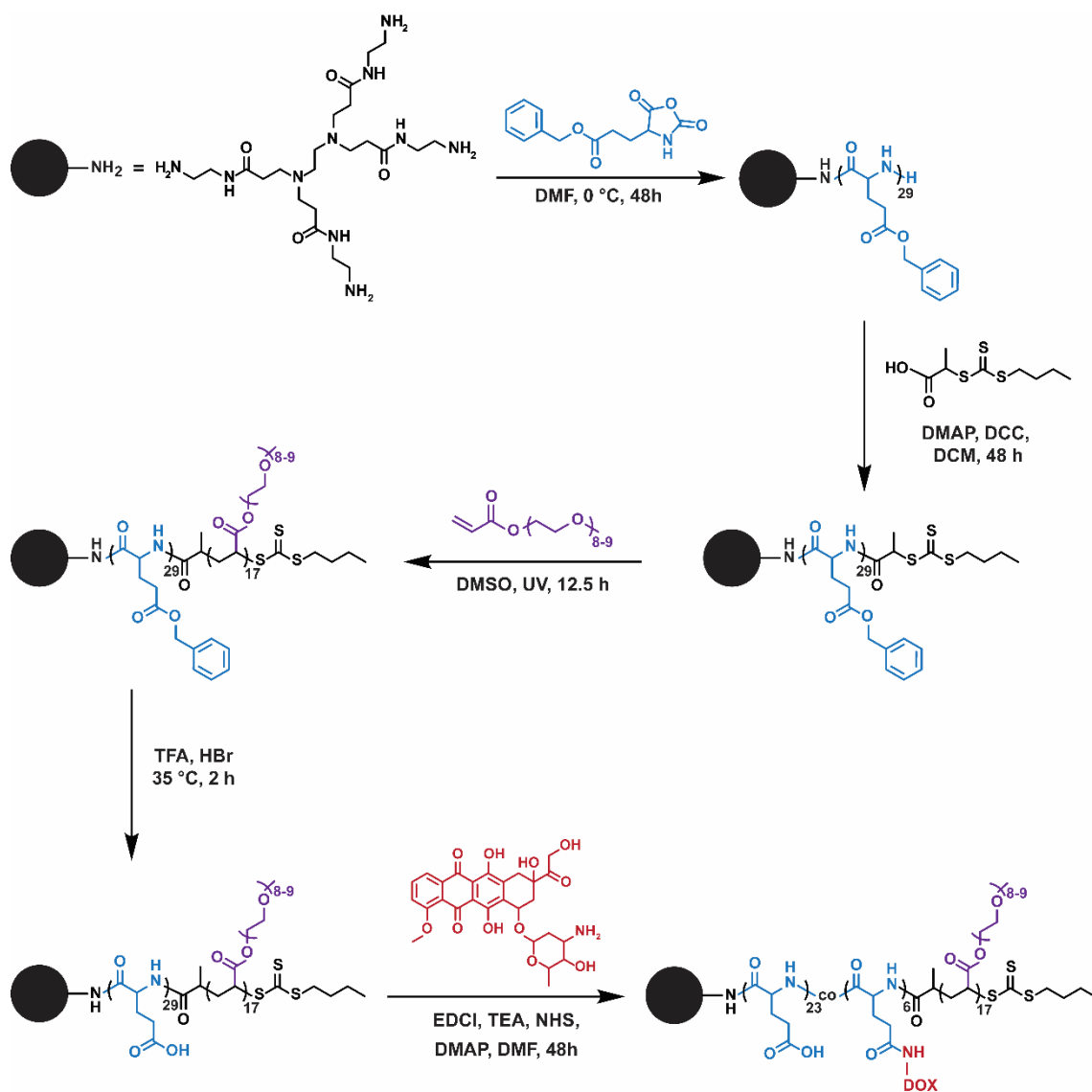


Figure 4.3 Synthetic scheme for the degradable polypeptide-based star polymer.

To enable photoiniferter RAFT polymerization from the PAMAM-PBLG star, RAFT agent 2-(n-butylthiocarbonylthio) propionic acid (BTPA) was conjugated to the terminal amine of each polypeptide arm via dicyclohexyl carbodiimide (DCC) coupling. A 2× excess of BTPA was used to ensure functionalization of all 4 terminal amines. Photoiniferter RAFT polymerization of poly(ethylene glycol) methyl ether acrylate (PEGA, M_n 480 Da) was performed in DMSO under UV light irradiation. Monomer conversion was monitored via NMR, with 46% conversion achieved in 12.5 hours, resulting in a poly(PEGA) (PPEGA) brush corona and a theoretical molecular weight of 54.1 kDa. GPC analysis showed a monomodal peak with M_n 21.1 kDa, M_w 27.7 kDa, and a dispersity of 1.19 (Figure 4.4).

Star polymers are notoriously difficult to analyse using linear standards because the different architecture alters their interactions with the chromatography beads. Although both stars were analysed in comparison to poly(styrene) standards, they were analysed on different GPC systems, which had different columns and different solvents as eluents. Exacerbating the situation, the PPEGA corona is highly hydrophilic while the PBLG is hydrophobic, which results in very different column interactions. Therefore, the theoretical molecular weights from NMR conversion were considered most accurate. Despite the difficulty using linear standards to determine molecular weight from GPC, it is clear from the GPC traces that the star polymers are monomodal and with reasonable dispersities.

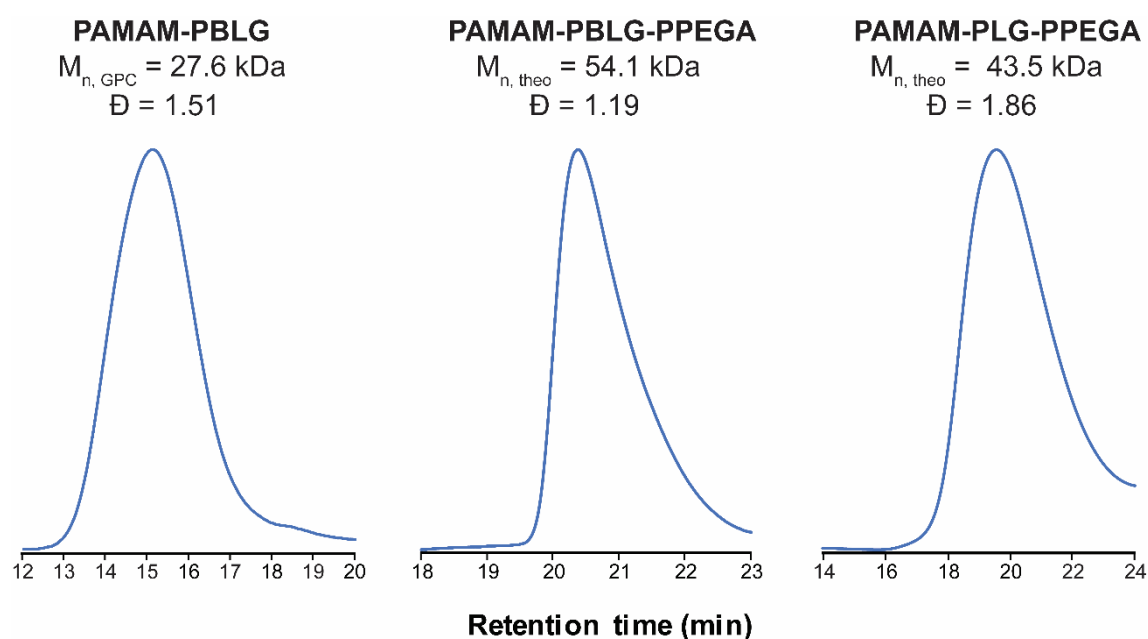


Figure 4.4 Gel permeation chromatogram of PAMAM-PBLG, PAMAM-PBLG-PPEGA, and PAMAM-PLG-PPEGA. $M_{n, GPC}$ and dispersity were determined using poly(styrene) standards for PAMAM-PBLG and PAMAM-PBLG-PPEGA, and PEG standards for PAMAM-PLG-PPEGA. $M_{n, theo}$ was determined from NMR conversion.

Upon completion of all polymerization steps, the γ -benzyl ester protecting group of the glutamic acid repeat units was removed under acidic conditions. GPC analysis of the isolate showed a monomodal albeit slightly broad peak, with a dispersity of 1.86 (Figure 4.4). Meanwhile, NMR analysis showed the peaks corresponding to the protons of the γ -benzyl ester protecting group at 5.1 and 7.3 ppm had disappeared, confirming all protecting groups were removed (Figure 4.5).

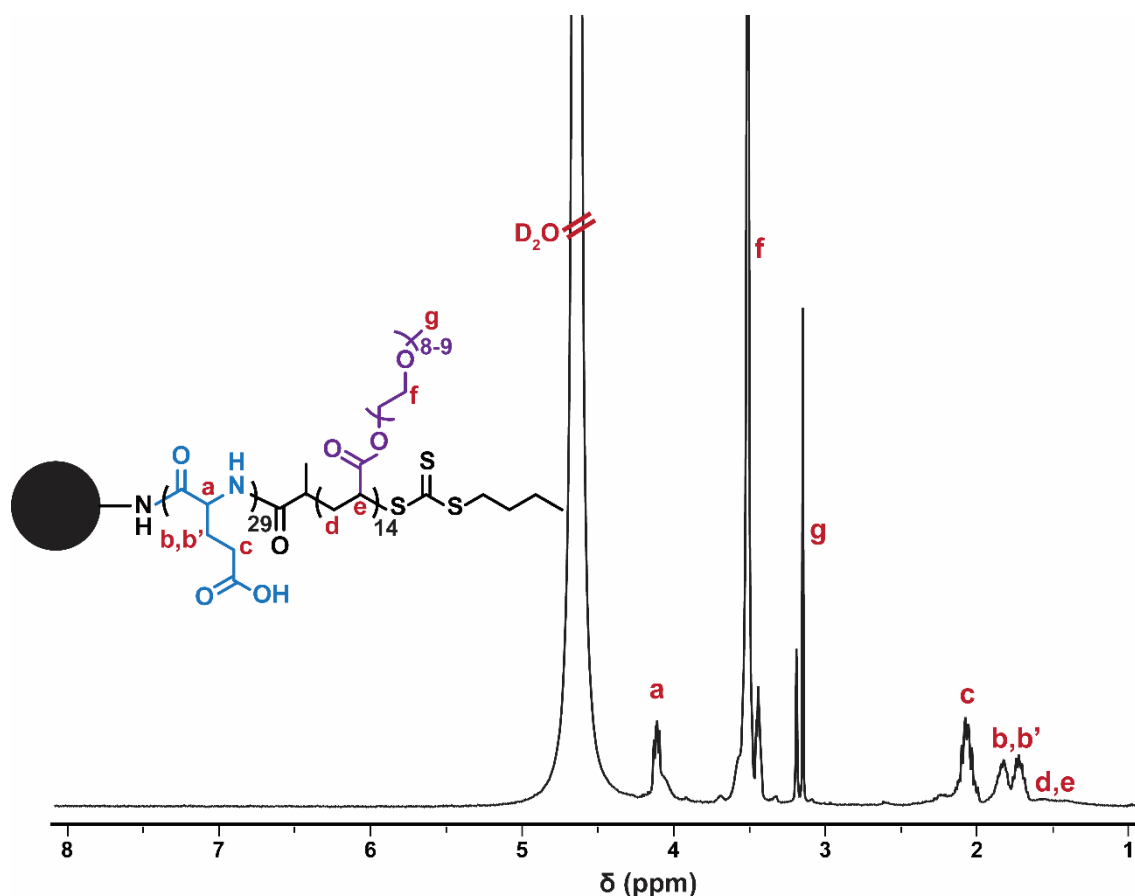


Figure 4.5 ^1H NMR spectrum of PAMAM-PLG-PPEGA isolate in D_2O . The chemical structure of PAMAM-PLG-PPEGA is shown with the corresponding protons and spectroscopy peaks labelled in red.

4.3.2 Nondegradable star

An analogous, nondegradable star polymer was synthesized for comparison to the polypeptide-based star polymer, following the synthetic pathway illustrated in Figure 4.6. Building on our success in Chapter 2, the nondegradable star was synthesized via photoiniferter RAFT polymerization using a core-first approach, employing the 4-XAN core reported in Chapter 2. The monomer tert-butyl acrylate (tBA) was chosen for the inner block because it can readily be converted to poly(acrylic acid), enabling DOX conjugation to the acid group using the same method as the polypeptide star. Exploiting the high conversion attainable with the core-first photoiniferter RAFT approach, polymerization of tBA and PEGA were performed “one-pot”. Polymerization of tBA (DP 50/arm) was first carried out under purple light irradiation with > 99% conversion observed after 18 h (XAN-PtBA). PEGA was added directly to the reaction mixture and polymerization was carried out under UV irradiation for 75 minutes, at which point 90% conversion was achieved (XAN-PtBA-PPEGA).

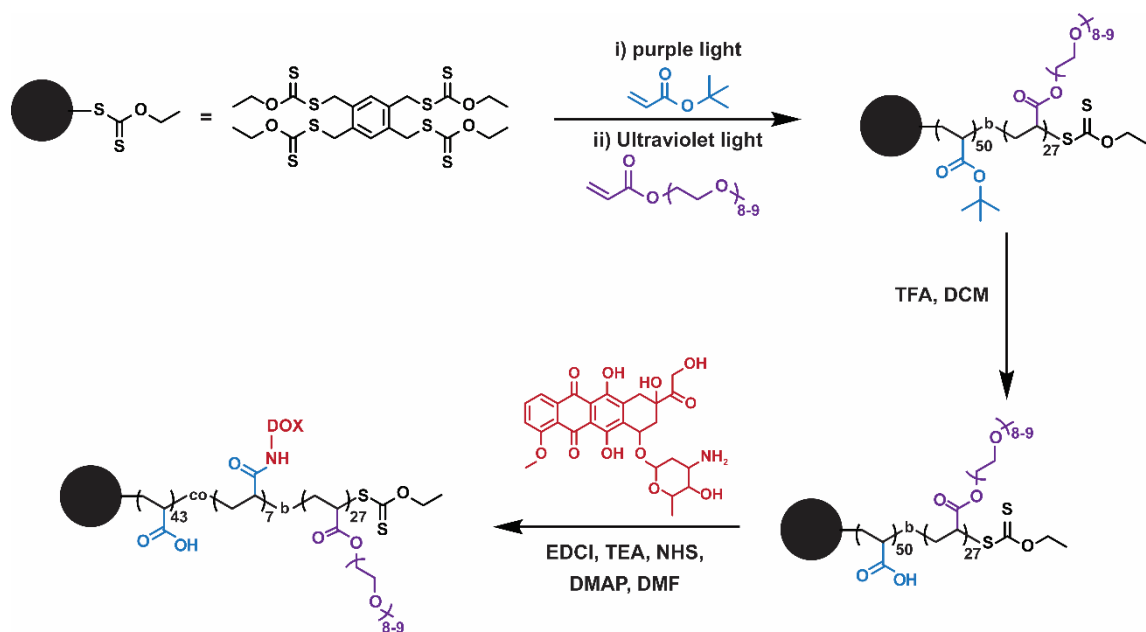


Figure 4.6 Schematic of the synthetic pathway of the nondegradable star polymer.

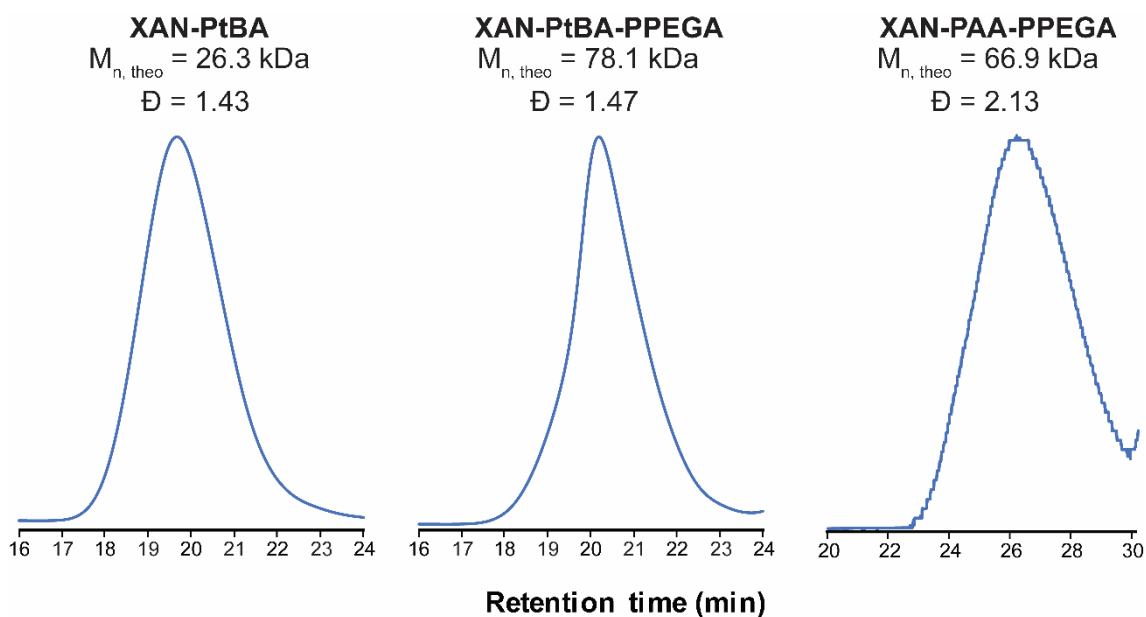


Figure 4.7 Gel permeation chromatograms of star-PtBA, star-PtBA-PPEGA, and star-PAA-PPEGA. $M_{n, \text{theo}}$ was determined from NMR conversion. Dispersity was determined using poly(styrene) standards for star-PtBA and star-PtBA-PPEGA, and PEG standards for star-PAA-PPEGA.

GPC analysis after each step demonstrated symmetrical, monomodal peaks (Figure 4.7). The dispersities of XAN-PtBA and XAN-PtBA-PPEGA were 1.43 and 1.47, respectively, and were consistent with previously reported RAFT polymerizations using xanthate chain transfer agents. Following the polymerization of PEGA, the tert-butyl protecting groups were removed in acidic conditions to yield a four-arm poly(acrylic acid)-block-PPEGA star polymer (XAN-PAA-PPEGA) (Figure 4.8). The measured dispersity of XAN-PAA-PPEGA was significantly higher at 2.13, but this is likely caused at least in part by the appearance of the solvent peak at 30 minutes, before the refractive index signal returned to baseline.

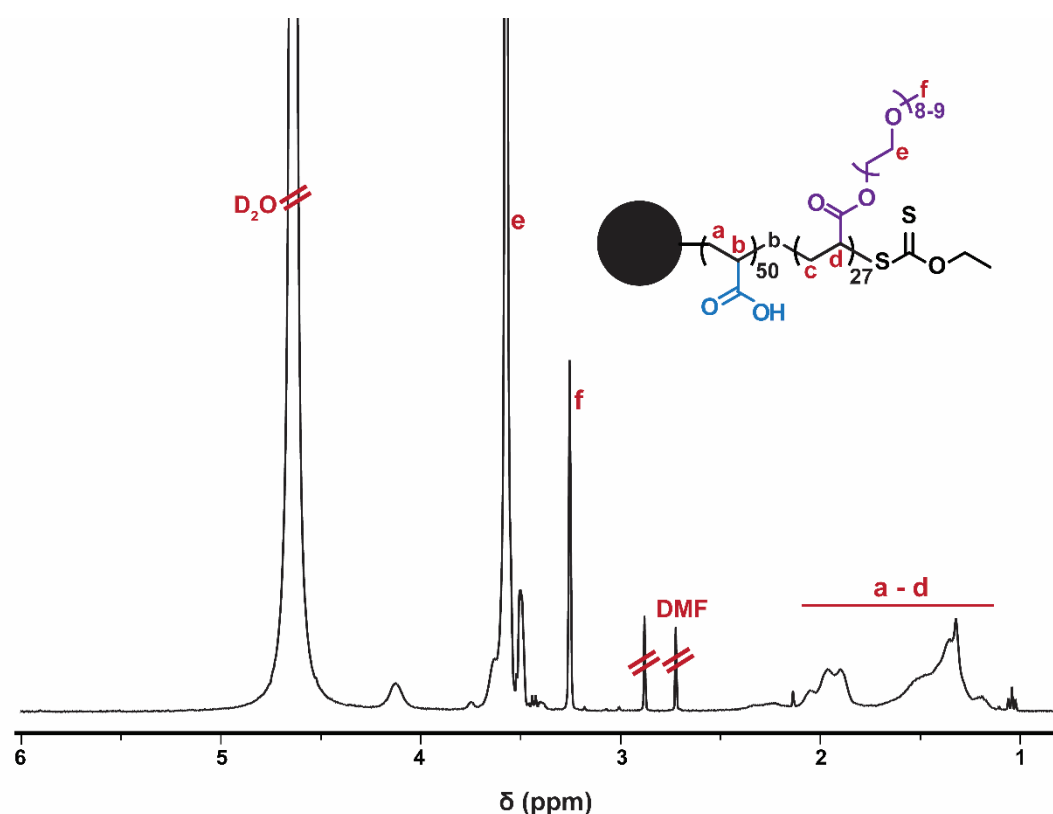


Figure 4.8 ^1H NMR spectrum of PAMAM-PLG-PPEGA isolate in D_2O . The chemical structure of PAMAM-PLG-PPEGA is shown with the corresponding protons and spectroscopy peaks labelled in red.

4.3.3 Drug conjugation to star polymers

Upon successful synthesis of the polypeptide and nondegradable star polymers, conjugation of DOX to the carboxylic acid side chains was performed via carbodiimide coupling using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI) hydrochloride. The conjugation of DOX was necessary to visualize cell uptake of the stars, transport to the lysosomes, and lysosomal escape, whilst also serving as a model drug.

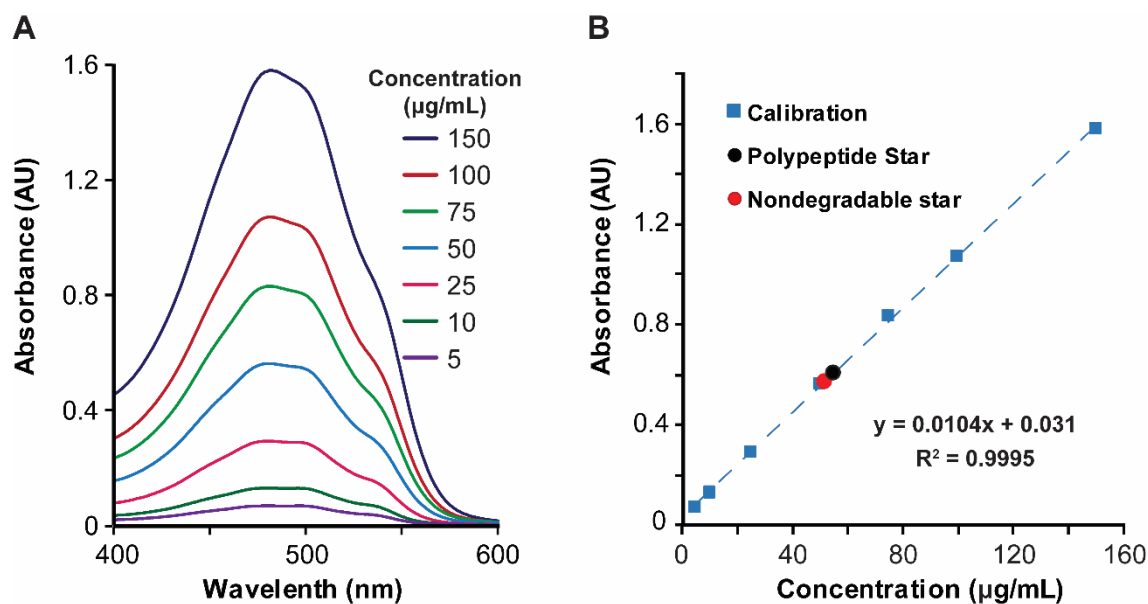


Figure 4.9 (A) Absorbance of free DOX at 480 nm was measured from 5-150 µg/mL to develop a standard curve. (B) Calibration curve of DOX based on absorbance at 480 nm, including the absorbance of the DOX-loaded star polymers at concentrations of 250 µg star/mL.

In order to quantify the amount of DOX conjugated per star polymer, a standard curve was made by measuring the absorbance of known concentrations of DOX in phosphate buffered saline (PBS) pH 7.4 at 480 nm, with concentrations ranging from 5 to 150 µg/mL (Figure 4.9A). The resulting standard curve was linear with an R^2 value of 0.9995 (Figure 3.7B). The DOX-loaded polypeptide and nondegradable stars were dissolved in PBS at concentrations of 250 µg/mL and their absorbance measured at 480 nm. Using the standard curve, the polypeptide and nondegradable star polymers were found to have 55.1 and 51.7 µg of DOX per 250 µg of star polymer, corresponding to DOX loadings of 22.0 and 20.7% by mass, respectively. Using the molecular weight of the star polymers, this corresponds to 22 DOX/star for polypeptide star and 28 DOX/star for the nondegradable star.

The properties of the DOX-loaded polypeptide and nondegradable star polymers are shown in Table 4.1. The DOX-loaded polypeptide and nondegradable stars have theoretical molecular weights of 55.5 and 82.1 kDa, respectively. The difference in molecular weight is due to the higher degree of polymerization of PEGA achieved with the nondegradable star. As previously mentioned, nanoparticles between 10 and 100 nm are most likely to accumulate within tumours, making both stars ideal for cancer therapy. Finally, both star polymers showed excellent DOX conjugation, with DOX representing approximately 20% of the star polymers by mass.

Table 4.1 The physical properties of the DOX-loaded polypeptide and nondegradable star polymers including theoretical molecular weight ($M_{n,theo}$), hydrodynamic diameter (d_h), and DOX loading as molecules per star and weight per cent (wt%).

Star Polymer		$M_{n,theo}$ (kDa)	d_h (nm)	DOX/star	DOX loading (wt%)
Polypeptide Star	PLG-g-DOX-PPEGA	55.5	16	22	22.0
Nondegradable star	PAA-g-DOX-PPEGA	82.1	21	28	20.7

4.3.4 Cell internalization and fate

Lysosomes are highly dynamic organelles containing over 50 hydrolytic enzymes, presenting a complex system difficult to accurately reproduce.⁹ To best understand how our star polymers would perform, we moved directly to cell assays.

In vitro assays were performed using a modified mouse fibroblast cell line to visualize cell internalization and trafficking of the DOX-loaded star polymers. These cells contained lysosomes transfected with a GFP-expressing lysosomal protein, so that all lysosomes can be visualized using fluorescent microscopy.

The uptake and cell fate of free DOX and the DOX-loaded nondegradable and polypeptide star polymers is shown in Figure 4.10. The first column shows DOX inside cells, which appears red, while in the second column the lysosomes are shown in green. The final column merges the first two columns, and the cell nuclei appear blue as a result of staining with Dapi. Colocalization of DOX and Dapi results in a purple colour, indicating DOX as reached the nucleus. Meanwhile, colocalization of DOX and lysosomes results in yellow, and clearly demonstrates where the DOX or DOX-loaded star polymers are within lysosomes.

As expected, treatment with free DOX results in DOX throughout the cell. DOX is a small molecule and can enter the cell via diffusion through the cell membrane.^{24,25} However, the fluorescent microscopy images of the DOX-loaded nondegradable star polymer differ significantly. The image in the first column shows less red throughout the cell, although some areas show a high density of red. The merged image shows very little red while yellow is ubiquitous, indicating colocalization of the nondegradable stars and lysosomes (Figure 4.11). This is likely caused by the stars accumulating within lysosomes. The lysosome is the terminal degradation centre of the cell, but the stars are not degradable and therefore remain trapped.

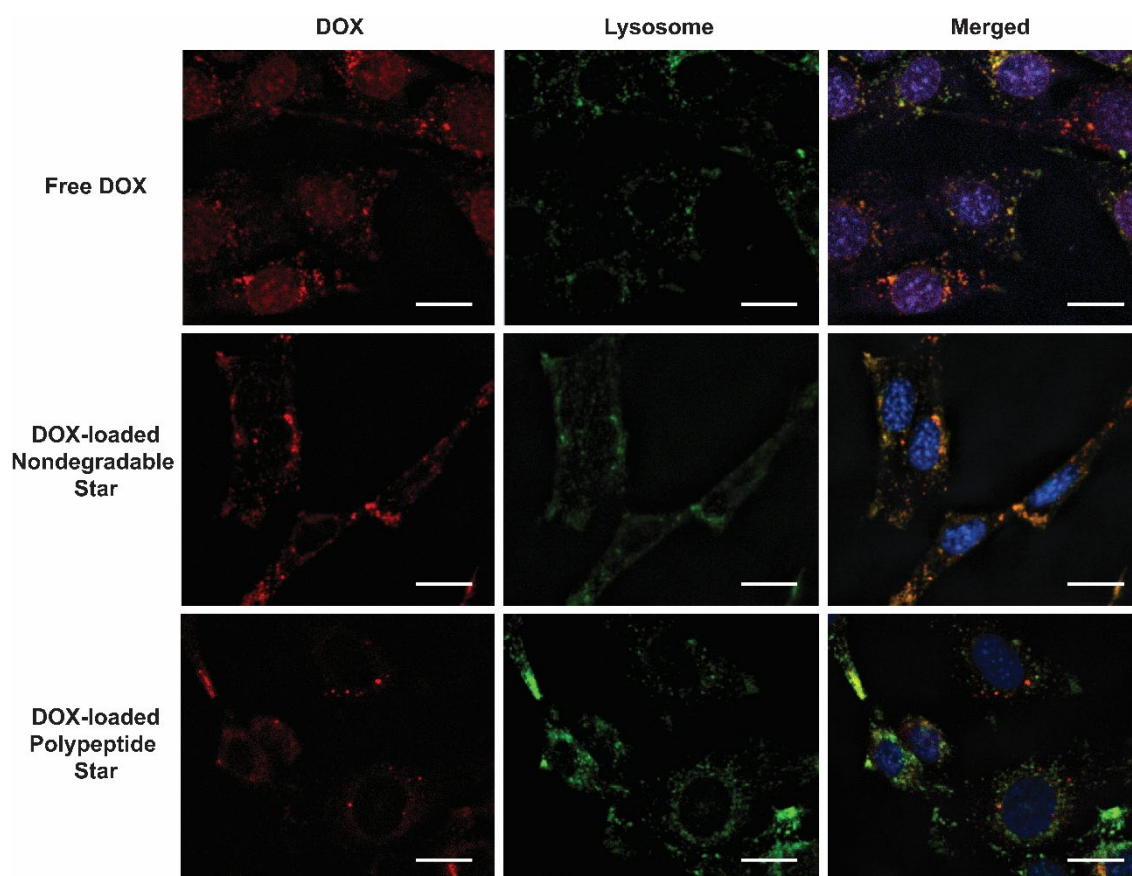


Figure 4.10 Fluorescent microscopy images of NIH 3T3 fibroblast cells treated with free DOX and DOX-loaded nondegradable and polypeptide star polymers. DOX is shown in red, lysosomes in green, and the nucleus blue. The scale bar represents 20 μm .

The final row shows the DOX-loaded polypeptide stars. The first column confirms that DOX-loaded polypeptide stars were taken up into the cells. The merged image clearly shows distinct red and green areas, with limited yellow, demonstrating that there is DOX in the cells that is not within the lysosome. An enlarged version of the merged image (Figure 4.12) shows miniscule red dots in between lysosomes and even within the nucleus. We believe this demonstrates degradation of the polypeptide backbone, releasing glutamic acid-DOX conjugates which can escape the lysosome via diffusion or amino acid effluxers.

The fluorescent images were taken 4 hours after the cells were treated with free DOX or DOX-loaded star polymers. Future works will include images taken at 1, 2, 4, and 6 hours to better understand how quickly the star polymers are degraded, as well as a greater number of images to quantitatively measure lysosomal and nuclear colocalization. In addition, methods of lysosomal amino acid efflux inhibition will be investigated to determine if the conjugated amino acids promote lysosomal escape.

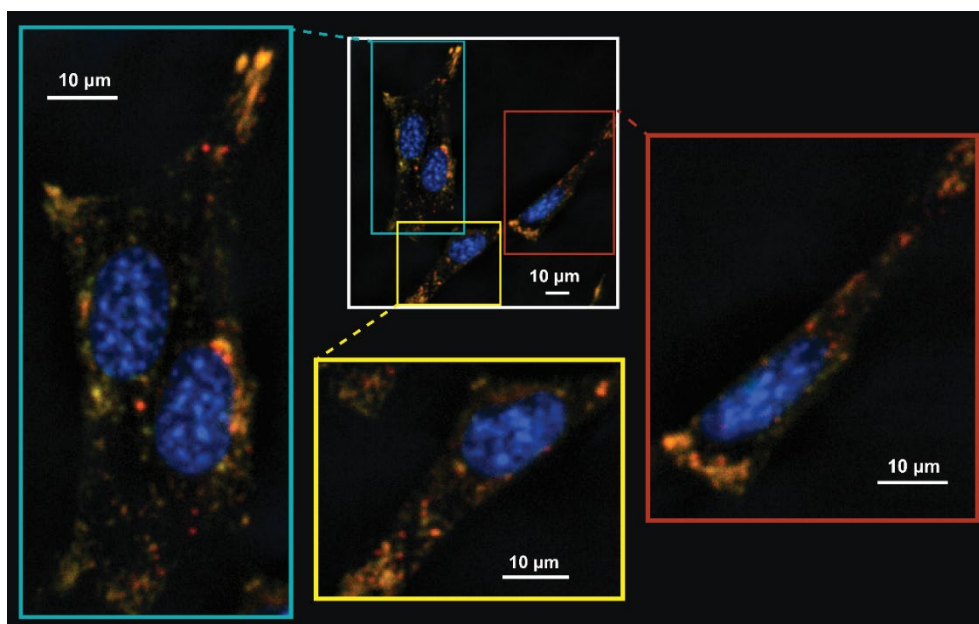


Figure 4.11 Enlarged fluorescent image of cells treated with the DOX-loaded nondegradable stars. The orange colour indicates the nondegradable stars are trapped in lysosomes.

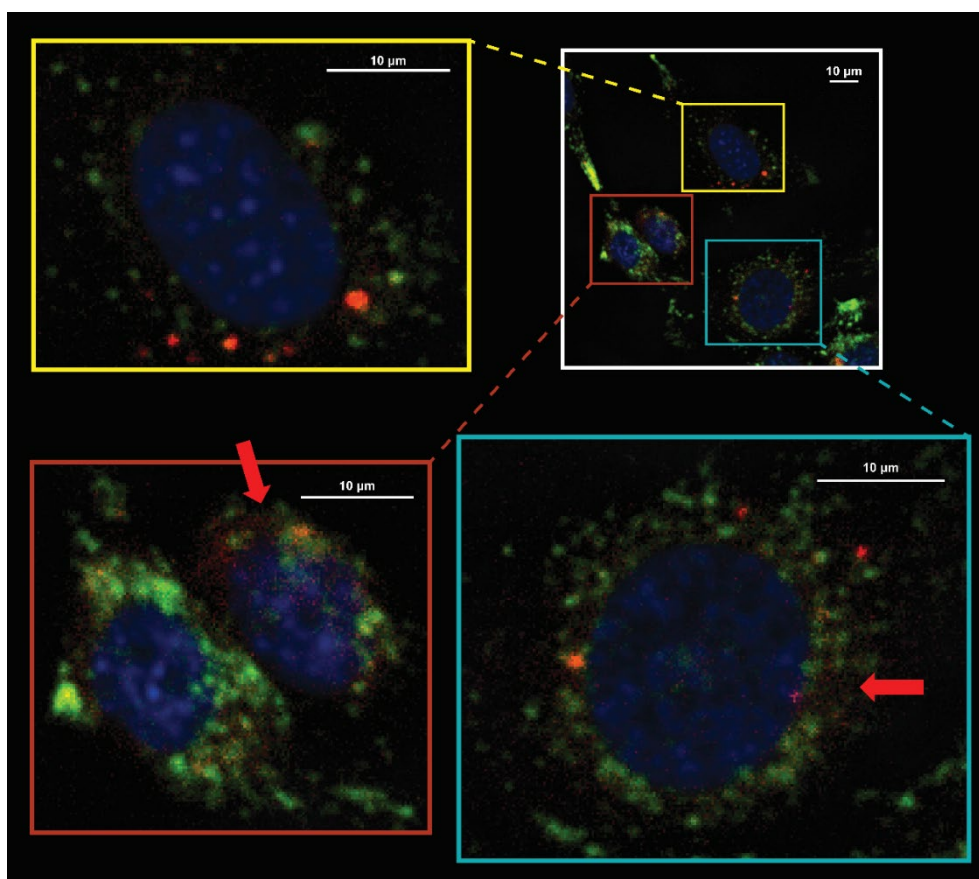


Figure 4.12 Enlarged fluorescent image of cells treated with the DOX-loaded polypeptide stars. Small red dots are visible throughout the cells, suggesting that Glu-DOX conjugates escaped the lysosome.

4.3.5 Cytotoxicity

The cytotoxicity of the DOX-loaded polypeptide and nondegradable stars was evaluated in comparison to free DOX in the human hepatoma cell line Huh7, to investigate the biocompatibility of the drug delivery systems. The cells were treated with equivalent doses of DOX, based on the amount of DOX in each star as determined from the DOX loading analysis. Cytotoxicity was evaluated at a range of concentrations, from 0.05 to 33.3 $\mu\text{g DOX/mL}$, in triplicate in three separate experiments (Figure 4.12). As expected, free DOX showed dose-dependent toxicity, with cell viability decreasing as the concentration of DOX increased.

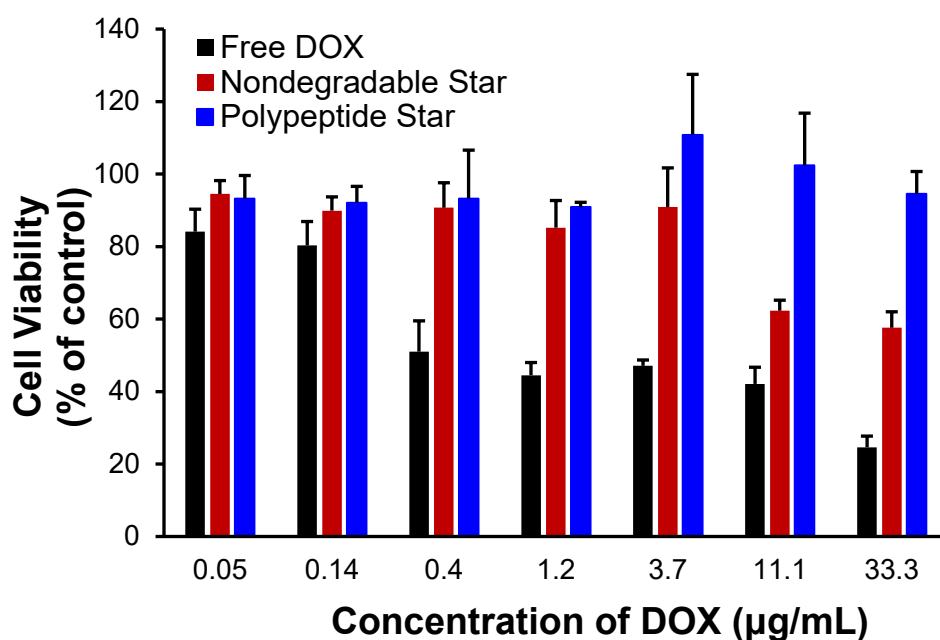


Figure 4.13 Cytotoxicity of free DOX, DOX-loaded nondegradable star, and the DOX-loaded polypeptide star in human hepatoma Huh7 cells as percent of control. Error bars represent standard deviation, n=9.

The toxicity of DOX conjugates is known to be highly dependent on the method of conjugation and whether DOX can be released from the conjugate. DOX conjugates are most commonly formed by two types of linkages; i) an amide bond, by conjugating the DOX primary amine to a carboxylic acid group, or ii) a pH responsive hydrazone bond using the non-cyclic ketone of DOX.^{26,27} Numerous studies have shown the amide linkage results in limited toxicity, especially in comparison to free DOX and the hydrazone linkage,²⁸⁻³⁰ most likely due to the essential role of the primary amine in DOX cytotoxicity. A single modification to DOX with an *N*-trifluoroacetyl group to protect the amine impairs the ability of DOX to bind to DNA, prevents disruption to DNA structure, and also reduced cytotoxicity by more than half in SXC01 human colon cancer cells *in vitro*.³¹

For our purposes, DOX served as a fluorescent dye to monitor the cell fate of the amino acid residues. The amide linkage was necessary to ensure that any lysosomal escape visualized was a result of the polypeptide backbone degradation. With this in mind, we expected DOX-loaded stars to demonstrate minimal cytotoxicity. The cell viability assay indicated the DOX-loaded polypeptide star is non-toxic, with cell viability greater than 90% observed at all concentrations. These results are consistent with previous reports where DOX was conjugated via an amide linkage. Although the cell tracking experiments demonstrate lysosomal escape, complete degradation of the polypeptide backbone would still result in DOX conjugated to a glutamic acid residue via the DOX amino group, limiting its therapeutic effect. These results demonstrate the biocompatibility of the polypeptide drug delivery system.

The nondegradable star polymer showed little to no toxicity from 0.05-3.7 $\mu\text{g DOX/mL}$. However, only 62.3 ± 2.9 and $57.6 \pm 4.4\%$ of cells survived treatment with 11.1 and 33.3 $\mu\text{g DOX/mL}$, respectively. We do not believe this toxicity was caused by the DOX, because our cell fate studies indicated the DOX-loaded nondegradable stars were trapped within the lysosome. We hypothesize the toxicity of the nondegradable star may be caused by its accumulation within lysosomes. As seen in Figure 3.7, the nondegradable star showed near complete colocalization with lysosomes. It is likely that accumulation of the stars in the lysosome are disrupting normal lysosome functions, leading to cell death. Numerous studies have shown that the accumulation of nanoparticles within lysosomes leads to lysosomal impairment, often *via* disruption of the autophagy-lysosomal pathway, and results in cell death.³²⁻³⁶

4.4 Conclusion

In this work we present a novel mechanism of endo-lysosomal escape, capitalizing on the protein recycling role of the lysosome. We synthesized a star polymer of an ideal size for cancer therapy, while incorporating an anti-fouling corona and degradable polypeptide arms to which fluorescent dye and chemotherapeutic agent DOX was conjugated. Fluorescent microscopy experiments with NIH 3T3 fibroblast cells demonstrated lysosomal escape of DOX into the cytoplasm, while the nondegradable control star remained trapped in the lysosome. Cytotoxicity studies with the human hepatoma cell line Huh7 clearly demonstrated the biocompatibility of the DOX-loaded polypeptide stars up to concentrations of 33 $\mu\text{g/mL}$, despite the toxicity of free DOX and the DOX-loaded nondegradable stars at an equivalent dose. This work represents an exciting development in endo-lysosomal escape that can be readily adapted for delivery of a wide range of chemotherapeutic agents. Future works will focus on gaining a better understanding of the lysosomal escape mechanism and expanding potential applications to nucleic acid and protein delivery.

4.5 Experimental Section

4.5.1 Materials

Monomers tert-butyl acrylate (98%) and poly(ethylene glycol) methyl ether acrylate, M_n 480 (PEGA), were purchased from Sigma and passed through a column of basic alumina to remove inhibitors. Potassium ethyl xanthate, 1,2,4,5-tetrakis(bromomethyl)benzene, triphosgene, triethylamine (TEA, $\geq 99.5\%$), 4-(dimethylamino)pyridine (DMAP), dicyclohexylcarbodiimide (DCC), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDCI), hydrobromic acid solution (HBr, 33wt % in acetic acid), trifluoroacetic acid (TFA, 99%), anhydrous *N,N'*-dimethylformamide (DMF, 99.8%), anhydrous pentane ($\geq 99\%$), dichloromethane (DCM), tetrahydrofuran (THF, $\geq 99.9\%$), deuterium oxide (D_2O) and doxorubicin hydrochloride (DOX) were purchased from Sigma and used as received. Dimethyl sulfoxide (DMSO, 99%, Chem-Supply), L-glutamic acid γ -benzyl ester (BLG, Mimotopes), G_0 poly(amidoamine) (PAMAM) dendrimer (37.97 wt %, Dendritech), and deuterated chloroform ($CDCl_3$, 99.5%, Cambridge Isotope Laboratories) were used as received. 2-(*n*-butylthiocarbonylthio) propionic acid (BTPA) was generously donated by Dulux. A Beaufly Nail Lamp was used as the UV light source (36 W, $\lambda_{max} = 365$ nm). The purple photoreactor was made using commercial strip lighting (60 LEDs/m 12V (DC) 5 m total strip length, working distance of approximately 5 cm) wrapped around the inside of a cardboard cylinder. The LED light emission spectrum ($\lambda_{max} = 401$ nm) was measured using a Maya 2000Pro spectrometer fitted with optical fibre (OceanOptics 100UV).

Characterization

4.5.1.1 Nuclear Magnetic Resonance (NMR) Spectroscopy

1H spectroscopic analysis was conducted using a Varian Unity Plus 400 MHz spectrometer operating at 400 MHz, with deuterated chloroform or deuterated water used as a reference. All samples were analysed at a concentration of approximately 10 mg/mL.

4.5.1.2 Gel Permeation Chromatography (GPC)

DMF GPC

GPC characterization of PAMAM-PBLG star polymers was carried out using HPLC grade DMF containing 0.05 M lithium bromide as eluent at a flow rate of 1 mL/min. GPC analysis was performed at 50°C using a modular Shimadzu system comprised of a Shimadzu RID-10A interferometric refractometer ($\lambda = 633$ nm), a Shimadzu SPD-20A UV-Vis detector, and a PostNova Analytics MALS detector ($\lambda = 658$ nm), with three phenogel columns (Phenomenex, 5 μ m bead size) in series. All samples were filtered through a 0.45 μ m nylon filter prior to injection.

THF GPC

GPC characterization of PAMAM-PBLG-PPEGA, PtBA, and PtBA-PPEGA, star polymers was carried out using HPLC grade tetrahydrofuran as eluent at a flow rate of 1 mL/min. GPC analysis was performed at 50 °C using a modular Shimadzu system comprised of a Shimadzu RID-10A interferometric refractometer ($\lambda = 633$ nm) using three Agilent MIXED-C columns (5 μ m bead size) operating at 45°C. All samples were filtered through a 0.45 μ m teflon filter prior to injection.

Aqueous GPC

GPC characterisation of PAMAM-PLG-PPEGA and PAA-PPEGA star polymers was carried out using Milli-Q water containing 0.1% (v/v) trifluoroacetic acid as eluent at a flow rate of 1 mL/min. GPC analysis was performed at 25 °C using a modular Shimadzu system comprised of a Shimadzu RID-10A interferometric refractometer ($\lambda = 633$ nm), Wyatt 3-angle light scattering detector, and three Waters Ultrahydrogel columns in series ((i) 250 Å porosity, 6 μ m bead size; (ii) and (iii) linear, 10 μ m bead size). All samples were filtered through a 0.45 μ m nylon filter prior to injection.

4.5.1.3 Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS) measurements were conducted using a Wyatt DynaPro NanoStar fitted with a 120 mW Ga–As laser operating at 658 nm; 100 mW was delivered to the sample cell. Analysis was performed at an angle of 90° at a constant temperature of 25 ± 0.01 °C. All sample concentrations were 0.1-1 mg/mL.

4.5.1.4 Fluorescent microscopy

Cells were imaged by fluorescence microscopy using a 60X 1.3 NA objective with a standard “Pinkel” DAPI/FITC/Cy3/Cy5 Filter set (Semrock). Emission was separated and captured using a 414/497/565/653 nm dichroic mirror and a quad-band bandpass emission filter. Images were analysed with Slidebook 6 (Intelligent Imaging Innovations, Denver, USA). For deconvolution, 10-16 slices were captured with a 0.33 μ m step size, exported and deconvolved using the Richard-Lucy algorithm^{37,38} with the CUDA³⁹ deconvolution plugin in ImageJ (Version 1.52g, Wayne Rasband, National Institute of Health, USA).

4.5.2 Methods

4.5.2.1 NCA Synthesis

γ -Benzyl glutamic acid (2 g, 8.43 mmol) was added to an oven-dried 250 mL double-necked round bottom flask under argon, followed by anhydrous THF (75 mL). Triphosgene (1.88 g, 6.32 mmol, 1.5 equivalents) was then added and the reaction was stirred under reflux at 60 °C for 2 h,

followed by 45 min sparging with argon into a saturated NaOH solution at room temperature. The solvent was removed under vacuum and the resulting residue recrystallized from anhydrous THF and anhydrous *n*-pentane overnight. Recrystallization was performed three more times the following day. The crystals were dissolved in minimal anhydrous THF and precipitated and washed with dry *n*-pentane to give white crystals with a yield of ~ 50%. ¹H NMR (400 MHz, CDCl₃) δH (ppm): 2.05-2.15 (m, 1H, CH₂CH₂CO), 2.22-2.32 (m, 1H, CH₂CH₂CO), 2.55-2.62 (t, 2H, CH₂CH₂CO), 4.33-4.38 (t, 1H, ring CHN), 5.12 (s, 2H, OCH₂C₆H₅), 6.43 (s, 1H, ring NH), 7.3-7.39 (m, 5H, C₆H₅).

4.5.2.2 Synthesis of degradable DOX-loaded PLG-PPEGA star polymer (PLG-g-DOX-PPEGA) *ROP of BLG NCA from G₀ PAMAM Dendrimer (PAMAM-PBLG)*

G₀ PAMAM dendrimer (37.97 wt% in methanol, 32.6 μL, 0.019 mmol) was added to a round bottom flask dried under vacuum. 10 mL anhydrous DMF was then added to the round bottom flask under argon with stirring until the dendrimer dissolved. BLG NCA (800 mg, 3.04 mmol) was dissolved in anhydrous DMF, added to the dendrimer solution, and stirred under argon for 48 h at 0 °C. DMF was removed under vacuum. The resulting residue was redissolved in minimal DCM and precipitated into cold diethyl ether. The precipitate was isolated and dried under vacuum to give a white powder. DMF GPC analysis indicated M_n 27.6 kDa, M_w 41.7 kDa, and Đ = 1.51, using poly(styrene) standards.

Conjugation of RAFT agent to PAMAM-PBLG (PAMAM-PBLG-TTC)

PAMAM-PBLG (1.26 g, 0.05 mmol), DMAP (25.9 mg, 0.212 mmol), and BTPA (96.1 mg, 0.40 mmol) were dissolved in 40 mL anhydrous DCM and degassed with argon from 15 min on ice. DCC was dissolved in 10 mL anhydrous DCM and added slowly to the reaction mixture, which was degassed for another 5 min. The reaction was left stirring under argon for 48 h, with the ice bath allowed to reach room temperature. The reaction mixture was covered with foil due to the light sensitivity of the BTPA.

After 48 h, the precipitate was removed via gravity filtration. The resulting solution was extracted 3 × each with 15 mL 0.5 M HCl and saturated bicarbonate solution. The solution was dried over magnesium sulphate and filtered via gravity filtration. The solvent was removed under vacuum and the resulting product precipitated and washed in cold diethyl ether. The precipitate was isolated and dried under vacuum, yield 280 mg.

RAFT polymerization of PEGA from PAMAM-PBLG-TTC (PAMAM-PBLG-PPEGA)

PAMAM-PBLG-TTC (150 mg, 0.006 mmol) and PEGA (353 mg, 0.74 mmol) were dissolved in 1 mL DMSO. After 12.5 h of UV irradiation, 46% monomer conversion was achieved, corresponding to 14 PEGA units/arm. The reaction mixture was precipitated and washed twice in cold diethyl ether. The precipitate was isolated and dried under vacuum, yield 116 mg. $M_{n, \text{theo}} = 54.1$ kDa. THF GPC analysis was performed using poly(styrene) standards, indicating M_n 21.2 kDa, M_w 27.7, and a dispersity of 1.19.

γ -Benzyl ester group deprotection (PAMAM-PLG-PPEGA)

PAMAM-PBLG-PPEGA (116 mg, 0.0023 mmol) was dissolved in 2 mL trifluoroacetic acid and 2 mL hydrobromic acid solution at 35 °C, with continuous stirring for 2 h. Once cooled, the product was precipitated and washed in cold diethyl ether. The precipitate was isolated and dried under vacuum, yield 76 mg. NMR spectroscopy of the isolated product showed complete removal of all γ -benzyl ester protecting groups (Figure 3.4).

DOX conjugation (PLG-g-DOX-PPEGA)

DOX (12.4 mg, 0.022 mmol) and TEA (12.2 mg, 0.12 mmol) were dissolved in 1 mL anhydrous DMF and stirred for 90 minutes. PAMAM-PLG-PPEGA (50 mg, 0.0011 mmol) was dissolved in 1 mL anhydrous DMF, to which NHS (16.7, 0.144 mmol) and EDCI (27.7, 0.144 mmol) were added. Solution containing DOX and TEA was added to the PAMAM-PLG-PPEGA solution and the vial washed twice with 0.5 mL anhydrous DMF. The reaction mixture was covered with foil and stirred continuously for 48 h. Product was dialyzed in 0.1 M NaHCO_3 aqueous solution overnight. The supernatant was replaced twice more with DI water, then isolated by freeze-drying.

4.5.2.3 Synthesis of nondegradable DOX-loaded PAA-PPEGA star polymer

4-arm xanthate initiator (4-XAN) (Same material as Chapter 2)

Potassium ethyl xanthogenate (2.68 g, 16.7 mmol) was added to a solution of 1,2,4,5-tetrakis(bromomethyl)benzene (1.5 g, 3.33 mmol) in acetone (30 mL) and stirred at room temperature for 20 h. The precipitate was filtered off and the organic solvent was removed under reduced pressure. The crude was dissolved in dichloromethane (DCM, 50 mL) and washed with water (20 mL $\times$ 3), brine (20 mL $\times$ 1), and dried over MgSO_4 . After solvent removal, the product was purified using column chromatography on silica gel (hexane: ethyl acetate 9:1) to give a light yellow solid (1.27 g, 62.13 % yield). NMR 400 MHz, ^1H NMR (CDCl_3); 7.37 (2H, s, $2\times\text{CH}$), 4.65 (8H, q, $J=7.1$ Hz, $4\times\text{CH}_2$), 4.39 (8H, s, $4\times\text{CH}_2$), 1.41 (12H, t, $J=7.1$ Hz, $4\times\text{CH}_3$).

RAFT polymerization of tBA and PEGA from 4-XAN (PtBA-PPEGA)

4-XAN (9.6 mg, 0.01561 mmol), and tBA (400 mg, 3.12 mg) were dissolved in 1.5 mL DMF (2M) in a Schlenk flask. The reaction mixture was deoxygenated by 3 freeze-pump-thaw cycles, then back-filled with ultra-high purity argon. The flask was placed in the photoreactor and left to stir under purple light irradiation for 18 h. The NMR spectroscopy indicated > 99% monomer conversion and a theoretical M_n of 26.3 kDa (50 repeat units per arm). GPC analysis using poly(styrene) standards indicated M_n of 36.4 kDa, M_w of 51.9 kDa, and a dispersity of 1.43.

PEGA (898 mg, 1.87 mmol) dissolved in DMF (890 μ L, 2M) was added to the flask containing the 4-XAN-PtBA star. A few drops of toluene were added as an internal standard to measure monomer conversion. Once again, the reaction mixture was deoxygenated by 3 freeze-pump-thaw cycles, then back-filled with ultra-high purity argon. The flask was placed in the photoreactor and left to stir under ultraviolet light irradiation for 75 min. NMR spectroscopy indicated 90% monomer conversion, equivalent to 27 PEGA units per arm. The reaction mixture was precipitated into cold diethyl ether. The precipitate was isolated and dried under vacuum. From NMR conversion, $M_{n,theo}$ is 78.1 kDa. GPC analysis using poly(styrene) standards indicated M_n of 24.9 kDa, M_w of 36.5 kDa, and a dispersity of 1.47.

Deprotection of tert-butyl group (PAA-PPEGA)

PtBA-PPEGA star polymer was dissolved 3 mL DCM, to which 3 mL TFA was added. The reaction mixture was stirred continuously at room temperature for 4 h. The solution was precipitated into cold diethyl ether. The precipitate was isolated and dried under vacuum. Theoretical M_n 66.9 kDa.

Doxorubicin conjugation (PAA-g-DOX-PPEGA)

DOX (17.3 mg, 0.03 mmol) and TEA (7.6 mg, 0.075 mmol) were dissolved in 1.5 mL anhydrous DMF and stirred for 90 minutes. PAA-PPEGA (100 mg, 0.0015 mmol) was dissolved in 2.5 mL anhydrous DMF, to which NHS (41.3 mg, 0.36 mmol) and EDCI (68.8 mg, 0.36 mmol) were added. Solution containing DOX and TEA was added to the PAA-PPEGA solution and the vial washed twice with 1 mL anhydrous DMF. The reaction mixture was covered with foil and stirred continuously for 44 h. Product was dialyzed in water adjusted to pH 10 with 2.5 M NaOH for 2 h, followed by 0.1 M NaHCO₃ aqueous solution overnight. The supernatant was replaced twice more with DI water, then isolated by freeze-drying.

4.5.2.4 Cell Culture

The human hepatoma cancer cell line Huh7 were cultured in Dulbecco's modified eagle medium containing 10% fetal calf serum at 37°C and 5% CO₂.

Mouse fibroblast cell line NIH 3T3 were transfected with the GFP-EEA1 wt plasmid (Addgene plasmid: 42307) using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instruction. Lentiviral transduction was performed using pCDH-EF1-mEmerald-Lysosomes-20-IRES-Puro and a third-generation lentiviral packaging system. NIH 3T3 cells were cultured in Dulbecco's modified eagle medium containing 10% fetal calf serum at 37°C and 5% CO₂.

4.5.2.5 Cell internalization and lysosomal escape

0.5 mg star polymer was reconstituted in 2.5 mL DMEM + 20% FBS and 0.5 mL 100 mM sodium bicarbonate to make 0.2 mg/mL solutions and were incubated overnight at room temperature, rotating. Free DOX at 2 mg/mL was diluted to 45 µg/mL in DMEM + 20% FBS.

3T3-NIH cells were seeded in 8 well chamber slides at 40,000 cells/well in 400 µL DMEM + 20% FBS one day prior to the experiment. Media was removed and replaced with the star polymer or free DOX solutions and allowed to incubate for 4 hours at 37° C. Cells were washed three times with DMEM + 20% FBS. Before imaging, the media was replaced with 400 µL FluoroBrite DMEM + 10% FBS and 5 µg/mL Hoechst 33342. Cells were then imaged by fluorescence microscopy and analysed as described in section 3.7.1.4.

4.5.2.6 Cytotoxicity Studies

Huh7 cells were seeded in a 96-well plate at a density of 5000 cells/well with 200 µL medium/well, and allowed to adhere overnight. Cells were treated with various concentrations of free DOX, DOX-loaded polypeptide stars (PLG-g-DOX-PPEGA), and DOX-loaded nondegradable stars (PAA-g-DOX-PPEGA) ranging from 0.05 to 33.3 µg/mL and incubated at 37°C for 48 hours. Three separate experiments were performed in triplicate. To each well 20 µL MTT reagent (Sigma) was added, then incubated for 4 hours, after which the supernatant was removed and 150 µL DMSO was added to each well to dissolve the formazan crystals. After 10 minutes shaking, the optical density at 570 nm (OD₅₇₀) was measured using a Victor TM X5 plate reader. The cell viability was calculated using the following formula, where the blank well contains only medium and the control well contains untreated cells:

$$\text{Cell viability (\%)} = \left(\frac{OD_{treated} - OD_{blank}}{OD_{control} - OD_{blank}} \right) \times 100\%$$

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Chapter 5

Conclusions and Future Perspectives

5.1 Conclusions

Star polymers are versatile materials with incredible potential in a wide range of applications but their study lags well behind that of their linear counterparts. RAFT polymerization offers exquisite control of polymer molecular weight, dispersity, and architecture, with photo-mediation offering unparalleled spatiotemporal control. In addition, the widespread availability of light makes photo-mediated RAFT polymerization very accessible. In this thesis, we sought to gain a better understanding of how photo-mediated RAFT polymerization can be applied to the synthesis of star polymers and their use in biomedical applications.

In **Chapter 2**, we investigated the synthesis of star polymers using a core-first, photoiniferter RAFT approach. The resulting star polymers were highly living, as demonstrated through polymerization kinetics and chain extension experiments. Based on our success and current interest in UHMW polymers, we synthesized UHMW star polymers under UV and visible light irradiation, achieving degrees of polymerization in excess of 10 000 repeat units per arm. Polymerization from a functionalized β -cyclodextrin core resulted in star polymers with molecular weights of 20 MDa, the largest star polymers synthesized to date.

In **Chapter 3** we reported a novel photocatalyst for broadband- and NIR-mediated polymerization in aqueous conditions via PET-RAFT polymerization. The broad absorbance of the photocatalyst (SA-TCPP) facilitated polymerization under white, blue, green, red, and NIR light irradiation. SA-TCPP was compared to three other photocatalysts that were previously reported for hydrogen evolution and showed the highest rate of polymerization for every wavelength used. We further demonstrated the versatility of our system by synthesizing a star polymer under NIR irradiation, using the 21-XAN core from Chapter 2. In addition, preliminary investigations with SA-TCPP resulted in the successful microlitre-scale polymerization of PEGMA in the presence of mammalian cells in complete cell culture medium.

Finally, in **Chapter 4** we synthesized dye-loaded star polymers to probe lysosomal escape from polypeptide nanoparticles. Star polymers were synthesized for these experiments because their unimolecular architecture and nanometre size make them promising candidates for cancer therapy. An inner polypeptide block was synthesized by NCA ROP, while a protective corona was synthesized via photoiniferter RAFT polymerization of PEGA. The straightforward nature

of photoiniferter RAFT polymerization enabled the facile synthesis of a nondegradable control star polymer. Conjugation of a fluorescent dye to the inner block side chains allowed us to monitor lysosomal escape. Fluorescent microscopy demonstrated that lysosomal escape only occurred when using the polypeptide-containing star, with nondegradable stars remaining trapped in the lysosome.

5.2 Future Perspectives

In **Chapter 2**, we reported the synthesis of UHMW star polymers, reaching the highest molecular weights reported via CRP techniques. As an initial investigative study, only dimethylacrylamide was used. Future work should broaden the class of monomers used. Xanthates are best suited to the polymerization of less activated monomers (LAMs) such as vinyl acetate, *N*-vinylpyrrolidone, and *N*-vinylcarbazole.¹ The synthesis of poly(vinyl acetate) (PVAc) is of particular interest; it is used industrially worldwide in adhesives, coatings, and paints, and is also the precursor for poly(vinyl alcohol) (PVA).² PVA is used across numerous industries and can be degraded by specialized microbes, making it a promising material as focus turns toward more environmentally friendly sources of plastic.^{3,4} UHMW star and linear PVAc and PVA polymers could therefore be of intense interest and their synthesis and physical properties require investigation.

Chapter 2 demonstrated that PDMA star polymers with DP 11 000 per arm could be readily synthesized but discrepancies between theoretical and experimental molecular weights were observed as higher DPs were attempted. We hypothesized this was due to extreme dilution of the photoactive species, in this case the xanthate RAFT agent. It would be interesting to see if a PET-RAFT approach could be used to increase the concentration of the photoactive species and lead to improved outcomes when attempting DPs greater than 11 000. Recent reports have demonstrated PET-RAFT polymerization can be used to synthesize UHMW linear polymers,^{5,6} so it seems probable this approach would also work for UHMW star polymers.

Finally, synthesis of materials from UHMW star polymers should be further investigated. Physically crosslinked gels and films can be synthesized from UHMW polymers due to entanglement of the polymer chains.⁷ The use of star polymers in these materials, where each arm is as long as most linear polymers, could therefore have very interesting effects.

The industrial application of RAFT polymerization has been slow, despite the widespread academic interest. This is most likely due to the cost of RAFT agent and the lack of necessity for

controlled molecular weight and low dispersity afforded by RAFT polymerization in many applications. I believe that the current developments in photo-mediated UHMW RAFT polymerization mean the time is ripe for industrial use of RAFT polymerization. UHMW cannot be synthesized using conventional radical polymerization, therefore use of a controlled radical polymerization method is essential. In addition, the amount of RAFT agent needed is dramatically reduced in the synthesis of UHMW polymers, greatly reducing the costs associated with RAFT polymerization. These factors together mean the use of RAFT polymerization is now more accessible and appealing for industry than ever before.

Chapter 3 employed a PET-RAFT approach to overcome the oxygen sensitivity and wavelength limitations of photoiniferter RAFT. The development of broadband and NIR-mediated polymerizations in aqueous conditions is significant due to potential biomedical applications. There is significant interest in being able to modify the surface of cells to manipulate cell behaviour or modify interactions. Recent work by the Hawker group demonstrated the first visible-light mediated polymerization from the surface of yeast and human Jurkat cells *via* PET-RAFT polymerization under blue light irradiation.⁸ The authors reported excellent biocompatibility, with 90% of cells surviving polymerization.

The use of a NIR-mediated system could allow cell surface modifications to occur *in vivo*. Therefore, improving the biocompatibility of the SA-TCPP system is an important future step. A NIR-mediated polymerization system could also be applied to *in vivo* crosslinking, offering true spatiotemporal control over gelation for *in situ* forming hydrogels. Although small molecule monomers and crosslinkers are often toxic, a non-toxic star or brush macro-crosslinker should improve biocompatibility. This type of externally controlled *in vivo* crosslinking could be an exciting development for tissue engineering and drug delivery applications.

While **Chapters 2 and 3** focused on synthetic developments of photo-mediated RAFT for star polymer synthesis, **Chapter 4** investigated a biomedical application. We demonstrated effective escape of a fluorescent dye from the lysosome when using a star polymer with a polypeptide component. Further works are needed to better understand this mechanism of lysosomal escape, such as whether the dye is escaping through diffusion or active transport. Does conjugation to an amino acid residue promote escape, or would the free dye escape equally well? Would the use of different amino acids promote escape? The polypeptide star synthesized in **Chapter 4** in conjunction with the NIH 3T3 cells with fluorescent lysosomes provide a straightforward platform for these studies. Once the system is better understood and optimized, the conjugation

of chemotherapeutic drugs such as cisplatin, camptothecin, and methotrexate can be investigated, as well as the incorporation of pH-responsive linkers to enable the release of functional DOX.

The use of RAFT polymerization to synthesize the protective corona also means that the PPEGA corona could readily be replaced with another polymer. Zwitterionic polymers such as polybetaines have shown anti-fouling properties comparable or even better than PEG-based materials.⁹⁻¹¹ It is important to keep in mind that although we do not want protein fouling to occur while the star polymers circulate in the blood stream, our system does require that enzymes be able to cleave the polypeptide backbone, so enzymes must be able to access the polypeptide segment. This means a balance between non-fouling and enzyme action is necessary, and it may be possible that a linear outer block will prove more accessible to lysosomal proteases than the bulky PEG-brush corona.

Finally, the presence of the RAFT agent at the star periphery lends itself to the conjugation of targeting ligands. Thiocarbonylthio groups are readily cleaved with primary amines to yield thiol-terminated polymers.¹² The resulting thiol groups could readily be conjugated to targeting ligands such as antibodies or aptamers using click chemistry to promote cancer cell targeting,^{13,14} or to form a gel for localized or long-term drug release.^{15,16}

5.3 References

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Star Polymers by RAFT Polymerization

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X.1 Star Polymers

Star polymers are a class of branched polymers in which three or more linear arms extend outwards from a central core. The dense, compact nature of star polymers can result in unique chemical, thermal, and rheological properties compared with their linear counterparts. In addition, the unique architecture of star polymers results in distinct core, arm, and peripheral regions that offer great versatility for customized structures.¹ As shown in Figure X.1, each region of the star can be altered and optimized, enabling endless possibilities.

Star polymers can be synthesized using numerous polymerization techniques with the most common being ring-opening polymerization (ROP), ring-opening metathesis polymerization (ROMP), and the controlled radical polymerization (CRP) methods atom transfer radical polymerization (ATRP), single electron transfer living radical polymerization (SET-LRP), nitroxide-mediated living radical polymerization (NMP), and reversible addition fragmentation-deactivation chain transfer (RAFT) polymerization.

ROP involves the ring-opening of cyclic monomers such as cyclic esters and O- and N-carboxyanhydrides (OCA and NCA, respectively) resulting in poly(esters) from cyclic ester and OCA monomers and poly(amides) from NCA monomers. Due to the biodegradable nature of the resulting polymers, ROP techniques are frequently used to synthesize polymers for biomedical applications. ROP typically requires the use of organic acids/bases or organometallic complexes to achieve fast reaction times and/or low dispersities. Unlike CRP, ROP is not sensitive to oxygen scavengers however protic contaminants such as water can initiate polymerization and induce side reactions, and even terminate reactions during NCA and OCA ROP. In addition, incorporation of compatible side chain functionality is more difficult and the number of commercially available monomers more limited than for CRP. ROMP also proceeds via ring-opening of cyclic monomers and results in well-defined polymers with readily modified end groups. The development of novel ruthenium and molybdenum catalysts has led a wider pool of monomers with a greater range of functionalities, however the presence of transition metals is often undesirable.

CRP techniques maintain high chain-end fidelity which ensures high conversions and low dispersities, however stringent oxygen-free environments are generally required. NMP was the first type of CRP developed however is less frequently used since the developments of ATRP and RAFT due to high reaction temperatures, poor control over methacrylate and methacrylamide monomers, and comparatively lower chain-end fidelity. Transition metal-mediated CRP including ATRP and SET-LRP have been widely used for the synthesis of star polymers using both the arm-first and core-first approaches. Although compatible of with a large array of vinylic monomers and capable of conversions greater than 95%, the use of a transition metal is required and not ideal for certain applications.

RAFT polymerization readily lends itself to the synthesis of star polymers. Compatible with an enormous range of monomers and multiple possible synthetic approaches, RAFT is a leading approach for star polymer synthesis. In addition, the development of photo-mediated RAFT has eradicated the necessity of additional initiators or catalysts. Although requiring further optimization, recent investigations into the use xanthates to control radical ROP demonstrate that RAFT polymerization may also be used to synthesize degradable polymers.

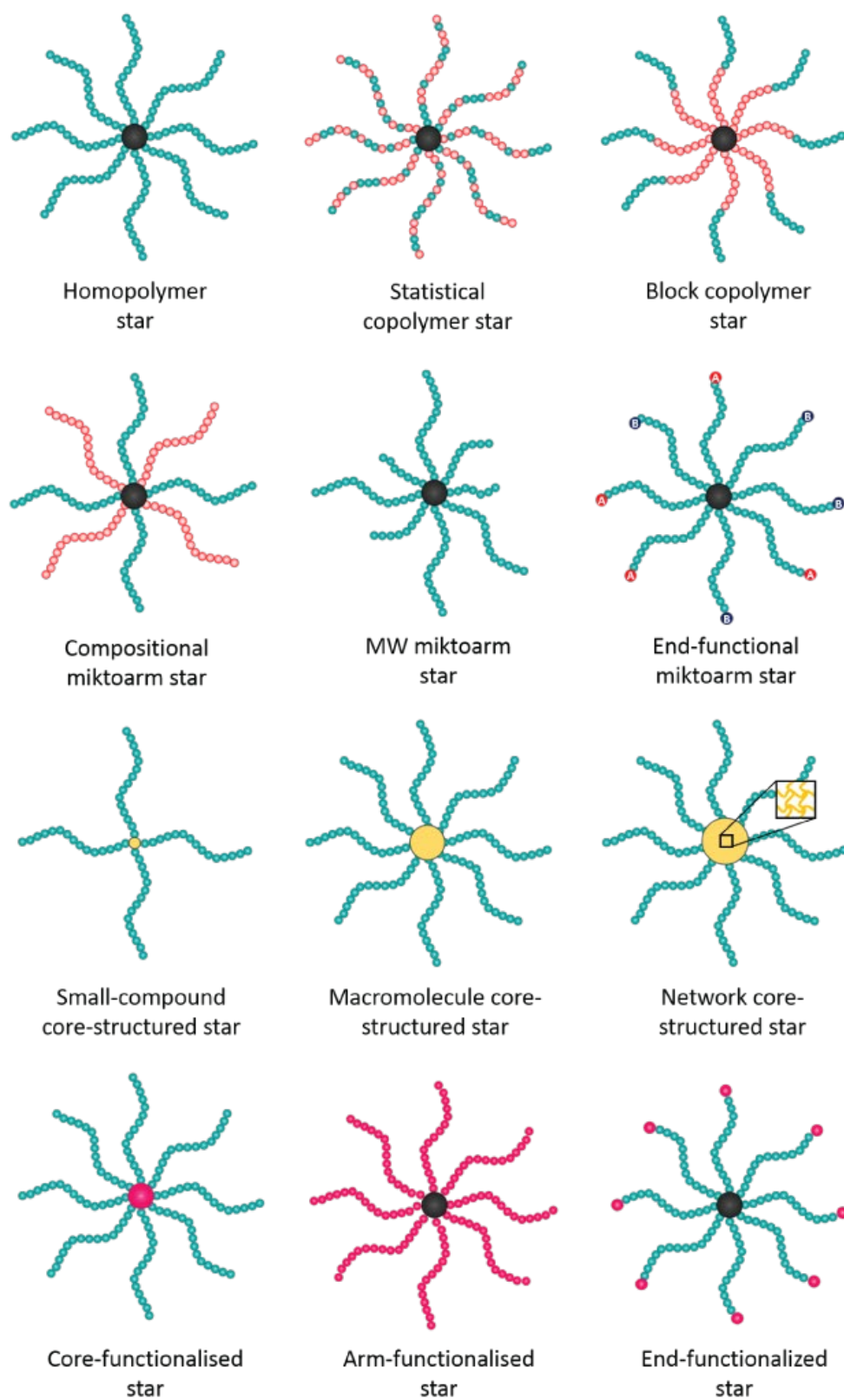


Figure X.1 An illustration of potential star polymer structures including stars with varied composition and sequence of arm polymer, possible miktoarm and core structures, and different region of functionalization.

X.2 Synthesis of Star Polymers via RAFT Polymerization

RAFT polymerization offers exceptional control over polymer molecular weights, dispersities, and is compatible with a wide range of monomers, making it an efficient and widely used method for the synthesis of star polymers. The two approaches to star polymer synthesis via RAFT polymerization are known as the core-first and arm-first approach. The advantages, disadvantages, and major developments for both approaches will be thoroughly discussed.

X.2.1 Core-First Approach

The core-first approach requires a multifunctional initiator from which arms can be polymerized. Any molecule that can be functionalized with 3 or more RAFT agents can serve as a core, enabling the use of a wide array of compounds and a great amount of freedom. Cores as varied as cyclodextrins,² porphyrins,³ hyperbranched polymers,⁴ metal-organic polyhedrons (MOPs),⁵ metal-organic macrocycles (MOMs),⁶ and quantum dots⁷ have been used for the synthesis of star polymers, with benzene- and pentaerythritol-based cores among the most commonly used.

When using the core-first approach, the number of arms is equal to the number of initiating sites and is therefore known prior to polymerization (assuming 100% initiation). This varies greatly from the arm-first approach, where the number of arms cannot be known until after polymerization and is not easily controlled. To achieve star polymers with low molecular weight dispersities, all initiating sites must have equal reactivity and 100% initiation efficiency. It is also important that the rate of initiation is greater than the rate of propagation and that either no or minimal chain termination occurs, both of which result in arms that contain different numbers of repeat units. The primary advantages of the core-first approach are high yields and facile purification, as low molecular weight monomers are easily separated from the resulting star polymers.¹

The core-first approach does suffer from some limitations. Star arms cannot be directly analysed unless cleaved from the core. The synthesis of star polymers containing more than one type of arm, known as miktoarm stars, is difficult as different initiating groups are required, although it has been demonstrated.^{8,9} Star polymers synthesized via the core-first approach generally have a smaller core domain and lower number of arms than star polymers synthesized using the arm-first approach, however the use of hyperbranched cores such as dendrimers has somewhat alleviated these limitations.

Perhaps the most challenging aspect of the core-first approach is the synthesis of the multifunctional core. Cores should have the same number of initiating sites to ensure the resulting star polymers have the same number of arms, arm molecular weight, and total molecular weight, any of which could impact the star properties. Purification is generally required after synthesis of the core, inhibiting one-pot star polymer synthesis. While the synthesis of cores is more commonly performed through conjugation chemistries, RAFT polymerization has been employed as a facile method of core synthesis.

Zhang et al reported the use of self-condensing vinyl polymerization (SCVP)-RAFT to synthesize a core using a polymerizable RAFT agent followed by polymerization from these RAFT agents to synthesize the star arms (Figure X.2).¹⁰ To form the hyperbranched macro chain transfer agent (CTA) core, a RAFT agent containing a styryl group was copolymerized with methyl acrylate or styrene. Branching points occurred when both the styryl and trithiocarbonate groups in one molecule participated in RAFT polymerization. A wide range of conditions were investigated and the authors concluded that the degree of branching, end group composition, and CTA functionality could be adjusted by altering the RAFT-monomer and comonomer ratios and reaction time. Although initial macroCTA cores had dispersities greater than 2, the authors were able to adjust conditions to achieve cores with dispersities as low as 1.16.

Hyperbranched macroCTA cores with approximately 7, 13, or 56 functional RAFT agents were subsequently used to polymerize styrene or *tert*-butyl acrylate to form star polymers. Theoretical and observed molecular weights were similar and dispersities ranged from 1.21-1.88, indicating controlled star polymers could be achieved using this approach. Subsequent works using RAFT to synthesize hyperbranched cores and subsequent polymerization of arms has further shown the versatility and ease of core-first star polymerization performed using RAFT.¹¹⁻¹³

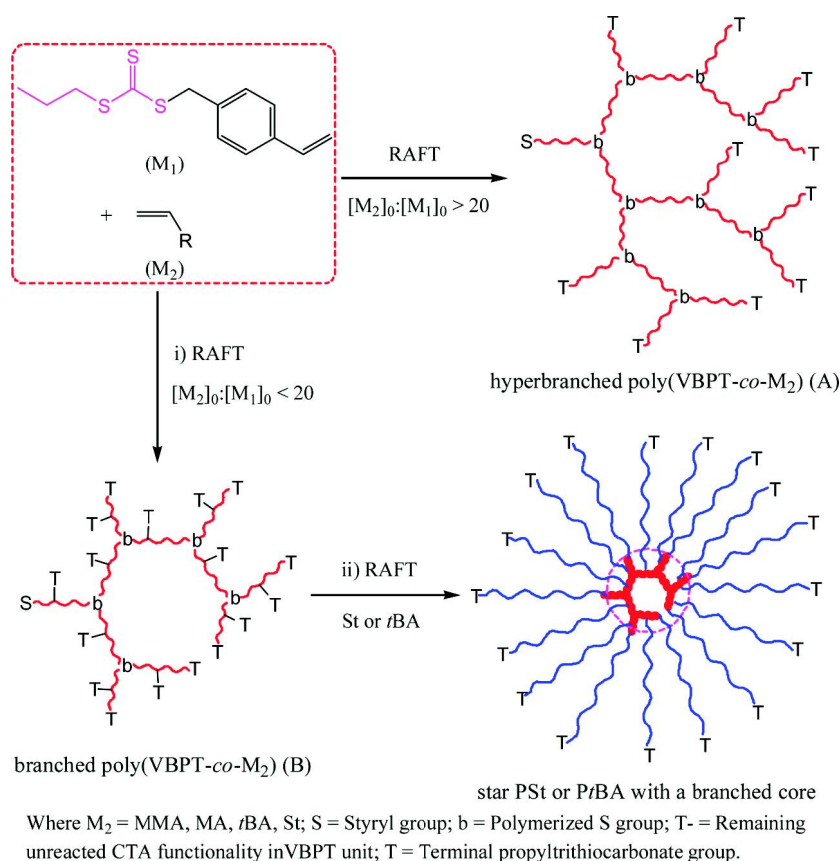


Figure X.2 Synthesis of hyperbranched cores using a styryl-RAFT monomer (M₁) and styrene or methyl acrylate (M₂). Once the core synthesis was optimized, subsequent polymerization from the core via RAFT resulted in well-defined star polymers.¹⁰ Reprinted with permission from *Macromolecules* 2011, 44, 7, 2034-2049. Copyright 2011 American Chemical Society.

While the core-first approach to star polymer synthesis is compatible with a wide range of polymerization techniques, star polymer synthesis via RAFT polymerization is unique due to the asymmetrical nature of RAFT agents, which contain a Z- and R-group (Figure X.3). Which group is conjugated to the core has important implications for the synthesis and functionality of the star polymer and should be considered prior to synthesis of the multifunctional core. Both approaches have advantages and disadvantages which have been extensively studied and will be discussed.^{1, 14, 15}

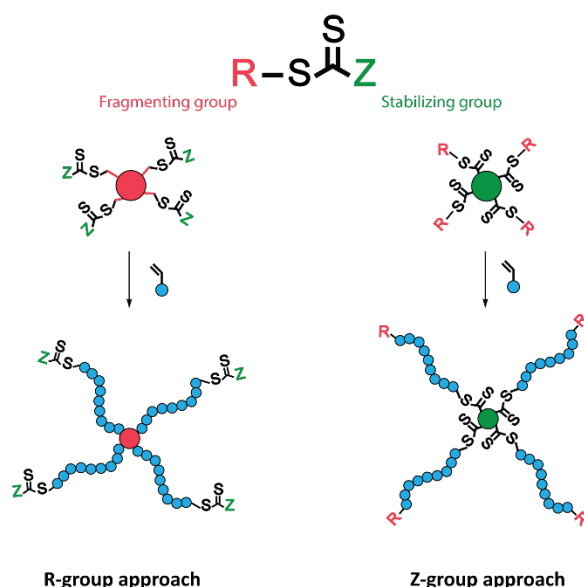


Figure X.3 RAFT polymerization of star polymers using the core-first R- and Z-group approaches.

X.2.1.1 Z-Group Approach

The Z-group approach is used when the Z-group of the RAFT agent is conjugated to the core. The R-group remains at the periphery of the star polymer while the thiocarbonylthio moiety remains at the core. As polymerization proceeds, a linear macroradical is formed away from the star polymer core. Because the propagating radical is not attached to the star core, star-star coupling is prevented. This avoidance of star-star coupling initially made the Z-group approach the preferred method of star polymer synthesis via the core-first approach. Termination events can still occur when two macroradicals react, producing a low molecular weight linear byproduct and resulting in the loss of at least one living arm from a star. However, this linear byproduct is much easier to purify from the desired star polymer than the high molecular weight byproducts resulting from star-star coupling. The mechanism of polymerization via the Z-group core-first approach is illustrated in Figure X.4.

The mechanism of polymerization does lead to certain limitations. Macroradical arms must reattach to the core, which becomes more difficult as the molecular weight of the arms and the number of arms is increased.^{2, 4} The location of the thiocarbonylthio group in the star core must also be carefully considered. The thiocarbonylthio group is sensitive to nucleophiles and radicals, both of which can cause destruction of the RAFT agent. With the RAFT agent located at the core, this means the destruction of a RAFT agent will lead to the loss of an arm. This could be intentionally exploited if a degradable star is desired or to analyse the arms following star polymer synthesis.^{16, 17} It is important to consider prior to synthesis if exposure to nucleophiles and radicals is likely during the intended application of the star polymer.

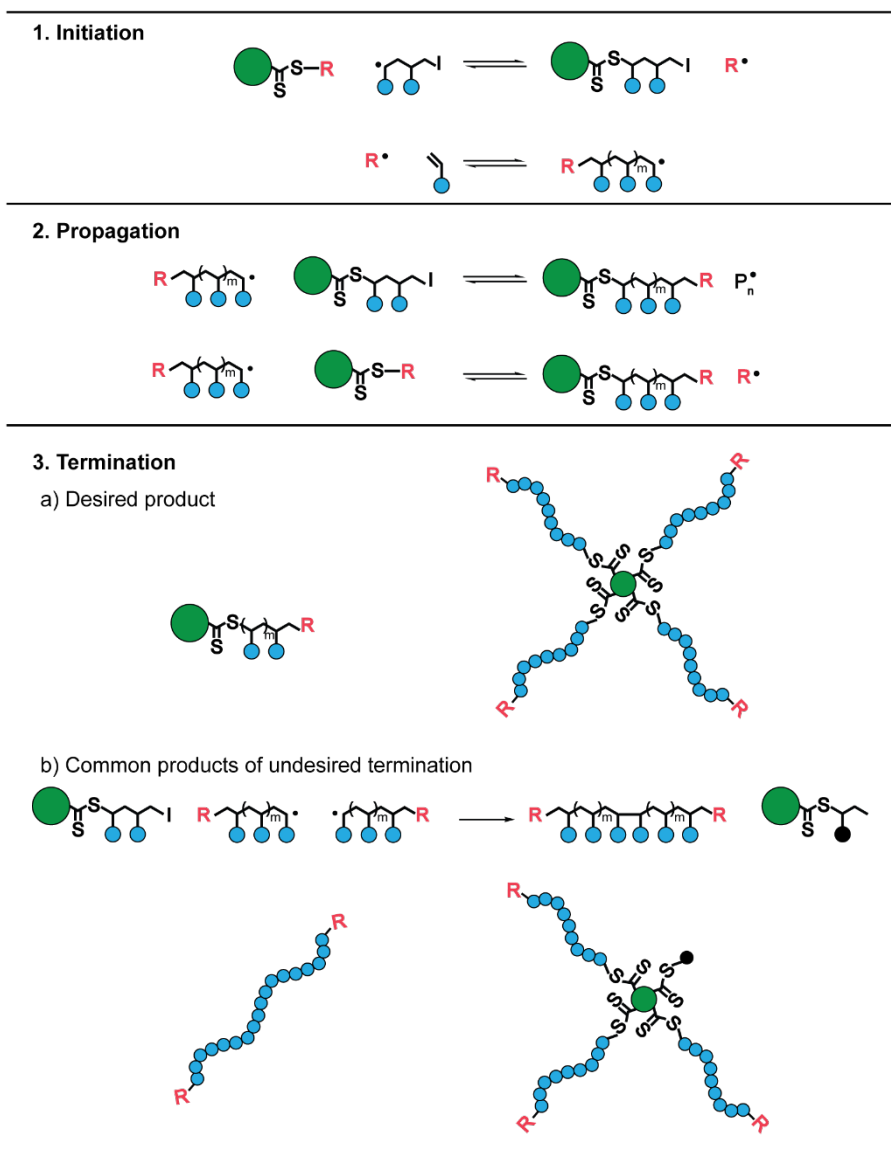


Figure X.4 RAFT polymerization of star polymers using the Z-group core-first approach. The thiocarbonylthio moiety remains attached to the core while the macroradical arms propagate away from the arm, then reattach. Unwanted termination can occur when macroradical arms react with each other, resulting in a linear byproduct and stars that have dead chain ends (black circle).

X.2.1.2 R-Group Approach

The R-group approach is used when the R-group of the RAFT agent is conjugated to the core. Due to the orientation of the RAFT agent, the thiocarbonylthio group is situated at the periphery of the star and the propagating radical moves outwards as polymerization progresses (Figure X.5). This has important implications for possible side and termination reactions throughout the polymerization. Because the propagating radical is at the star periphery, termination reactions between the growing arms can result in intra-star termination causing two dead arms, or inter-star termination, also known as star-star coupling. Both inter- and intra-star termination reactions will lead to high dispersities, however inter-star termination results in a high molecular weight product up to twice the intended molecular weight that can be difficult to remove.

The primary advantage of the R-group approach is that the thiocarbonylthio group remains at the periphery of the star. RAFT agents are often coloured, resulting in coloured products which is often undesired. The location of the RAFT agent at the exterior enables destruction of the RAFT agent to remove colour or to introduce additional functionality. The conversion of RAFT agents to functional groups has been extensively reviewed, with conversion to groups that can participate in click reactions being the most appealing.¹⁸⁻²⁰

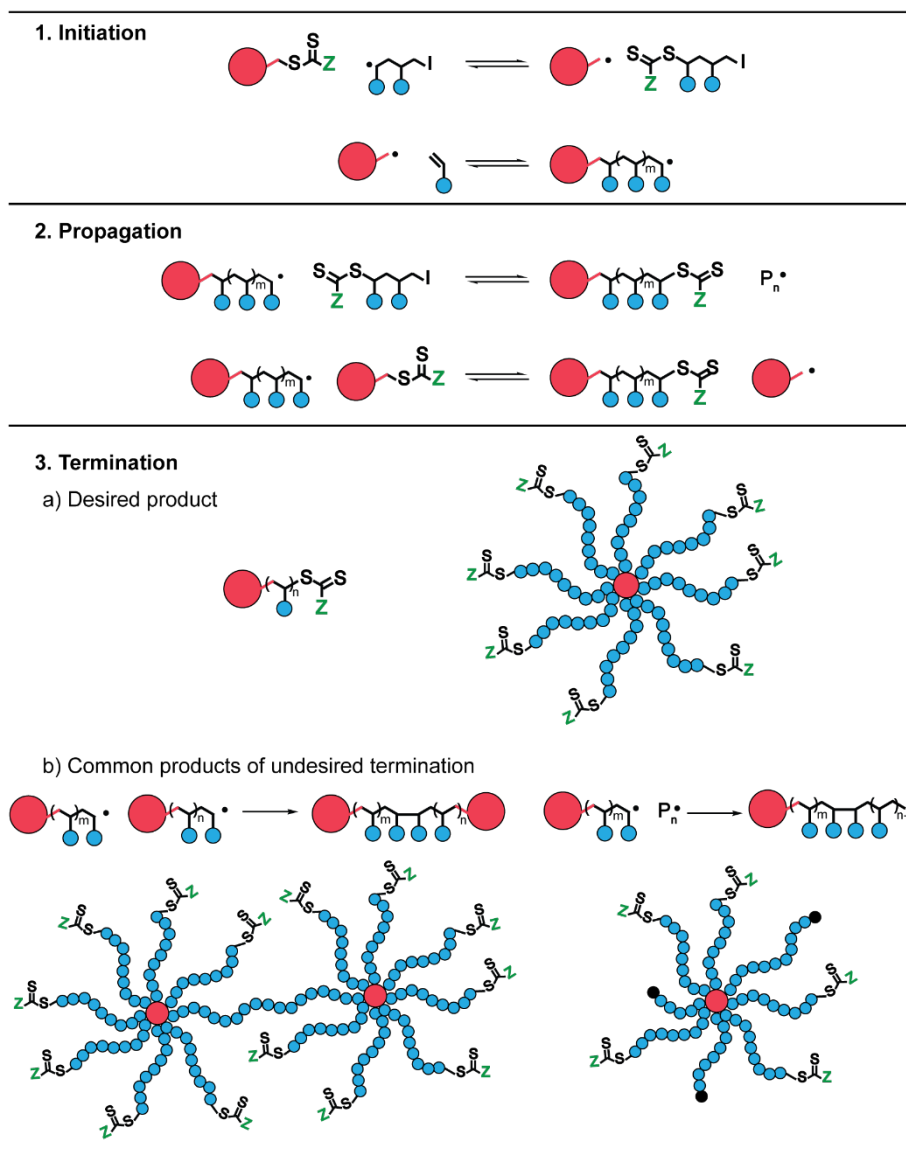


Figure X.5 RAFT polymerization of star polymers using the R-group core-first approach, where the propagating radical remains at the star periphery. Unwanted termination reactions can result in star-star coupling and dead polymer arms that no longer contain the thiocarbonylthio moiety.

The Maynard group exploited the terminal RAFT groups resulting from the R-group core-first approach to synthesize star poly(*N*-isopropylacrylamide)(PNIPAAm)-lysozyme conjugates.²¹ The polymerization of 4-arm PNIPAAm stars was stopped at 52% conversion to limit star-star coupling and ensure retention of the RAFT end group. Using a mechanism previously reported by Perrier et al,²² the RAFT agents were reacted with a symmetrical azo-initiator containing furan-protected maleimide groups to achieve star polymers with terminal protected maleimide groups. Once deprotected, the enzyme

lysozyme was conjugated to the star polymer via thiol-maleimide click chemistry.²¹ More recently, Wang et al synthesized 4-arm PNIPAAm stars and converted the RAFT group to an alkyne via one-pot aminolysis and subsequent Michael addition of propargyl acrylate (Figure X.6).²³ The 4-arm PNIPAAm star was then reacted with azide-functionalized β -cyclodextrin, forming a temperature-responsive PNIPAAm hydrogel. These examples highlight the ease with which star polymers synthesized using the R-group approach can undergo end-group functionalization.

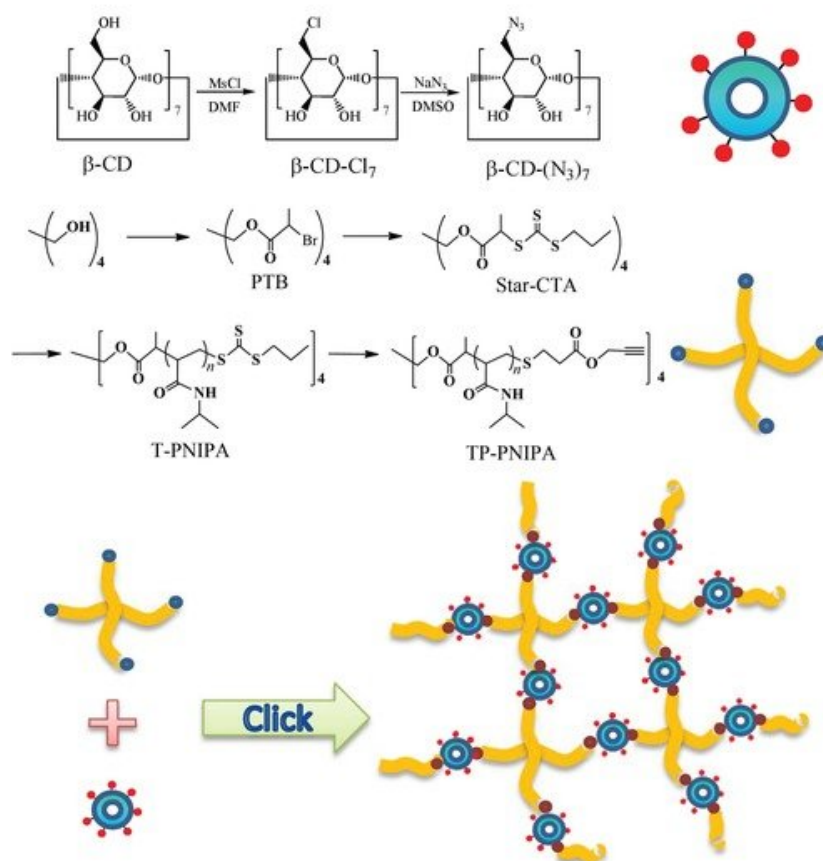


Figure X.6 Synthesis of star polymers via R-group core-first approach using RAFT polymerization. Terminal thiocarbonylthio groups were converted to alkynes, facilitating hydrogel synthesis through click chemistry.²³ Reprinted with permission from *Polymers* 2016, 8, 93.

X.2.1.3 Developments in Synthesis

While the R- and Z-group approaches for core-first star polymers has been well studied, there have been few developments that have altered the way the RAFT core-first approach is conducted since its inception. Most reactions are still carried out using thermal initiators such as AIBN and VA-044. Therefore the recent usage of photo-mediated RAFT polymerization for the synthesis of star polymers using the core-first approach has been a welcome and exciting development. Photoinduced electron/energy (PET)-RAFT polymerization involves a photoredox reaction between the thiocarbonylthio group and a photoredox catalyst.²⁴ The use of a photoredox catalyst provides temporal control over the polymerization, meaning reactions can be stopped and restarted at will. In addition, PET-RAFT can proceed in the presence of air, thereby eliminating the need for degassing and stringent oxygen-free conditions typically required for RAFT polymerizations.

The Boyer group reported the use of PET-RAFT to synthesize 3- and 4-armed stars with photocatalyst zinc tetraphenylporphyrin (ZnTPP).²⁵ The star polymers were reasonably well controlled with

dispersities ranging from 1.1 to 1.5 and used for screening structure-activity relationships. More recently, the Boyer and Lim groups used PET-RAFT to modify lanthanide-doped upconversion nanoparticles (UCNPs) which have unique photoluminescent properties with potential biomedical applications.²⁶ UCNPs were coated with silica and functionalized with RAFT agent, from which poly (oligoethylene glycol methyl ether methacrylate) (POEGMA) was polymerized. The addition of the POEGMA shell rendered the UCNPs water-soluble without affecting the upconversion emission spectrum of the particles.

Photo-mediated RAFT polymerization can also be performed via direct photolysis of the RAFT agent, a method known as photoiniferter RAFT polymerization. Linear polymers synthesized via photoiniferter RAFT polymerization have been shown to exhibit exceptional chain-end fidelity, leading to monomer conversions greater than 95% as well as multiblock and ultra-high molecular weight (UHMW) polymers.^{27, 28} Exploiting the high chain-end fidelity resulting from photoiniferter RAFT polymerization, the Qiao group synthesized UHMW star polymers with molecular weights in excess of 20 MDa using the R-group core-first approach.²⁹ The authors first demonstrated the controlled synthesis of poly(dimethylacrylamide) (PDMA) star polymers from tetrafunctional trithiocarbonate (4-TTC) and xanthate (4-XAN) cores under blue and purple light, respectively. Further experiments with the 4-XAN core resulted in star polymers with molecular weights of 3.8 MDa, equivalent to 0.96 MDa per arm, using both purple and UV light. Finally, a modified β -cyclodextrin core containing 21 RAFT agents was synthesized. A molecular weight of 23 MDa was targeted, with 96% conversion achieved after 18 hours of UV irradiation for a theoretical molecular weight of 21.9 MDa. This finding was corroborated by static light scattering as well as GPC analysis of the star polymer arms following their cleavage from the core. The authors reported difficulties synthesizing star polymers when molecular weights greater than 1 MDa per arm were targeted and attributed these difficulties to the extreme dilution of the chain transfer agent. A summary of the UHMW PDMA star polymers synthesized is shown in Table X.1.

Table X.1. Summary of UHMW PDMA star polymers synthesized via photoiniferter RAFT polymerization using the R-group, core-first approach.²⁹ Adapted with permission from *ACS Macro Lett.*, 2019, 8, 10, 1291-1295. Copyright 2019 American Chemical Society.

Number of arms	DP per arm	Target MW (MDa)	[M] ₀ (M)	Time (h)	Conversion (%) ^a	M _{n(theo)} (MDa) ^b	M _{W(SLS)} (MDa) ^c	R _G (nm) ^c
4	10 100	4	1	6	96	3.8	2.5	71.1
4 ^d	10 100	4	1	102	96	3.8	3.8	74.7
4	20 200	8	3	6	94	7.5	6.3	124.1
21	1 900	4	1	3.5	95	3.8	4.6	105.7
21	11 000	23	0.5	18	96	21.9	20.4	205.1
21	22 000	46	0.5	18	94	43.3	3.2	79.6

^a Conversion was determined using ¹H NMR spectroscopy.

^b Theoretical molecular weights (M_{n(theo)}) were determined via monomer conversion values.

^c M_{W(SLS)} and R_G were determined by static light scattering.

^d This polymerization was performed using purple light irradiation, while all others were irradiated with ultraviolet light. Polymers where experimental and theoretical results were in agreement are highlighted in purple. The largest reported RDRP star polymer, with M_{W(SLS)} of 20.4 MDa, is highlighted in dark purple and bold text.

The synthesis of UHMW diblock stars polymers was reported in 2019 by Dao, Cameron, and Saito.³⁰ Using a tetrafunctional CTA, the authors synthesized star polymers containing a neutral poly (acrylamide) inner block and a negatively charged poly (acrylic acid) outer block via the Z-group core-first approach. Size exclusion chromatograms of the star polymers showed bimodal distributions following polymerization of the first block and multimodal distributions following polymerization of the second block, indicating a loss of control and termination events. Despite the large dispersities and multimodal distributions observed, the synthesis of UHMW block star polymers is an exciting development in the field of polymer chemistry. In addition, the block star polymers showed promise as polymeric flocculants, highlighting potential applications of UHMW star polymers.

X.2.2 Arm-First Approach

The arm-first approach to star polymer synthesis requires the crosslinking of linear arms using di- or multi-functional crosslinking or coupling agents. Unlike the core-first approach, where the molecular weight of the core is often negligible, the arm-first approach results in a core that is a crosslinked network and typically represents approximately 30% of the star polymer's total molecular weight. Stars synthesized using the arm-first approach are often referred to as core-crosslinked stars (CCS), distinguishing them from stars synthesized using the core-first or grafting-onto approaches.¹

The arm-first approach offers several advantages over the core-first approach. Most importantly, the arm-first approach does not require synthesis of a multifunctional core, enabling one-pot synthesis. Unlike the core-first approach, the polymer arms are easily characterized prior to crosslinking. The unique nature of the core resulting from the arm-first approach lends itself to further functionalization, including the loading of functional compounds. In addition, miktoarm star polymers are more easily synthesized using the arm-first approach. There are however some disadvantages. Although a greater number of arms can be achieved with the arm first approach, the number of arms can only be determined after synthesis and controlling the number of arms and star molecular weight is difficult. Star polymers can also be difficult to purify if not all arms are incorporated into the star polymer, since the arms are of a high molecular weight and have similar properties to the star. Star-star coupling can also occur if the growing cores of separate stars come into contact.

The arm-first approach via RAFT polymerization can be further divided into the macroRAFT and macromonomer routes (Figure X.7). The macroRAFT method, also referred to as the macro chain transfer agent (macroCTA) method, is most common. Following RAFT polymerization of the arm, the incorporated RAFT agent then acts as the chain transfer agent during the crosslinking polymerization step. This method is dependent on the presence of living RAFT agents in the polymer and achieving near complete monomer conversion. Any dead chains will not be crosslinked and therefore not incorporated into the star polymer, so maintaining livingness of the RAFT end group is essential. Following arm synthesis, crosslinker can be added directly to the reaction mixture enabling a facile, one-pot approach to obtain well-defined star polymers. The macroRAFT approach can also be used with arms synthesized through alternative polymerization techniques provided a RAFT agent is incorporated into the arms prior to crosslinking, allowing RAFT star polymerization of arms synthesized using other polymerization techniques.

The macromonomer route was first demonstrated by the Matyjaszewski group using ATRP and requires an arm with a polymerizable vinyl end group.³¹ A small molecule RAFT agent is used and the arm is incorporated into the star as a macromonomer during polymerization. The macromonomer route allows for a lower number of initiating sites than the macroinitiator approach, reducing the likelihood of star-star coupling.³¹

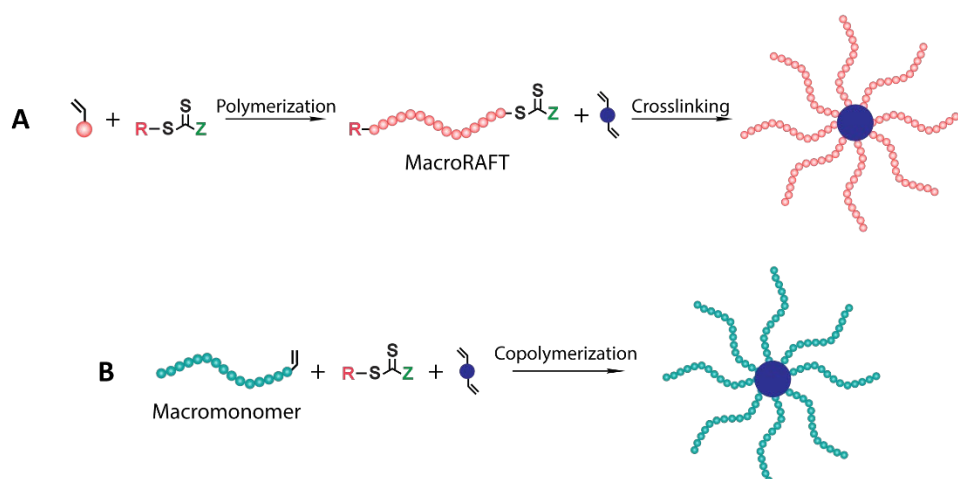


Figure X.7 Synthesis of star polymers using the arm-first approach via RAFT polymerization can be performed using the A) macroRAFT and B) macromonomer approaches.

The An group used commercially available 2 kDa PEG methacrylate (PEGMA), highlighting that synthesis of a macroRAFT agent either through RAFT polymerization of the arm or conjugation of RAFT agent to the arm was not required for this approach.³² The PEGMA arm, RAFT agent, and radical initiator were soluble in phosphate buffer pH 7 while the crosslinker and optional comonomer formed oil droplets. As polymerization proceeded a random copolymer of the macromonomer arm, crosslinker, and spacer comonomer (if used) was produced. This is in contrast to the macroRAFT approach where polymerization results in a block copolymer. The resulting amphiphilic copolymers then self-assembled with the hydrophobic portions forming the core while the PEGMA chains radiated outwards. The authors investigated each component of the system, varying the concentration of phosphate buffer and macromonomer, ratio of crosslinker, presence of comonomer, and molecular weight of the macromonomer. Most star polymers formed showed greater than 90% arm incorporation, narrow size distributions and dispersities below 1.2. However, poor star formation was observed when the molecular weight of the arm was increased to 4 kDa.³²

This method is ideally suited for the crosslinking of arms that were synthesized using polymerization techniques other than RAFT. The Heise group synthesized poly(glutamic acid) (PGA) via NCA ROP initiated by 4-vinylbenzylamine, ensuring that all polymer chains contained a vinyl end group.³³ Initial star polymers formed via RAFT polymerization were well-defined with a dispersity of 1.2, although arm incorporation was only 14%. The authors found arm incorporation could be increased by periodically adding more crosslinker to the reaction although this also led to an increase in polydispersity. Despite the low arm incorporation, this work demonstrated the versatility of RAFT polymerization for the crosslinking of star polymers, even when the arms were prepared using other techniques.

X.2.2.1 Developments in Synthesis

The synthesis of star polymers via the arm-first approach has undergone a number of developments in the last decade that have led to improved polymers. During the early days of RAFT polymerization, the arm-first approach was plagued by low arm incorporation into the star resulting in high polydispersities. Low incorporation of arms was thought to be due to “dead” chain ends that could not participate in further polymerization. However, major developments by the Boyer, Davis, An, and Qiao groups have made the arm-first approach the new method of choice.

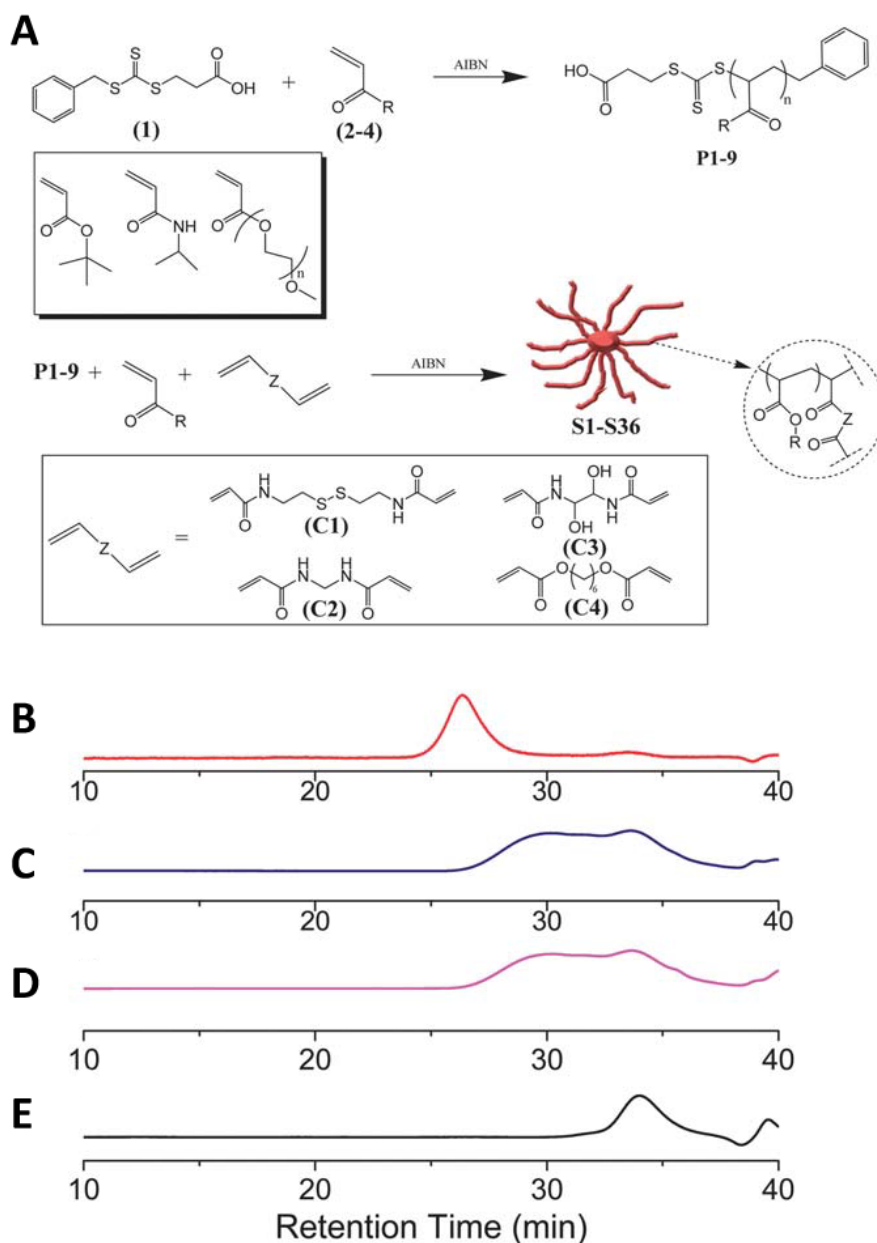


Figure X.8 (A) Synthesis of star polymers with varying arm compositions and crosslinkers in a range of solvents to investigate the impact of crosslinker solubility on star formation. GPC traces of star polymers obtained by RAFT, using *N,N'*-bis(acryloyl)cystamine (BAC) as the cross-linker, in (B) toluene, (C) DMF, (D) acetonitrile and (E) arm polymer used in this study before crosslinking. BAC is poorly soluble in toluene leading to well-defined stars and is soluble in DMF and acetonitrile where limited star formation was observed.³⁴ Adapted from Ref. 34 with permission from the Royal Society of Chemistry.

In a seminal paper by Boyer and Davis, the synthesis of well-defined star polymers with low molecular weight dispersities and high arm incorporation were reported using the arm-first approach by selecting poorly soluble crosslinkers.³⁴ Star polymers with POEGA arms and *N,N'*-bis(acryloyl)cystamine or 1,6-hexanediol diacrylate as crosslinker were synthesized using DMF, acetonitrile, and toluene as solvent to investigate the effect of crosslinker solubility on star formation (Figure X.8A). 1,6-hexanediol diacrylate is soluble in all three solvents while *N,N'*-

bis(acryloyl)cystamine is soluble in DMF and acetonitrile but only partially soluble in toluene. POEGA arms crosslinked with *N,N'*-bis(acryloyl)cystamine in toluene resulted in well-defined star polymers with a dispersity of 1.12 and greater than 90% arm incorporation, a dramatic improvement over previously reported star polymers. All other reactions resulted in dispersities greater than 1.5 demonstrating the importance of crosslinker and solvent choice (Figure X.8B-E). The authors hypothesized the improved star formation using *N,N'*-bis(acryloyl)cystamine in toluene was a result of nano-phase separation that induced compartmentalization of the crosslinking. Further experiments demonstrated high arm incorporation and low dispersity could be achieved using arms of varying composition and molecular weight as well as other poorly soluble crosslinkers.³⁴

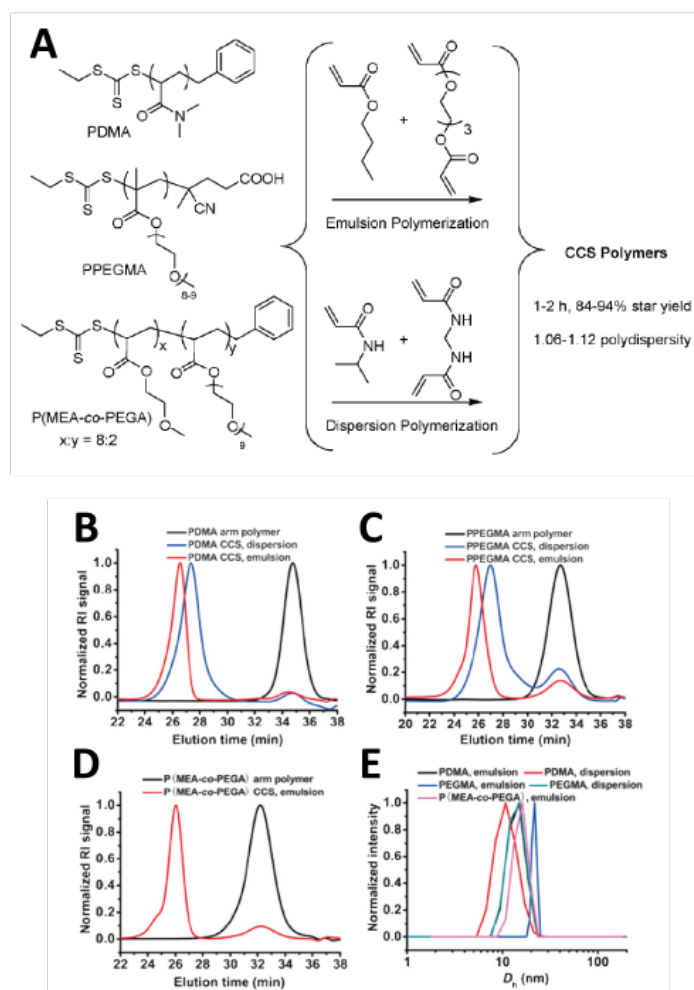


Figure X.9. Core-crosslinked stars synthesized using emulsion and dispersion RAFT polymerization.

(A) Monomers and crosslinkers used for emulsion and dispersion polymerization. GPC chromatograms of (B) PDMA, (C) PPEGMA, (D) P(MEA-co-PEGA) arm and star polymers. (E) Dynamic light scattering chromatogram showing the hydrodynamic radius of star polymers synthesized using emulsion and dispersion polymerization.³⁵ Adapted from Ref. 35 with permission from the Royal Society of Chemistry.

Applying a similar concept, the An group has developed and thoroughly investigated the use of heterogeneous RAFT polymerization systems to develop well-defined star polymers by emulsion and dispersion RAFT.^{32, 35-42} First reported in 2011, the An group demonstrated the synthesis of CCS polymers in water with a solid content of 10% w/v via emulsion and dispersion polymerization and

that these star polymers stabilized emulsions during particle synthesis.³⁵ The versatility of the method was shown using poly(*N,N'*-dimethylacrylamide) (PDMA), poly(poly(ethylene glycol) methyl ether methacrylate) (PPEGMA), and poly(2-methoxyethyl acrylate-co-poly(ethylene glycol) methyl ether acrylate) (P(MEA-co-PEGA) arms. The emulsion system was conducted with spacing monomer butyl acrylate and crosslinker poly(ethylene glycol) diacrylate (PEGDA) while the dispersion system contained *N*-isopropylacrylamide (NIPAAm) and methylene bisacrylamide (MBA) as the spacing monomer and the cross-linker, respectively (Figure X.9A). In all cases a high degree of arm incorporation and dispersities of 1.12 and below were observed from GPC analysis. Interestingly, emulsion polymerization led to greater arm incorporation, higher molecular weight, and lower dispersity than dispersion polymerization (Figure X.9B-E).

The next major development was reported by the Qiao group when they used photoiniferter RAFT polymerization in the synthesis of star polymers via the arm-first approach.⁴³ The synthesis of linear polymers via visible light-mediated RAFT without the use of exogenous radical sources or catalysts was first reported in 2015 and resulted in improved livingness at monomer conversions greater than 90%, demonstrated using MALDI-ToF and the one-pot synthesis of a pseudohexablock copolymer.²⁷ Irradiation with blue light was shown to directly activate trithiocarbonates, eliminating the need for additional radical sources. The improved livingness is likely due to the significantly lower concentration of radicals present in the reaction mixture, resulting in less termination reactions.

The highly living nature of polymers synthesized via photoiniferter RAFT made them excellent candidates for the synthesis of star polymers. Using a two-step, one-pot approach, McKenzie et al synthesized linear poly(methyl acrylate) (PMA) which was then crosslinked using ethylene glycol diacrylate (EGDA) (Figure X.10A). The ratio of crosslinker to arm was investigated with a ratio of 10:1 resulting in the best star formation. A ratio of 5:1 resulted in incomplete star formation while a ratio of 15:1 led to a broader size distribution. The degree of polymerization (DP) of the arm was also important in the synthesis of star polymers. The crosslinker to arm ratio was held at 10:1 while PMA arms with DPs of 60, 80, and 120 were crosslinked. GPC analysis of the stars formed from PMA DP 60 were bimodal, with the higher molecular weight product likely the result of coupling between two star cores due to the small arm length. A DP of 80 resulted in well-defined star polymers with a dispersity of 1.41 and arm incorporation of 94%. The largest arms, with DP 120, also resulting in well-defined stars albeit with a slightly higher dispersity and only 89% arm conversion. The lower conversion of DP 120 arms into the star is thought to be caused by reduced access to the core due to the greater arm length.⁴³

Importantly, the RAFT chain ends were shown to still be living following star polymerization. An in/out star was synthesized, with monomer added to the already formed stars. Further irradiation with blue light resulted in the polymerization of new arms from the star core (Figure X.10B). The number of arms in the initial star was calculated to be 31 and the number of arms following the in/out polymerization was found to be 60. The kinetics of monomer conversion during the in/out chain extension were compared to a control synthesis of linear PMA, with the apparent rate constant indicating the efficiency of polymerization from the core was 95% and that the functionality of most RAFT agents was maintained.⁴³

In 2017 the Qiao and Kamigaito groups demonstrated the synthesis of novel star polymers using a mechanistic transformation from cationic RAFT polymerization to radical RAFT polymerization.⁴⁴ Cationic RAFT was first reported by the Kamigaito and co-workers, who demonstrated polymerization of electron-rich vinyl ether monomers that are not readily polymerized via radical RAFT polymerization to obtain linear polymers with dispersities below 1.1.⁴⁵ Synthesis of star polymers via cationic RAFT was demonstrated in a one-pot approach, with linear poly(isobutyl vinyl ether) (PIBVE) prepared and then crosslinked using a divinyl ether crosslinker. A mechanistic transformation to radical RAFT was

also possible with PIVBE arms being crosslinked using a diacrylate crosslinker. Finally, the authors showed that a block copolymer of PIBVE and a heterobifunctional monomer with structure vinyl ether-R-acrylate (eg. 2-(vinylloxy)ethyl acrylate) could be synthesized via cationic polymerization of the vinyl ether groups and then crosslinked to form a star polymer through radical RAFT polymerization of the pendant acrylate groups. This mechanistic switch enables the synthesis of star polymers containing monomers not easily synthesized by cationic or radical RAFT polymerization alone and resulted in the synthesis of well-defined star polymers with high arm incorporation and dispersities as low as 1.18.⁴⁵

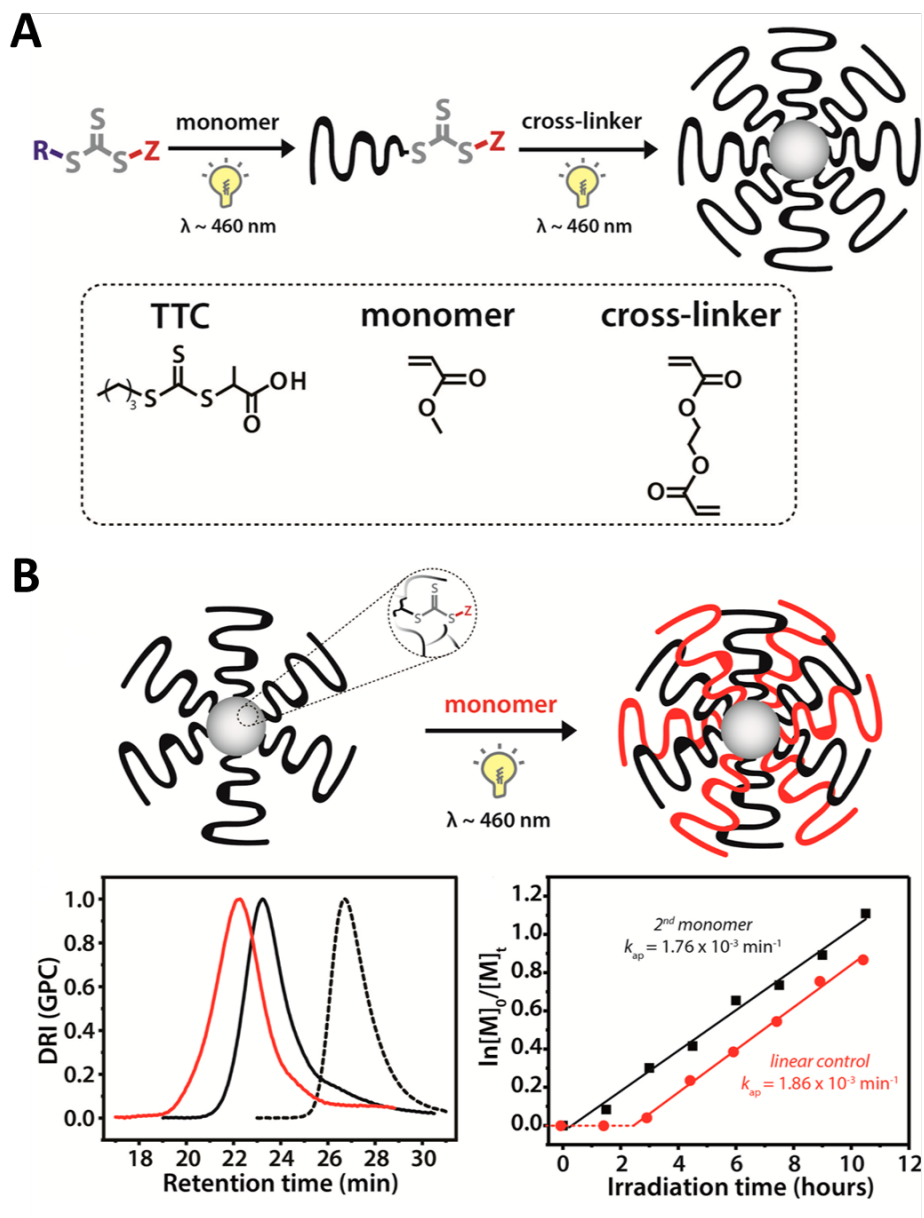


Figure X.10 Photo-mediated RAFT polymerization for the synthesis of star polymers via the (A) arm-first approach and (B) from the resulting star core using the in/out technique. GPC chromatograms show the RI trace of the arm, CCS polymer, and star polymer following the in/out reaction. The apparent rate of polymerization of the second monomer compared to the linear control indicated the efficiency of polymerization from the core was 95%.⁴³ Adapted with permission from *ACS Macro Lett.* 2015, 4, 9, 1012-1016.

The development of simple, rapid, high-throughput techniques to synthesize well-defined polymers of all architectures has been an important area in recent years. In 2017 the Cooper-White group reported the development of rapid one-pot sequential aqueous RAFT (rosa-RAFT) for the high-throughput synthesis of star polymers (Figure X.11).⁴⁶ Polymerizations of acrylate and acrylamide monomers were performed in an water/dioxane mixture (80/20 v/v) using water-soluble thermal radical initiator VA-044. Crucially, these polymerizations did not require degassing. Arm polymerization and crosslinking steps only required 3 minutes each at 100 °C in a thermocycler. The high-throughput technique enabled the rapid synthesis of a library of star polymers using different crosslinkers, crosslinking ratios, and mixing of macroRAFT arms to synthesis miktoarm star polymers, marking an important development in the synthesis of star polymers.

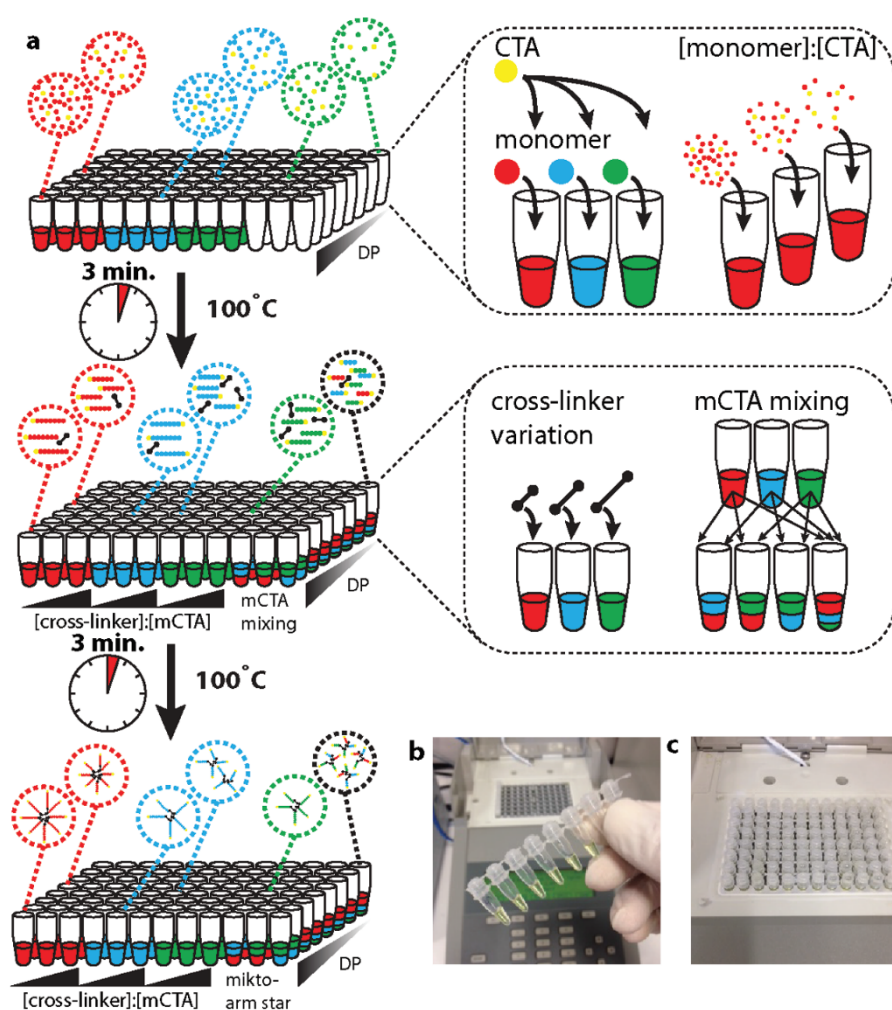


Figure X.11 Synthesis of star polymers via high-throughput rosa-RAFT. MacroCTA arms were synthesized in 3 min followed by 3 minutes for the crosslinking step. The species and ratio of crosslinker could be varied as well as mixing of the macroCTA arms to rapidly synthesis a large star polymer library.⁴⁶ Reprinted with permission from Ref. 46. Copyright 2017 John Wiley and Sons.

X.2.3 Grafting-to Approach

A third method of star polymer synthesis is known as the grafting to approach and involves the conjugation of arms onto a functional core (Figure X.12). This approach allows for excellent characterization as both the core and arm can be analyzed prior to conjugation. It is important to note

that although the linear arms can be synthesized via RAFT polymerization, the actual formation of the star is a result of chemical conjugation. Similar to end group functionalization of star polymers synthesized by the R-group core-first approach, RAFT agents can be readily modified to functional groups that can participate in coupling reactions.

Like the core-first approach, the number of possible arms is pre-determined. However, ensuring 100% conjugation can be difficult. Achieving quantitative conjugation becomes more difficult as the number of arms and the molecular weight of arms increases due to congestion at the core.

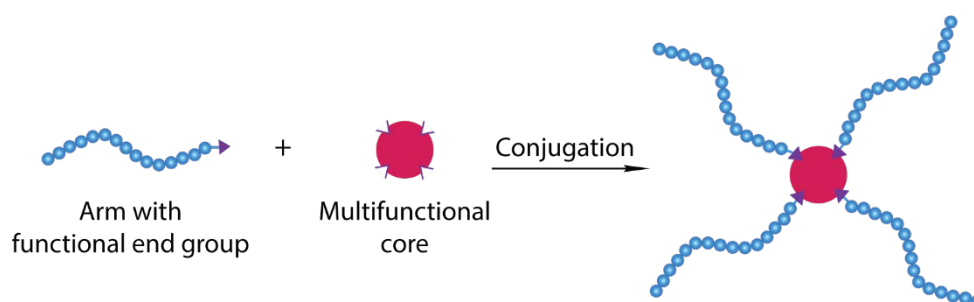


Figure X.12. The grafting-to approach requires the conjugation of arms with a functional end group to a multifunctional core.

X.3 Application of star polymers

The ease with which RAFT polymerization affords the preparation of various star sizes, compositions and functionalities has seen the development of a broad array of RAFT-mediated star polymers for targeted applications. The unimolecular structure of star polymers ensures they maintain their structure and are not susceptible to shear or disassembly under fluctuating or extreme conditions. The combined tolerance of RAFT polymerization to many functional groups and the distinct regions of star polymers facilitates the generation of core-, arm-, or end-functionalized star polymers.^{1, 47} By leveraging the unique property of star architectures, star polymers prepared via RAFT have found application in diverse fields; including as drug delivery and imaging contrast agents, viscosity modifiers, emulsion stabilization, and in advanced materials synthesis.

X.3.1 Star polymers in Biomedical Applications

Controlled delivery of therapeutics is one of the most common applications for star polymers synthesized by RAFT polymerization. Star polymers have several advantages that enable them to safely transport therapeutic agents to the appropriate site and control the rate of release. Star polymers are generally between 10 and 100 nm in diameter which is often considered the ideal size of nanoparticles for cancer therapy. Particles greater than 10 nm avoid the removal from circulation by the renal system experienced by smaller particles while nanoparticles smaller than 100 nm are thought to passively accumulate in tumours due to the irregular blood vessel structure in tumours that results from abnormal gene expression and rapid growth. The core-shell structure of star polymers can shield loaded therapeutics or imaging agents from unwanted degradation and uptake by the body's immune system, resulting in increased delivery efficiency while also facilitating functionalization of the star surface with specific targeting moieties such as peptides, antibodies, and sugars, thus improving delivery to the target site or tissue. In addition, intelligent design of star polymers can result in therapeutic release over a desired timescale or upon exposure to a particular stimulus. Due to their advantages and versatility, star polymers synthesized by RAFT polymerization are promising candidates for delivery of drug, gene, biomolecule, protein, and imaging agents. As with

all materials designed for biomedical applications, it is essential to ensure the star polymers and their possible degradation products do not induce inflammatory response or toxicity before, during, or after delivery.^{1, 48-50}

The Whittaker and Davis groups designed and synthesized star polymers by RAFT polymerization for biomedical applications including the delivery of chemotherapeutic agents, genes, imaging agents, protein inhibitors, and signalling molecules.⁵⁰ In order to prepare star polymers which are soluble in water and the biological environment, hydrophilic monomers such as poly(oligo(ethylene glycol) methyl ether acrylate/methacrylate) (POEGA/MA) are most commonly used to form the star polymer arms. Star polymers comprising POEGA/MA arms have demonstrated reduced protein adsorption and also provide stability to star polymers during circulation as they are not susceptible to dissociation in the same way as self-assembled structures.⁵¹⁻⁵³ *In vivo* and *in vitro* studies have shown that the POEGA star polymers with different molecular weights (49, 64, and 94 kDa), and therefore hydrodynamic volumes, exhibit different pharmacokinetics and tumour disposition behaviours, with the largest star polymers (94 kDa) displaying longer plasma exposure and improved uptake into solid tumours.⁵² The star polymers may include stimuli-responsive functionalities such as thermoresponsive monomer NIPAAm⁵⁴ and pH-responsive monomer 2-(dimethylamino)ethyl methacrylate (DMAEMA),^{53, 55} which were used in the arm or as secondary monomer in the core. These star polymers change their size when exposed to changing temperature or pH and stimulate the release of the drug at the target site.

Stars polymers for biomedical applications should be large enough to avoid removal by the renal system, leading to improved circulation half-lives. However, star degradation is desirable after cell internalization to improve therapeutic delivery or after delivery to prevent bioaccumulation. To achieve this end, degradable crosslinkers such as acid-labile crosslinkers^{56, 57} and reduction-sensitive disulphide crosslinkers are utilized in core structure.^{53, 58, 59} These crosslinkers are readily hydrolysed in the acidic environment of tumour tissue or reduced by glutathione upon exposure to the glutathione-rich intracellular environment, resulting in degradation of the core and therefore the star polymer.

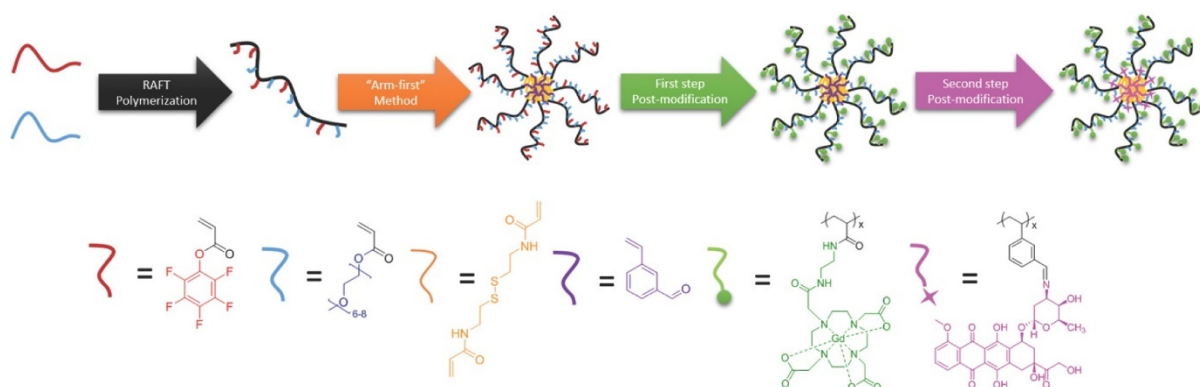


Figure X.13. Schematic illustration of the synthesis of multifunctional core cross-linked star polymers functionalized with MRI contrast agent and chemotherapeutic drug.⁶⁰ Reprinted with permission from Ref. 60. Copyright 2017 John Wiley and Sons.

The unique structure of star polymers facilitates the functionalization of the core, arm, or peripheral regions and can be accomplished by incorporating functional groups into the crosslinkers, monomers, and RAFT agents used. Common functionalities include therapeutic molecules, imaging agents, and dyes. N-hydroxysuccinimide (NHS) functionalized RAFT agents have been used to prepare end-functionalized star polymers.⁶¹ Arm-functionalized star polymers were synthesized by incorporating

activated ester-based monomers such as pentafluorophenyl acrylate (PFPA)^{60, 61} and 4-vinylbenzyl chloride (VBC)⁶² into the arms. PFPA^{61, 63} and vinyl benzaldehyde (VBA)^{51, 64} have been incorporated into cores as secondary monomers during crosslinking to generate core-functionalized star polymers. It has also been shown that two types of functional groups could be incorporated into star polymers to functionalize them with both chemotherapeutic drug and MRI contrast agent. Star polymers were synthesized by incorporating aldehyde-functional monomer VBA into the core and activated-ester monomer PFPA in the arm (Figure X.13). The VBA and PFPA monomers were subsequently modified with chemotherapeutic drug Doxorubicin and an MRI contrast agent based on disparate reactivities of VBA and PFPA.⁶⁰

For gene delivery applications, cationic monomers or cationizable monomers such as 2-(dimethylamino)ethyl acrylate/methacrylate (DMAEA/MA) were incorporated into star polymers.⁶⁵⁻⁶⁸ These monomers form electrostatic interactions with negatively charged genetic molecules such as DNA or RNA, resulting in complexes called polyplexes. This electrostatic complexation protects genes from enzymatic degradation during circulation and ensures the necessary compaction to enter to the target cells. In addition, star polymers synthesized by RAFT polymerization have been used for micelle and vesicle formation for drug delivery and imaging,^{69, 70} bone regeneration,⁷¹ cell sheet engineering,⁷² lectin binding,⁷² delivery of nitric oxide to prevent biofilm formation,⁷² and targeting primary human immune cell subsets,⁷³ demonstrating their versatility in biomedical applications.

X.3.2 Star Polymers in Other Applications

The unique properties of star polymers allow tunable interactions with other species in solution. The compact nature of star polymers leads to significantly different properties than linear polymers of the same molecular weight, while their flexible arms lead to different behaviours than particles of the same size. Such interactions can be leveraged for controlling the reactivity of these other species, leading to altered chemical pathways and affording the preparation of advanced materials. This section will present a selection of applications in which star polymers were essential for improved performance.

X.3.2.1 Emulsion Stabilization

Since reporting the synthesis of core-crosslinked star (CCS) polymers via emulsion and dispersion polymerization in 2011,³⁵ An and co-workers have pioneered the use of CCS polymers as emulsion stabilizers.^{39, 40, 42, 74, 75} Emulsion systems have wide industrial relevance, including in food, cosmetics, pharmaceuticals, coatings, oil recovery, and chemical processing. Qiu et al demonstrated the use of a fluorescein-tagged PDMA CCS that adsorbed to the interface of a toluene/water emulsion with excess PDMA stars remaining the aqueous phase, shown in Figure X.14A, and Nile red-stained toluene droplets shown in Figure X.14B. Stabilized emulsions were used as templates for the synthesis of spherical polystyrene particles inside the oil droplets. Following solvent evaporation, aggregation of PDMA stars around the particles (Figure X.14C) and Nile red-stained poly(styrene) particles were observed (Figure X.14D).

Exploiting the versatility of RAFT polymerization, the An group developed reversible emulsification/demulsification systems readily triggered by external stimuli, including heat/salt/pH through targeted interactions between stars and between star arms and solvents (Figure X.15).^{39, 40, 42, 74} While previous work had been carried out using amphiphilic star polymers to stabilize emulsions by having them act as surfactants, An and co-workers focused on CCS polymers with entirely hydrophilic arms and the structural properties of star polymers that may cause this stabilizing behaviour. Star polymers are similar in size to particulate emulsifiers but display greater flexibility, which Chen et al hypothesized would allow the CCS polymers to adopt favourable configurations once adsorbed on to the surface and aid their residence at the surface.³⁹

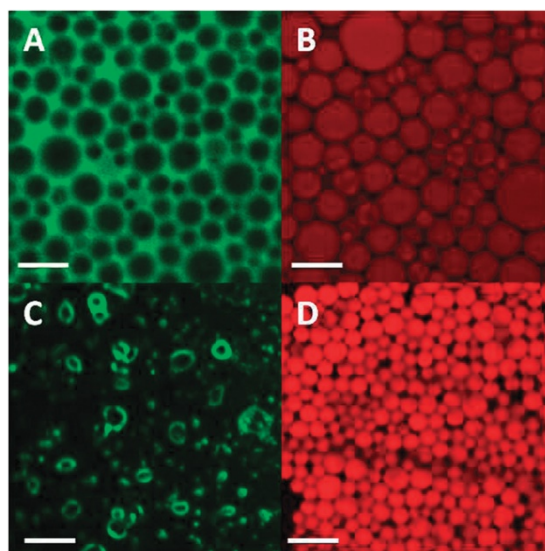


Figure X.14. Confocal images of water/toluene (60/40 v/v%) emulsions containing (A) 0.2wt% fluorescein-tagged PDMA stars and (B) Nile red stained toluene demonstrating the emulsion stabilizing activity of the PDMA stars. Following solvent evaporation, (C) PDMA stars at particle surface and (D) particles synthesized using emulsion templating. Scale bar is 20 microns.³⁵ Adapted from Ref. 35 with permission from the Royal Society of Chemistry.

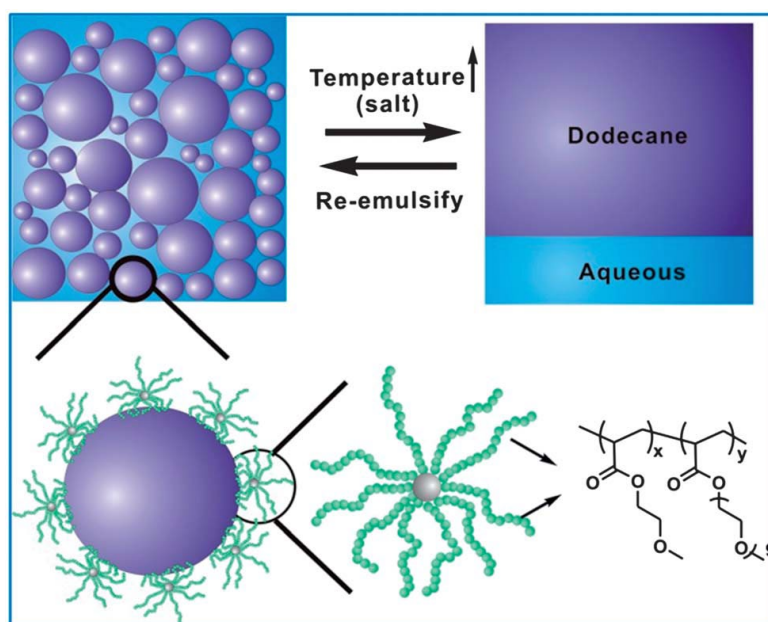


Figure X.15. High internal phase emulsion (HIPE) stabilized by poly(MEA_x-co-PEGA_y) star co-polymers exhibit on-demand demulsification triggered by salt and temperature.⁴² Adapted from Ref. 42 with permission from the Royal Society of Chemistry.

Star polymers have also been recently used as dispersants of multiwalled carbon nanotubes (MWNTs) in aqueous and acetone solutions.⁷⁶ Gao et al reported the synthesis of an amphiphilic “octopus” star polymer with a RAFT agent-functionalized silsesquioxane cage using the core-first approach. Star polymers were shown to produce more stable MWNT dispersions than linear polymers, which the

authors suggested was due to hydrophobic core anchoring to the nanotube surface while the hydrophilic arms extended outwards and reduced the hydrophobic area of the MWNT surface (Figure X.16).⁷⁶

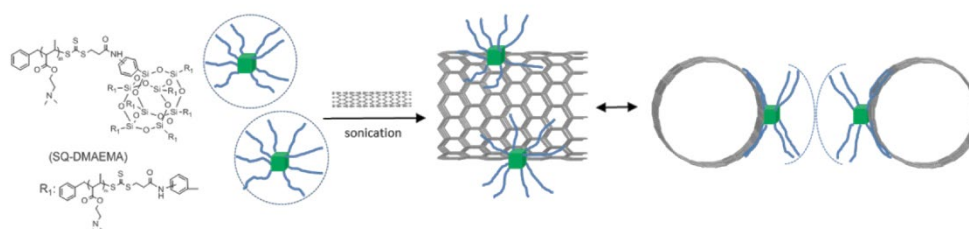


Figure X.16. Star polymers with POSS core and hydrophilic arms stabilized MWNTs in aqueous solvent.⁷⁶ Adapted from Ref. 76 with permission from the Royal Society of Chemistry.

X.3.2.2 Advanced Materials

Numerous applications have been reported in which use of star polymers resulted in improved properties in comparison to linear polymers. Hendrich and Vana synthesized linear and star-shaped polymers that underwent redistribution of copolymer blocks due to the incorporation of bifunctional RAFT agents.⁷⁷ Tensile testing demonstrated that inclusion of as little as 5 mol% star polymer led to higher ultimate strength and material toughness compare to films of pure linear multiblock polymers. Zheng et al reported improved resolution using a novel star polymer photoresist for krypton fluoride (KrF) photolithography.⁷⁸ Star copolymers were synthesized from a tetra-RAFT functionalized pentaerythritol core using *tert*-butyl acrylate and *p*-acetoxystyrene as monomers. Use of the star copolymers resulting in a pattern resolution of 200 nm, demonstrating greater resolution than analogous linear photoresists.

Star polymers have also shown superior properties as single-ion conduction polymer electrolytes (SCPE) which are valued for their potential use in energy storage applications.⁹ Miktoarm star polymers were composed of a polyhedral oligomeric silsesquioxane (POSS) core and poly (ethylene glycol) (PEG) and lithium poly ([styrene-4-sulfonyltrifluoromethyl-sulfonyl] imide) (PSTF-Li⁺) arms. The lithium-ion conducting stars demonstrated high thermal stability and tunable dc-conductivity. The improved thermal stability of the star polymer in comparison to a linear analogue was thought to be a result of the restricted motion of the star polymer, which is less sensitive to heat. PEG-*b*-PSTF linear copolymers were found to have greater dc-conductivity than the star polymer because of the star's higher T_g value, however the addition of more PEG arms led to significantly increased conductivity, highlighting the tunability of star polymer composition and performance.⁹

Star polymers can also be used in situations where the use of linear polymers is not possible. Lin and co-workers synthesized degradable star polymers through a combination of ring-opening polymerization (ROP) and RAFT polymerization.⁷⁹ ROP of ϵ -caprolactone was performed from a β -cyclodextrin core and R-group of RAFT agents were conjugated to the resulting terminal alcohol groups. RAFT polymerization of 4-chloromethylstyrene was performed followed by azide-functionalization by reacting sodium azide with the chlorine groups. The star polymers were irradiated with UV under dilute conditions to crosslink the azide groups, resulting in monodisperse nanoparticles with a crosslinked shell (Figure X.17). The authors were able to tailor the nanoparticle size and shell thickness by altering the size of each block and could control the density of shell crosslinking by varying the duration of UV irradiation. Finally, degradation of the poly(caprolactone) core was possible under acidic conditions.⁷⁹

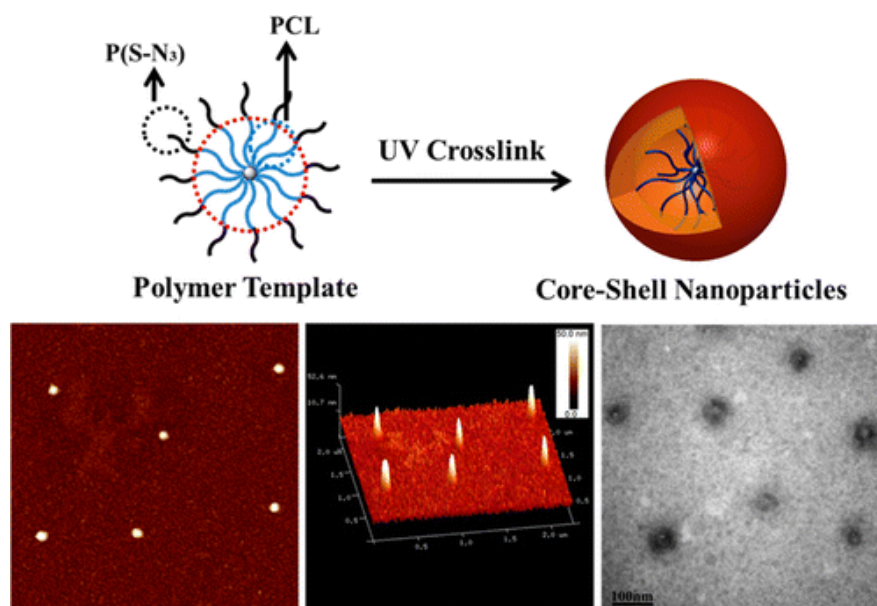


Figure X.17. Star polymers with degradable inner block and crosslinkable shell were synthesized using ROP and subsequent RAFT polymerization. Crosslinking of outer block and degradation of polyester block resulted in formation of hollow nanoparticles.⁷⁹ Reprinted with permission from *Chemistry of Materials*, 2014, 26, 20, 6058-6067. Copyright 2014 American Chemical Society.

X.4 Conclusion

Star polymers are highly valued due to their unique architecture and physical properties. Although star polymers can be synthesized using a variety of polymerization techniques, RAFT polymerization provides excellent control and a greater degree of tunability and functionalization than most. The multiple synthetic approaches available facilitate the facile synthesis of star polymers, with varied arm and core compositions readily produced.

Continued development of RAFT polymerization, in particular novel mechanisms of initiation and RAFT agents offering improved control over a wider range of monomers, will result in more controlled and more complex star polymers opening up new materials and applications. As greater spatial and temporal control develops along with reduced dependence on oxygen-free environments, the application of star polymers synthesized via RAFT polymerization will continue to expand and transition towards industrial use.

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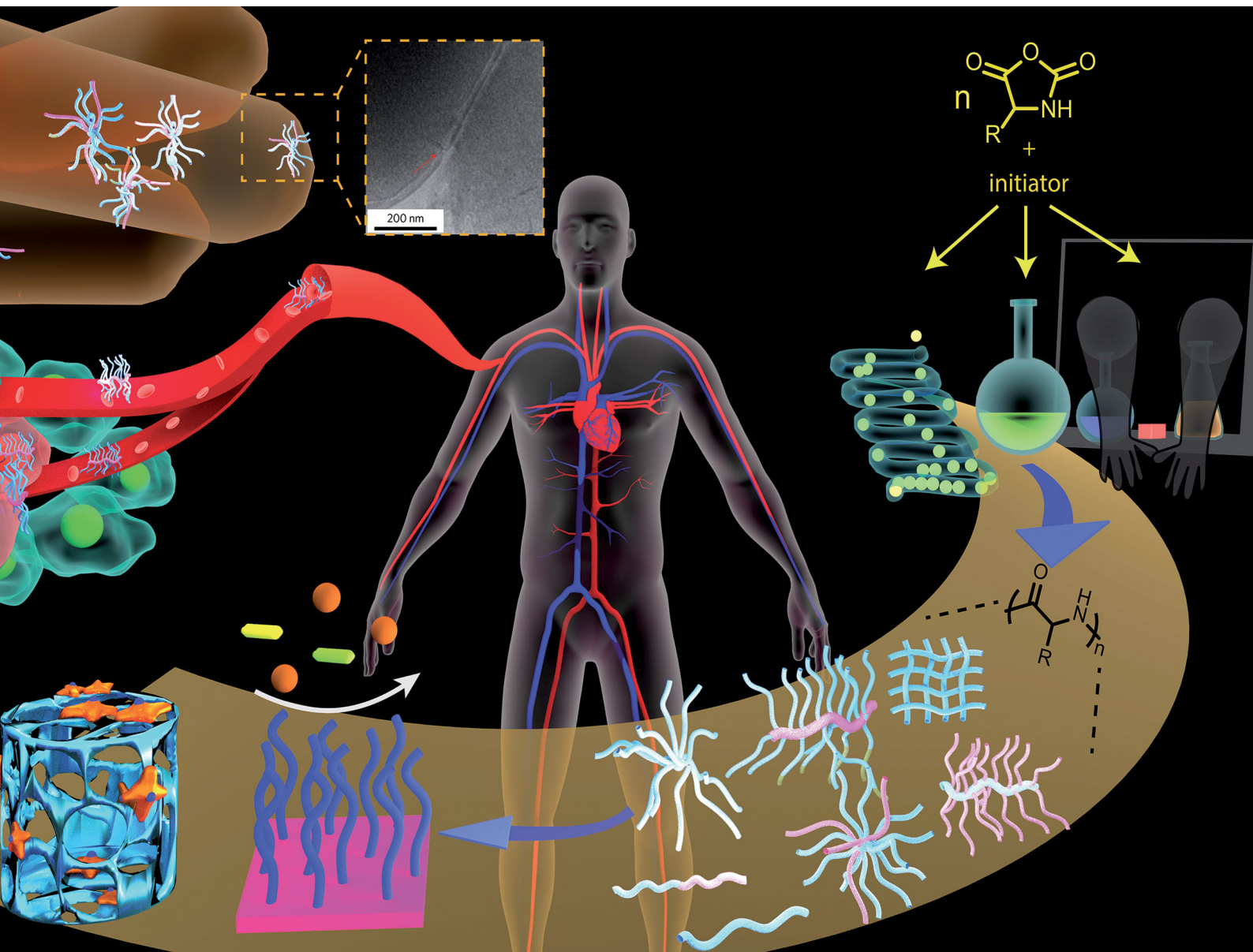
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ISSN 0306-0012

REVIEW ARTICLE

Greg G. Qiao *et al.*

Ring opening polymerization of α -amino acids: advances in synthesis, architecture and applications of polypeptides and their hybrids



Cite this: *Chem. Soc. Rev.*, 2020, 49, 4737

Ring opening polymerization of α -amino acids: advances in synthesis, architecture and applications of polypeptides and their hybrids

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Polypeptides have attracted considerable attention in recent decades due to their inherent biodegradability and tunable cytocompatibility. Macromolecular design in conjunction with rational monomer composition can direct architecture, self-assembly and chemical behavior, ultimately guiding the choice of appropriate application within the biomedical field. This review focuses on the applications of polypeptides alongside the synthetic advances in the ring opening polymerization of α -amino acid *N*-carboxyanhydrides achieved in the past five years. Key architectures obtained through NCA ROP or in combination with other polymerization methods are reviewed, as these play an important role in the wide range of applications towards which polypeptides have been applied.

Received 1st February 2020

DOI: 10.1039/c9cs00738e

rsc.li/chem-soc-rev

1. Introduction

Polypeptide-based materials are versatile, smart products with a wide range of applications in the biomedical fields due to

their inherent biocompatibility and biodegradability, properties which are afforded by the amide linkages forming the peptide backbone. Synthetic polypeptides are most commonly obtained in one of three ways: by ring opening polymerization (ROP) of *N*-carboxyanhydrides derived from α -amino acids (NCAs), solid-phase peptide synthesis (SPPS) or recombinant peptide synthesis. SPPS is a powerful technique that allows the automated synthesis of relatively complex sequences through convenient isolation and purifications steps, yet the size limitation is typically <50 mers. Recombinant techniques for expressing peptides in microorganisms are scalable and yield high molecular weight, sequence-defined polypeptides but require specialized equipment that is not readily available

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in most synthetic laboratories, in addition to being time and cost-intensive. NCA ROP enables the formation of bioactive and high molecular weight polypeptides composed of natural or non-proteinogenic amino acid residues in a facile and expedient manner. Despite the lack of sequence control, its synthetic advantages make it an attractive and economical method to synthesize polypeptides in large quantities.

The expanding field of NCA ROP dates its origins to 1906, when Hermann Leuchs first reported the synthesis of α -amino acid *N*-carboxyanhydrides.¹ This discovery effectively opened the frontier of synthetic polypeptides, though work would not commence in earnest until 1921, once the detrimental effects of the First World War had eased and Staudinger's concept of polymers had become more widely accepted by the scientific community.² In the nearly one hundred years since, vital contributions by numerous research teams have taken this once largely unknown chemistry to a position where polypeptides can

now be formulated on demand with precise control over molecular weight, architecture, polymer dispersity and end group fidelity. Of note are research efforts that led to optimal synthetic procedures and purification techniques to furnish NCA monomers with the necessary reagent purity for polymerization.^{3–7} Additionally, the controlled ring opening polymerization was greatly aided by Deming's pioneering work employing organonickel initiators which provided access to high molecular weight homo- and block copolymers with excellent living characteristics.⁸ The discovery of effective metal catalysts for NCA ROP propelled the field forward in the late 1990s,^{8,9} inciting renewed interest in this chemistry. Intensive research has been devoted in the last twenty years to a wide range of avenues for progress: novel initiators and mechanisms of polymerization, manipulation of polymerization conditions, polypeptide architectures, secondary structuring, self-assembly behavior in aqueous environments, and application of NCA ROP-derived polymers to various biomedical



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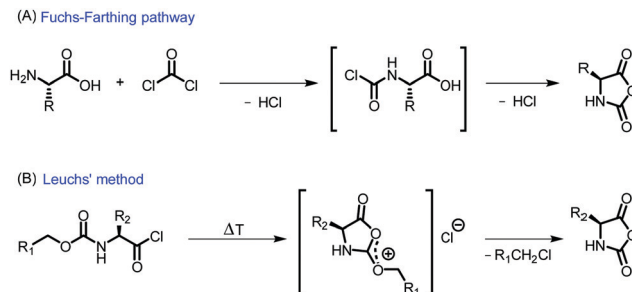
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challenges. To date, more than 200 NCA monomers have been described in the literature, creating ample supply of functions for biomedical applications.⁷ In all these cases, interest has been motivated by the desirable characteristics achieved with polypeptide materials: from their relatively inexpensive synthesis with potential for commercial scale, to their protein-mimicking ability. The choice of NCA monomers and polymerization order can direct the secondary structure, self-assembly and ultimate application of polypeptides. The inclusion of polymer segments can further help to modulate architecture, activity, selectivity and interaction with medically-relevant fluids and metabolites.

In the present review, focus is placed on the recent applications of NCA ROP polypeptide materials and their hybrids. To this end, the most recent work published within the past five years in the fields of antibacterial polypeptides, drug and nucleic acid delivery, tissue engineering, biosensors and antifouling surfaces will be presented. To further understand the potential applications and how these materials are synthesized, the synthetic advances realized since recent reviews^{10,11} are firstly summarized. The synthetic developments are supplemented with a discussion and overview of select molecular architectures that can be attained through NCA ROP exclusively or in combination with other cutting-edge polymerization techniques to produce polypeptide hybrids with particular promise in the biomedical fields. This review article will present recent advances in NCA polymer chemistry and an in-depth study on their applications and will prove to be a valuable resource in conjunction with excellent reviews recently published.^{12–20}

2. Recent developments towards the synthesis of well-controlled polypeptides

The past five years have seen great strides in the synthesis of polypeptides, with a number of approaches reported for



Scheme 1 Proposed mechanisms for the synthesis of α -amino acid N-carboxyanhydrides.

obtaining controlled polypeptides from varied initiators in shorter timeframes. The desire to speed up the polymerization has also translated to the synthesis of NCA monomers and the employment of certain polymerization methodologies which allow for the use of crude NCAs, thereby fast-tracking the process.

2.1 Developments in NCA synthesis

NCAs or Leuchs' anhydrides are most commonly synthesized following the Fuchs–Farthing procedure (Scheme 1A), which employs phosgene or a stable substitute to cyclize the amino acid residue of interest.^{3,21} In some cases, scavengers such as the cyclic monoterpenes α -pinene and limonene may be added in the cyclization step to reduce the amount of HCl in solution,²² though due to their lipophilicity this addition may complicate the workup. Alternatively, N-alkyloxycarbonylamino acids with halogenating compounds can be used in accordance to the Leuchs' method (Scheme 1B).^{23–25} In addition to these two methodologies, there is considerable interest in developing greener and safer NCA syntheses owing to the highly toxic nature of phosgene and HCl or alkyl halide by-products. Endo and co-workers have extensively examined the use of bisaryl-carbonates and activated urethane derivatives of α -amino acids



Neil M. O'Brien-Simpson

Neil O'Brien-Simpson graduated from Edinburgh Napier University in 1992 with a BSc with honours in Science and Management Studies. He moved to Australia and completed a PhD in peptide-polymer vaccines in 1998 at The University of Melbourne. He is currently a Professor and his research interests include antimicrobial peptides/materials, nanoparticles for peptide and vaccine delivery and bacterial outer membrane vesicles-host interactions. He has several editorial duties and is the current president of the Peptide Users Group.

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Greg G. Qiao

Professor Greg Qiao received his PhD at the University of Queensland in 1996. He joined the University of Melbourne in 1996 and became a full Professor in 2009. He was an Australian Research Council's professorial Future Fellow (2012–2015) and the Chair of Polymer Division of the Royal Australia Chemical Institute (RACI) (2015–2016). He received the Applied Research Award in 2017, ExxonMobil Award in 2015, RACI's Polymer Division Citation in both 2011 & 2019, and Freehills Award in 2010. He has published >250 journal papers and is a co-inventor of >20 patents. His key research interests are in polymeric architectures, new activation methods for RAFT, peptide polymers, tissue scaffolds, and gas membranes.

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since these syntheses proceed with less toxic cyclization agents, and lead to less corrosive by-products.^{26,27} Their approach involves *N*-carbamoylation of α -amino acids followed by intramolecular cyclization to furnish the NCA, in some cases *in situ*. Very recently, Li and co-workers have also examined the efficacy of dimethylcarbonate (DMC) for the synthesis of γ -benzyl-L-glutamate (BLG) NCA.²⁸ The authors first produced the intermediate *N*-methoxycarbonyl-BLG from the reaction between DMC and amino acid (2 eq. DMC supplied, 100 °C, *N,N*-dimethylformamide (DMF)). After purification, the intermediate underwent cyclization with glacial acetic acid (90 °C, 8 h) to furnish the NCA. It is worth noting that further optimization of this method would be required to achieve NCA in as high yields as traditional methods.

2.1.1 Relaxed NCA purity requirements for emulsion polymerizations. Regardless of the synthetic pathway undertaken for NCA synthesis, it is important to ensure a high monomer purity as this parameter largely dictates the outcome of NCA ROP. Notably, recent polypeptide syntheses in emulsion^{29,30} provide an exciting exception to the stringent purity requirement. The Cheng group has demonstrated it is possible to polymerize crude NCA without adversely affecting the polymerization kinetics or the final quality of the polypeptides, since the HCl impurities present in the crude monomer preferentially partition into the aqueous phase of dichloromethane (DCM)/water emulsions.²⁹ Nonetheless, several purification methods can be chosen to remove HCl, acid chloride and isocyanatoacyl chloride impurities from the crude NCA; these include the widely employed repetitive crystallizations under inert atmosphere or vacuum,³¹ rapid extractions with 0 °C water and 0.5% w/v sodium bicarbonate followed by extensive drying,⁵ the use of flash chromatography⁶ or as reported recently, filtrations with celite.³²

In 2017, Semple *et al.* reported the filtration of a crude solution of NCA in DCM through a bed of celite to be an effective method for removal of HCl in large scale syntheses (>100 g).³² Purification of large amounts of NCAs entails unique challenges associated with scale given that several of the aforementioned purification techniques may be challenging or time consuming, hence celite filtration offers a convenient alternative. Proton-induced X-ray emission (PIXE) analysis of L-Tyr(OBn) NCA purified with this method revealed celite could reduce the chlorine content from 733 to 115 ppm in addition to reducing trace amounts of metals, minimizing the recrystallization steps. Celite filtration has also been employed by others for the synthesis of NCA monomers of superior quality for kinetic screening.³³ Contamination of NCA by HCl and the HCl salts of unreacted amino acids can be easily detected by examination of the monomer's melting point, since impure samples exhibit a depression in said value. Barz and co-workers studied NCA purity by correlation of melting point data with single crystal and powder X-ray crystallography, further demonstrating the importance of highly pure NCAs in the well-controlled synthesis of polypeptides from amine initiators.³⁴

2.1.2 Flow chemistry as a means to synthesize NCAs. Flow chemistry has been recently employed by the Fuse group to

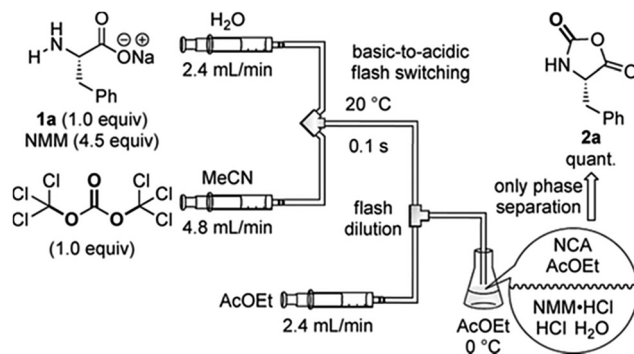


Fig. 1 Basic-to-acidic flash switching by means of microfluidic device to produce an arsenal of NCAs in reduced timeframes. Reproduced with permission from ref. 35. Copyright 2018 John Wiley and Sons.

synthesize a wide range of functional NCAs in reduced timeframes. The work presented by Otake *et al.* was made possible by a microfluidic device which enabled a rapid basic-to-acidic switching, affording NCAs with various side chains and protecting groups such as acid sensitive silyl, *tert*-butyl, trityl, Boc, allyl, propargyl and acetonide (Fig. 1).³⁵ The rapid synthesis (0.1 s) begins with a biphasic system; sodium salts of the desired amino acid are dissolved in an aqueous solution containing *N*-methyl morpholine (NMM). Rapid mixing of this solution with triphosgene dissolved in acetonitrile inside a V-shape mixer affords the desired NCA in under one second. A flash dilution with ethyl acetate immediately downstream allows the separation of the monomer from its more water-soluble impurities before it can hydrolyze. NMM is a mild base ($pK_a = 7.4$) well suited to the flow procedure as it neutralizes the HCl generated but is not active enough to abstract the acidic proton of the NCA, which would result in undesired polymerization. A screening of bases revealed those with a higher pK_a than NMM cause an undesired polymerization of the NCA. Additionally, optimization of the reagent dosing suggested 4.5 equivalents of NMM with respect to triphosgene are optimal for high yields. Despite a large excess of triphosgene being employed in the synthesis, water and base combine to decompose this reactant. The authors reported a large amount of the NCAs they screened were of sufficient quality for polymerization after the liquid extraction, with a select few requiring a further crystallization step. Furthermore, the methodology is suitable for the generation of proline NCA without emergence of proline dimers. Overall, the employment of flow chemistry for NCA synthesis is a promising advancement, with the possibility for automation and scale up. It provides advantages over the conventional Fuchs–Farthing synthetic procedure by shortening the reaction time whilst also allowing the reaction to proceed at room temperature. Additionally, the by-products generated are water soluble and easily removed in the liquid extraction step. Nonetheless, the flash dilution with ethyl acetate is a variant of the ice-cold extraction disseminated by Poché and co-workers, which may raise concerns for long term storage if any water traces remain.

2.1.3 Investigations into other NCA analogues. While this review article focuses exclusively on the advances in the

synthesis and polymerization of α -amino acid NCAs, it is worth noting that other NCA analogues are increasingly being investigated.³⁶ The ring opening of *N*-substituted NCAs yields polypeptoids that exhibit different properties to traditional polypeptides such as thermal processability and proteolytic stability. Another set of analogues, the *N*-thiocarboxyanhydrides (NTAs), are afforded high air and thermal stability and therefore long-shelf life, allowing their polymerizations to be carried out under open air conditions.³⁷ Interested readers are referred to the following review articles.^{38,39}

2.2 Developments in the ring opening polymerization of NCAs

Ring opening polymerizations of α -amino acid NCAs have been traditionally initiated by primary amines and their less nucleophilic derivatives such as amine hydrochloride⁴⁰ and tetrafluoroborate salts.⁴¹ Tertiary and secondary amines, alkoxides, thiols,⁴² hexamethyldisilazane (HMDS),⁴³ trimethylsilyl-amines,⁴⁴ and rare earth metal complexes⁴⁵ have also been utilized to successfully initiate the polymerization. The use of transition metal catalysts provides an alternative synthetic strategy that eliminates many of the side reactions present in the conventional polymerization of NCAs.^{8,9,46}

Herein we describe the scientific developments reported from 2014 until the time of publication for the synthesis of well-controlled polypeptides. These works highlight the scientific community's aim of streamlining a ring opening polymerization which often needs stringent moisture-free conditions to produce pure, living, and well-controlled polypeptide architectures. For the reader's benefit and to aid in contextualizing the NCA ROP developments of the past five years, Fig. 2 and 3 outline the most commonly employed NCA ROP pathways and proposed mechanisms, established and new. The normal amine mechanism (NAM) and activated monomer mechanisms (AMM) can take place simultaneously and often compete with each other in classic amine polymerizations, decreasing control over molecular weight and molecular weight distributions (Fig. 2A and B). Basic initiators with reduced nucleophilicity follow the activated monomer mechanism as depicted in Fig. 2B. To aid with control of amine-initiated polymerizations, Schlaad⁴⁰ and Vicent⁴¹ examined the use of protonated amine salt initiators in 2003 and 2013, respectively (Fig. 2D). The reduced nucleophilicity of amine salt initiators can be exploited to achieve more controlled polymerizations in a manner similar to reversible-deactivation radical polymerization (RDRP) techniques, where reversible deactivation and activation of monomer chains can greatly enhance the homogeneity of the polymerization products. In this case, control is achieved through an equilibrium between dormant amine salt and active amine sites. The reaction temperature ($40 < T < 80$ °C) affects the equilibrium, with higher temperatures favoring a shift towards higher concentrations of free primary amines. This polymerization method can be utilized to obtain polypeptides with molecular weight distributions as low as 1.03, though extended reaction times are necessary (3 days) and incomplete polymerization may occur due to the low reactivity of the active sites.

Transition metal initiators offer remarkable polymerization control and fast kinetic rates. The highly efficient nickel and cobalt initiators developed by Deming react identically across the anhydride bonds of NCAs by oxidative addition (Fig. 2C). Initially a 6-membered amido-alkyl metallacycle is formed, which contracts to a 5-membered amido-amidate metallacycle upon reaction with further NCA monomer and loss of CO₂. Proton migration and further insertion into the anhydride ring furnish polypeptides with tailorable molecular weight and narrow molecular weight distributions. To install C-terminal end group functionality Curtin and Deming further proposed allyloxycarbonylaminoamides as universal precursors to the active zerovalent metal complex through tandem oxidative reactions (Fig. 2C(ii)).⁴⁷ Metal catalysis is thus highly useful in the synthesis of block and hybrid polypeptides, though it still requires careful choice of ligand, solvent and temperature conditions in order to access the chelating metallacyclic intermediates that afford a living polymerization. Additionally, concerns associated with the removal of the residual metal initiators have somewhat curtailed the widespread use of metal catalysts for NCA ROP.

A further established method of polymerization depicted in Fig. 2E are the silazane derivatives developed by Lu and Cheng. Hexamethyldisilazane was shown to polymerize BLG NCA in a controlled manner in 2007.⁴³ Additional investigations have demonstrated the versatility of organic silane initiators towards achieving well-controlled polypeptides, with a wide range of derivatized primary amines successfully employed.⁴⁴ The improved control of silazanes in comparison to certain primary amine polymerizations may be explained by the differentiated mechanistic pathway. The mechanism differs from NAM or AMM as there is a concerted transfer of the trimethylsilyl (TMS) moiety to CO-1 which frees up the primary amine *in situ*. The formation of the TMS carbamate is key to the control, as the migration of TMS occurs with each monomer addition to the growing chain, affording greater control over the propagation. New developments pertaining to this class of initiators are presented in Section 2.2.3.

2.2.1 Ring opening polymerization from novel amine initiating systems. Free primary amines are the most commonly employed initiators for NCA ROP. The polymerization carried out in DMF is typically controlled by cooling down the reaction mixture,⁴⁸ with nitrogen flow,⁴⁹ or through the use of high vacuum techniques developed by the Hadjichristidis group.³¹ Focus has recently been aimed at producing polypeptide products in shorter times or triggered by stimuli that allow control of initiator concentration over the polymerization. Table 1 offers an overview of the recent polymerization techniques summarized in this review that result in a kinetic enhancement, along with relevant experimental conditions.

An interesting find in the Hadjichristidis laboratory was the peculiar polymerization kinetics of triethylaminetriamine (TREN), an initiator containing a sterically hindered tertiary amine and three primary amines. TREN exhibited faster propagation speeds than primary amines yet did not give rise to uncontrolled high molecular products unlike other tertiary amines which promote

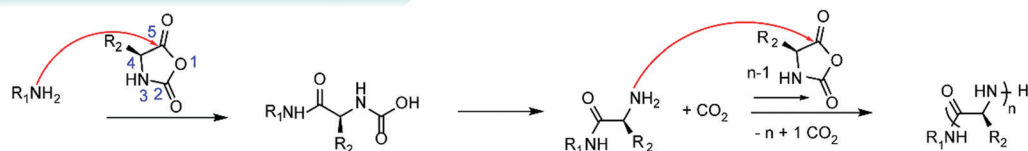
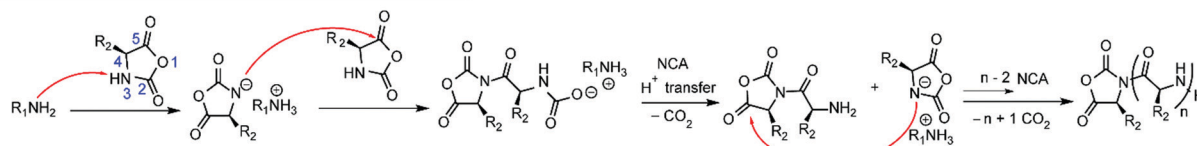
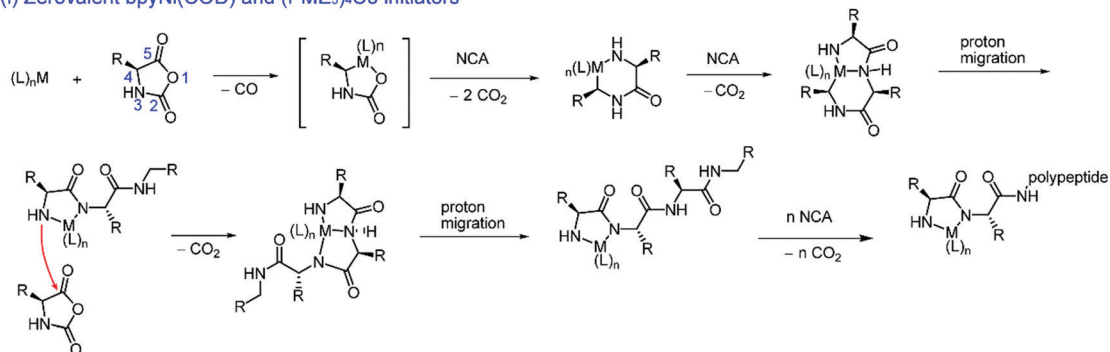
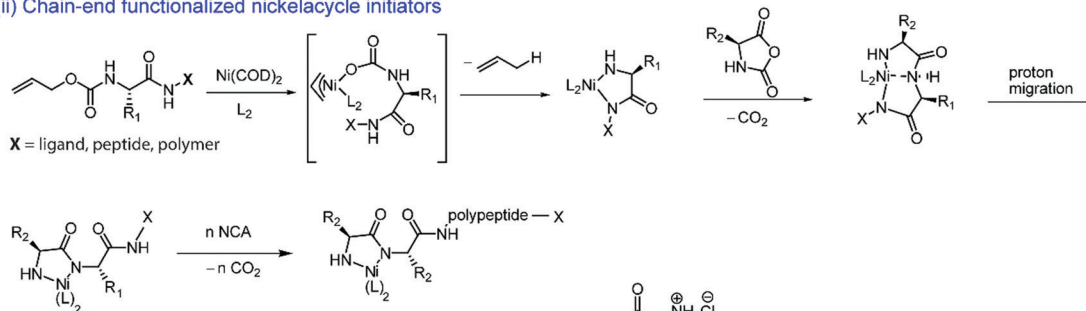
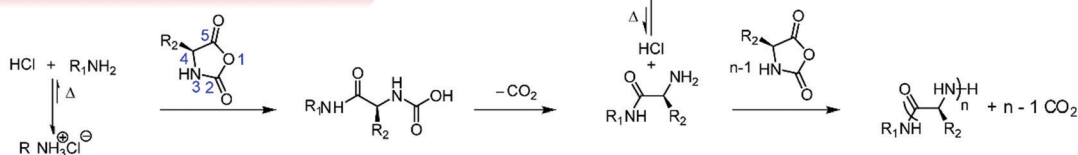
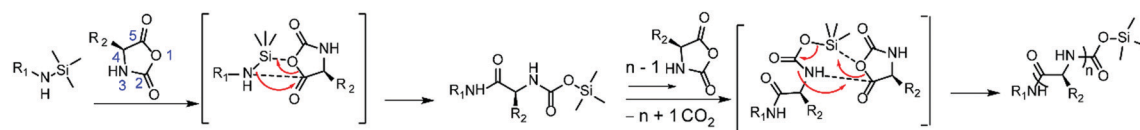
(A) Normal Amine Mechanism (NAM)**(B) Activated Monomer Mechanism (AMM)****(C) Transition Metal Initiators****(i) Zerovalent $bpyNi(COD)$ and $(PME_3)_4Co$ initiators****(ii) Chain-end functionalized nickelacycle initiators****(D) Protonated Amine Salt Initiators****(E) Silazane Derivatives - TMS Mechanism**

Fig. 2 Established methods of polymerization of N -carboxyanhydrides derived from α -amino acids and their mechanisms.

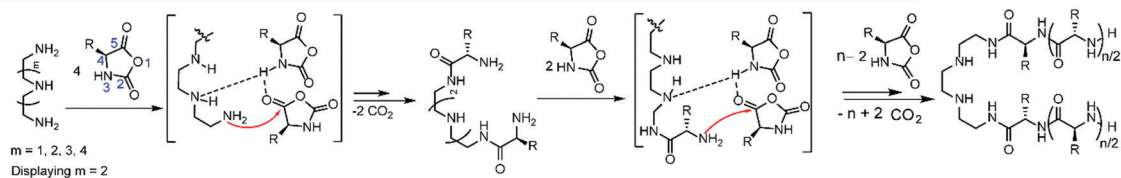
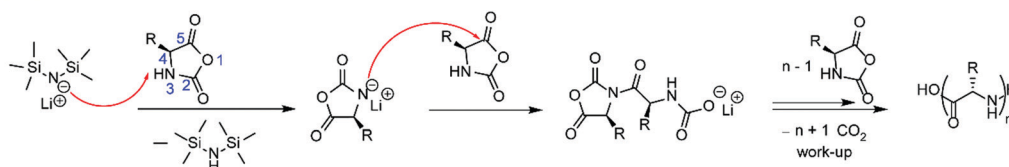
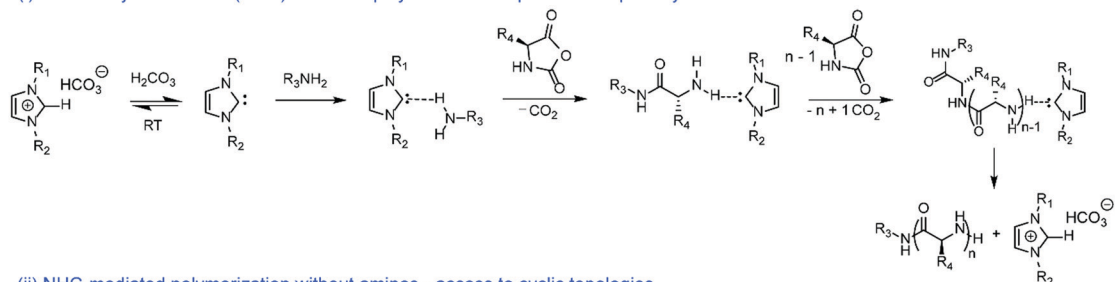
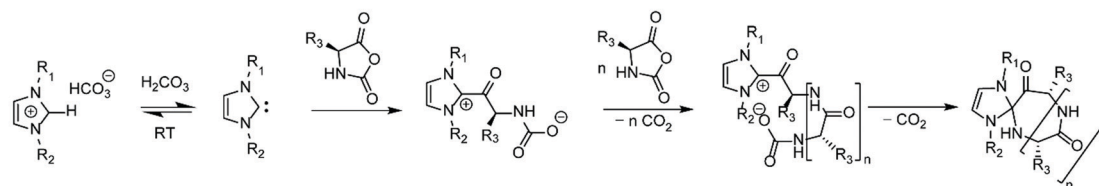
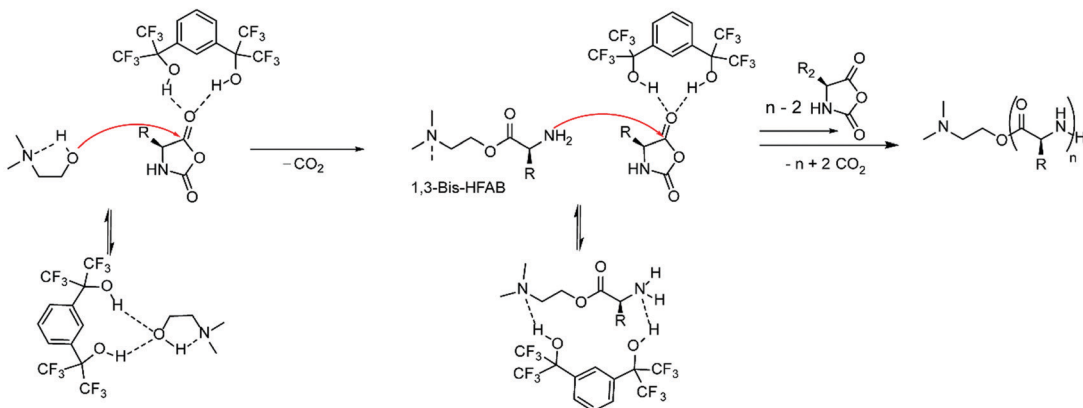
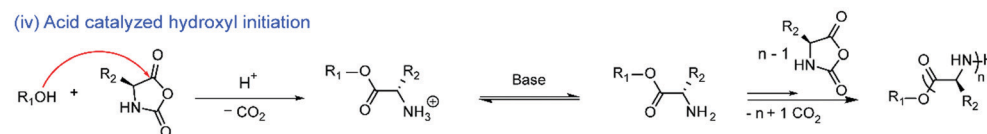
(A) Allied Amines - Accelerated Amine Mechanism through Monomer Activation (AAMMA)**(B) Lithium Hexamethyldisilazide****(C) Organocatalytic Ring Opening Polymerization****(i) N-heterocyclic carbene (NHC) mediated polymerization in presence of primary amines****(ii) NHC-mediated polymerization without amines - access to cyclic topologies****(iii) Fluorinated alcohol catalysis****(iv) Acid catalyzed hydroxyl initiation**Fig. 3 Recently developed methods of polymerization of *N*-carboxyanhydrides derived from α -amino acids.

Table 1 NCA polymerization systems with enhanced kinetic rates. Selected conditions have been summarized to provide a comparison between studies

Initiator	NCA ^a	Catalyst	$\frac{[\text{NCA}]_0}{[\text{I}]_0}$	$[\text{NCA}]_0$ (M)	Solvent	Temperature (°C)	Time (min)	M_n (kDa)		$\bar{D}$	Ref.
								SEC	Expected		
Ethylenediamine	BLG		50	0.10	DCM	R.T.	50 < <i>t</i> < 55	44.0	21.9	1.15	50
<i>N,N</i> -Dimethylethanolamine	BLG	TU-S	120	0.19	DCM	25	12	30.1	26.3	1.05	51
<i>N,N</i> -Dimethylethanolamine	BLG	1,3-Bis-HBAF	120	0.19	DCM	25	7	45.7	26.3	1.07	52
	ZLL						9	33.5	31.5	1.08	
	Alkynyl						8	23.1	20.1	1.05	
Poly(norbornene-TMS) ₁₀₀	BLG		100	0.05	DCM	23	45 < <i>t</i> < 50	2.29 × 10 ³	2.18 × 10 ³	1.02	33
PEG-PBLG in emulsion	BLG		100	0.050	DCM/ H ₂ O	R.T.	16	37.4	36.8	1.06	29
	ZLL						13	44.3	41.1	1.07	
	ELG						30	29.9	30.6	1.06	
Allied amines (triethylenetetramine)	BLG		120	0.19	DMF	25	120	27.7	26.0	1.17	53
Imidazolium hydrogen carbonate	BLG		100	0.20	DMF	R.T.	10	23.6	22.1	1.18	54
<i>n</i> -Hexylamine	BLG	Imidazolium hydrogen carbonate	100	0.20	DMF	R.T.	10	21.3	22.0	1.10	54
TREN	BLG		100	0.19	DMF	25	96	22.2	21.9	1.15	55
Trimethylstannyl phenyl sulfide	ZLL		100	0.16	DMF	25	180	17.7	22.3	1.17	56
Lithiumhexamethyldisilazide	BLG		100		THF	R.T.	< 120	78.1		1.24	57
	BDLL						7 h	36.3		1.23	

^a BDLL (Boc-D,L-lysine NCA), alkynyl (γ -propargyl-L-glutamate NCA), ELG (γ -ethyl-L-glutamate NCA).

the AMM pathway (Fig. 4).⁵⁵ The authors postulated presence of the tertiary amine at the core was key, as this amine was not basic enough to abstract the acidic proton from the NCA but could nonetheless activate its amido group, allowing for faster propagation. The authors termed this phenomenon the accelerated amine mechanism by monomer activation (AAMMA). In a further study, the authors showed how primary and secondary amines can also act cooperatively and synergistically to elicit living polymerization of NCAs at room temperature.⁵³ The combination of primary and secondary amines in the initiating molecule speeds up the polymerization, whilst also preventing the AMM pathway from taking place (Fig. 3A). Interestingly, a dual initiator mixture of hexamethyldiamine (HMDA) and *N,N'*-dimethyl-1,2-ethanediamine (DMEDA), when combined in stoichiometric ratios, could also bring about the benefits exhibited in initiating molecules containing both types of amines, without losing 'livingness'.

The use of dual mixtures of initiator and base as catalyst was also investigated by Schlaad.^{58,59} In the latter study, amine hydrochloride or primary amine initiators were combined with triethylamine (TEA) or *N,N*-diisopropylethylamine (DIPEA). A limitation of this combination is that under the conditions

reported the AMM mechanism could not be excluded, in contrast to the AAMMA systems (*vide supra*). Presence of AMM is evidenced by the non-linear portion of the kinetic plot in the early polymerization stages (Fig. 5A).

Nonetheless, the polymerization of linear BLG NCA could be controlled based on the supplied ratio of initiator to TEA. The tertiary base helps to shift the equilibrium of the ω -chain ends toward more reactive propagating groups. With amine hydrochloride initiators the polymerization was controlled up to addition of 1.1 eq. of TEA, whereas free amine/TEA systems required the ratio to be kept below 0.8 eq. (Fig. 5B).

Combined, these studies provide important insight as to how to best achieve an equilibrium between fast kinetic rates and control over molecular weight and polydispersity index (PDI), which may be particularly important when designing complex architectures. A further dimension for control is adjustment of spatiotemporal parameters. Heise and co-workers showcased this novel methodology for amine polymerization, furnishing the first report of UV-initiated NCA ROP with nitrobenzyl-protected initiators.⁶⁰ Primary amines remain photocaged until a light stimulus is exerted, which leads to cleavage at the carbamate moiety and unmask the free amine necessary for

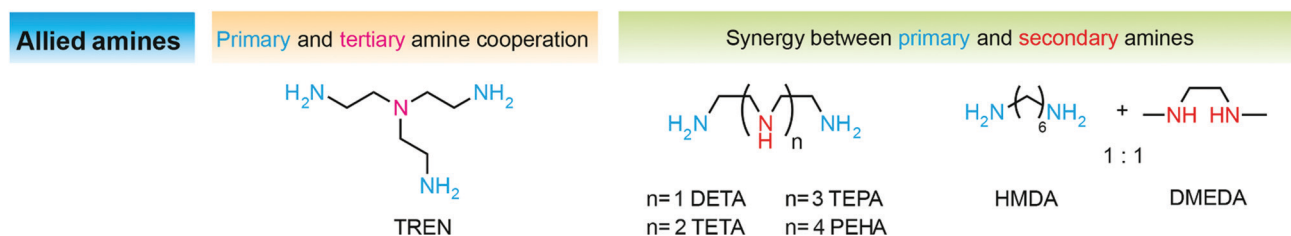


Fig. 4 Enhanced kinetic rates can be achieved in the NCA ROP of BLG and ZLL monomers using an alliance of amines.^{53,55}

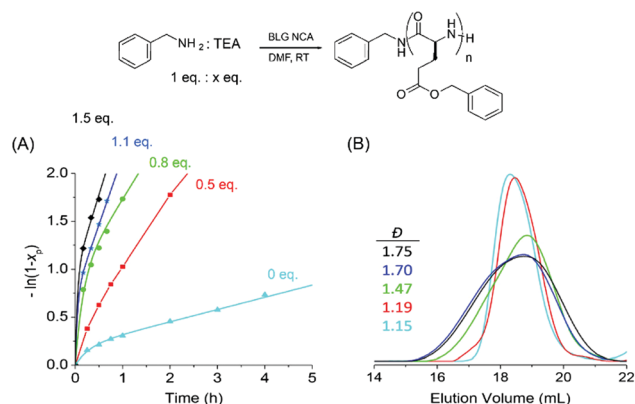


Fig. 5 (A) First order time-conversion plots for the ring opening polymerization of BLG-NCA (100 eq.) initiated by benzylamine (BnNH₂, cyan), BnNH₂:TEA = 1:0.5 (red), BnNH₂:TEA = 1:0.8 (green), BnNH₂:TEA = 1:1.1 (dark blue) and BnNH₂:TEA = 1:1.5 (black) at room temperature in DMF. (B) SEC-RI traces obtained for each of the polymerization systems after ~24 h of reaction. Reproduced with permission from ref. 59. Copyright 2017 Elsevier.

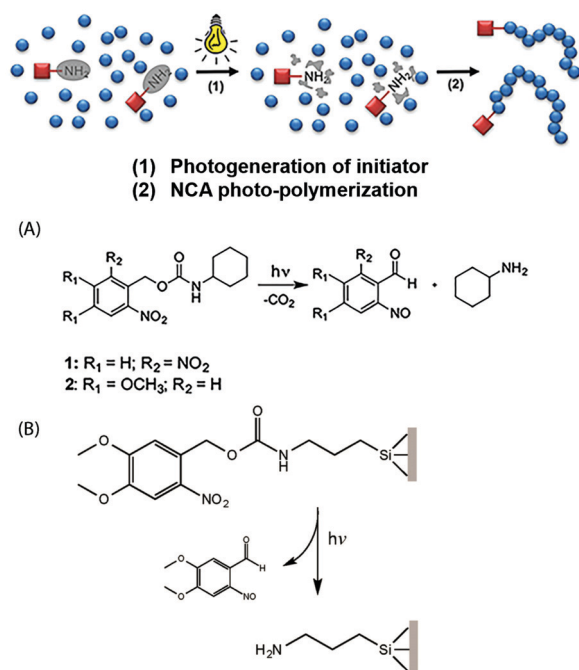


Fig. 6 Schematic of NCA ring opening photo-polymerization initiated by (A) photoamine generators in solution. Adapted from ref. 60 with permission from The Royal Society of Chemistry. Copyright 2016. (B) Masked amines anchored to a silicon wafer, and activated under UV-A irradiation.⁶¹ Adapted from ref. 61. Copyright 2018 John Wiley and Sons.

initiation (Fig. 6A). This approach allows for the modulation of molecular weight based on irradiation time or distance from the light source (effectively changing intensity), leading to polypeptide products of tailored length. In a follow-up publication to this proof of concept work, the authors further showed it is possible to apply this technique to immobilised amines on silicon wafer surfaces, producing polypeptide-decorated surfaces (Fig. 6B).⁶¹ The phototriggered UV-A initiated ROP of

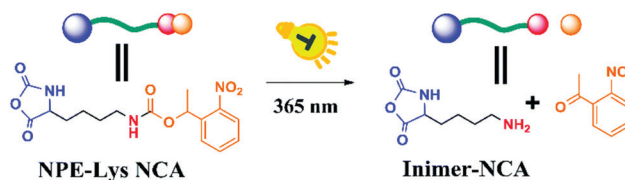


Fig. 7 Schematic representation of inimer and photocleaved products upon irradiation ($\lambda = 365$ nm). Adapted from ref. 64 with permission from The Royal Society of Chemistry. Copyright 2018.

NCA has been further explored utilizing caged NCA monomers with a photocleavable group in their side chain (Fig. 7). Deming⁶² and Dong⁶³ separately reported the synthesis of *N*_ε-(*o*-nitrobenzyloxycarbonyl)-L-lysine NCA, which the Dong group employed as an inimer (initiator and monomer) for the synthesis of hyperbranched polypeptides, in addition to linear architectures. Enhancements in architectural control were reported subsequently; these were achieved by substitution of the aforementioned lysine NCA with *N*_ε-(1-(2-nitrophenyl)ethoxycarbonyl)-L-lysine NCA.⁶⁴ The α -methyl substituent present on the nitrobenzyl group of this monomer yields nitrosoacetophenone upon cleavage, and largely reduces imination side reactions previously caused by the nitroso-benzaldehyde by-product. Imination is undesirable as it leads to a low degree of branching due to termination upon reaction with ϵ -NH₂. In this work polypeptides with varying degrees of branching, MW and secondary structure were obtained by variation of light intensity. Higher intensities led to lower molecular weights and hyperbranching due to the presence of more deprotected amine sites as well as more polypeptide molecules in random coil conformation. Nitrogen flow, which is known to increase the kinetics of the polymerization, also played a role in dictating the characteristics of the final products; less cyclic architectures were encountered due to the NCA favoring ring opening of another monomer over ring closure.

2.2.2 Ring opening polymerization facilitated by organocatalysts. Catalysis through hydrogen bonding is a powerful strategy to obtain well-controlled polypeptides due to the stabilization capacity hydrogen bonds offer. Organocatalysts can mediate polymerization control by activation of the NCA monomer and through fast and reversible exchange between dormant and active ω end groups of the growing polypeptide, in addition to mediating favourable interactions with the initiator.

N-Heterocyclic carbenes (NHC) are a particularly fascinating class of organocatalysts, having demonstrated excellent properties in the ROP of a variety of monomers to produce cyclic and linear polymers.^{65–68} Lu and co-workers employed 1,3-dimethylsilylimidazol-2-ylidene in catalytic amounts as an acyl transfer/catalytic agent (0.3 equiv. with respect to HMDS indicator) to improve the conversion in the otherwise sluggish polymerization of *O*-diethylphospho-L-tyrosine NCA.⁶⁹ Recently, NHCs have also been utilized by the Kim group for NCA ROP; Zhang *et al.* exploited the air stability of imidazolium hydrogen carbonate salts as precatalysts for the ring opening polymerization of a variety of NCA monomers.^{54,70} The active NHC catalyst can be released from the imidazolium hydrogen carbonate salts (IHC) by formal loss of H₂CO₃, kickstarting the polymerization

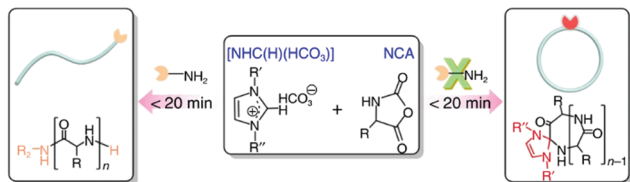


Fig. 8 N-Heterocyclic carbene-mediated ring opening polymerization with different topological outcomes. A variety of NCA monomers were successfully polymerized through implementation of $[NHC(H)(HCO_3)]$ with varied electronic and steric properties. Adapted from ref. 54. Copyright 2018 Springer Nature.

to furnish the controlled and selective polymerization of NCAs (Fig. 8). Linear architectures can be accessed through the inclusion of a primary amine initiator, whilst cyclic polypeptides are obtained in its absence. The NCA ROP mediated by IHC complexes in DMF proceeds rapidly, with polymerizations completed in under 20 min (Table 1). The proposed mechanism displayed in Fig. 3C(i) suggest the NHCs preferentially hydrogen bond with initiator and ω -terminus alike if an amine initiator is added. The NHC thus affords a living and controlled ring opening polymerization. In the absence of a primary amine initiator, the organocatalyst not only initiates the polymerization but also mediates the chain propagation as counterions, following a zwitterionic mechanism (Fig. 3C(ii)).

Another possible organocatalyst that may be used to mediate the well-defined polymerization of BLG NCA by aniline or other primary amines is the binaphthol-derived phosphoric acid organocatalyst discovered by Yang *et al.*⁷¹ (*S*)-1,1'-binaphthyl-2,2'-dimethoxyphosphoric acid provides a beneficial impact on the polymerization of BLG NCA initiated by aniline in DCM at room temperature. Several parameters are important in ensuring control. Firstly, the substituents on the binaphthyl skeleton are important. The electron donating methoxy groups provided more controllability than electron withdrawing groups, which were deemed detrimental based on the larger than expected molecular weights and higher PDIs obtained. A variant of the effective organocatalyst with *R* optical configuration also caused more disperse molecular weight distributions, highlighting the need to fine tune the organocatalyst's properties. Thirdly, solvent effects are prominent in coordinating solvents such as DMF and tetrahydrofuran (THF) and lead to extended polymerization times with deviation from expected molecular weights, hence DCM is advised. Interestingly, the polymerizations employing (*S*)-1,1'-binaphthyl-2,2'-dimethoxyphosphoric acid exhibited long induction periods, extending the time required from 0.8 to 2.5 h for a desired degree of polymerization (DP) of 50. This is perhaps due to phosphate ammonium salt formation where dissociation, though rapid and at room temperature compared to classic salt initiations, slows the propagation. Nonetheless 99% conversion of BLG was achieved and the polypeptides displayed an improvement in PDI from 1.36 to 1.08. The polypeptides also exhibited all the common features of a living polymerization based on successful chain extension experiments. Interestingly, this organocatalyst was not effective in activating a benzyl alcohol initiator.

Many NCA polymerizations are conducted employing a strong nucleophile initiator which attacks the electrophilic carbonyl at CO-5 (Fig. 2A), however less nucleophilic molecules can be utilized successfully under the right conditions. Hydroxyl functional groups are weakly nucleophilic, and were first studied decades ago as initiators for NCA ROP.⁷² The low nucleophilicity of hydroxyls compared to primary amines translates into a slow initiation which can pose challenges due to the fact that the propagation rate outpaces the rate of initiation. In such cases the resultant polypeptides are poorly defined and may contain a high oligomeric fraction. Despite this challenge hydroxyl functionality is present in many small organic molecules and polymers, making this initiating group highly desirable to access varied polypeptide architectures. Alcohols are widely used initiators for the ROP of heterocycles aided by catalysis with metal-based complexes, enzymes and small organic compounds or inorganic acids that enhance the nucleophilicity of the initiator and the propagating hydroxyl end group.⁷³ Extensive work by Waymouth and Hedrick has revealed hydrogen bonding interactions with functional thioureas, diazabicycloundecene, triazabicyclodecene and fluorinated alcohols can simultaneously enhance the nucleophilicity of the alcohol initiator/propagating end group and activate the monomer.^{74,75} Some of the important insights learnt from the controlled ROP of cyclic esters have been applied to NCA ROP.

2.2.2.1 Organocatalysis to facilitate ring opening from aminoalcohols. Hadjichristidis and co-workers demonstrated aminoalcohols can be successfully employed to ring open various NCAs with several organocatalysts. Initially glutamic acid and *Z* lysine NCAs were polymerized in the presence of *N,N'*-bis[3,5-bis(trifluoromethyl)phenyl]-thiourea (TU-S), a hydrogen bond organocatalyst.⁵¹ The chosen aminoalcohol initiators were interesting given that they contain primary hydroxyl and tertiary amine functionality. Normally, the tertiary amine would be problematic due to promotion of the AMM, however this pathway for polymerization is silenced by hydrogen bonding interactions with the thiourea organocatalyst. The presence of the tertiary amine in initiation is important as it aids to activate the hydroxyl initiating group. This was demonstrated by the inability of other aminoalcohol molecules containing secondary amine or amide functionality to completely activate the hydroxyl group, or polymerize, respectively. Importantly, the thiourea's action is three-pronged; in addition to the hydrogen bonding with the tertiary amine, it also affords the activation of the electrophilic carbonyl group in the NCA. Thirdly, it reversibly deactivates the growing amine end groups, providing excellent polymerization control (Fig. 9A). Optimized reaction conditions demonstrated superior results could be obtained in the non-hydrogen bonding solvent DCM, since more polar reaction solvents such as DMF and THF (which affect hydrogen bonding) resulted in bimodal or larger than expected molecular weights, respectively (Fig. 9B). The reported system was translatable to functional NCAs, as demonstrated by a subsequent publication where living homo- and copolymerization of γ -propargyl-L-glutamate (PLG) NCA was achieved.⁷⁶

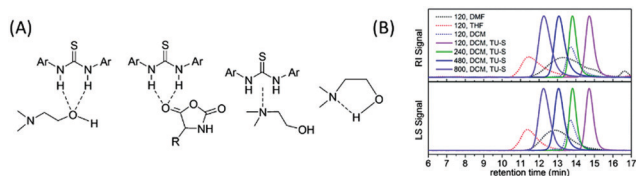


Fig. 9 (A) Control through different types of hydrogen bonding present throughout the polymerization of BLG by DMEA when *N,N'*-bis[3,5-bis(trifluoromethyl)phenyl]-thiourea organocatalyst mediates the polymerization and (B) SEC traces of resulting polypeptides for different targeted lengths and solvents of polymerization. Reproduced with permission from ref. 51. Copyright 2015 The Royal Society of Chemistry.

Very recently, 1,3-bis(2-hydroxyhexafluoroisopropyl)benzene (1,3-Bis-HFAB) was also discovered to mediate the polymerization from amino alcohol initiators in DCM with outstanding results.⁵² The fluorinated alcohol 1,3-Bis-HFAB has been previously combined with (–)-sparteine as a bicomponent catalytic system for the ROP of cyclic esters,⁷⁴ yet the catalysis of NCA ROP proceeds in excellent yields without requiring a cocatalyst. As shown in Fig. 3C(iii), 1,3-Bis-HFAB activates the initiating alcohol and also hydrogen bonds with the NCA (activation at CO-5) without inducing polymerization by itself. In the propagation stage it continues to act as a hydrogen donor to the NCA, and also silences the tertiary amine present in DMEA. It further facilitates a reversible exchange between active and inactive polymer chains which decreases the nucleophilic character of the chain-end primary amine group (Fig. 10). Kinetic screening of the polymerization of BLG NCA revealed a fast two stage kinetic profile, where the k_{app} constant of the second stage was several times larger than k_{1app} , a finding consistent with reports from the Cheng group (see Table 1). Overall 1,3-bis-HFAB could mediate the polymerization in a living fashion with high rate, selectivity and activity, affording polypeptides of high molecular weight (up to DP 1920 explored) and low *D*.

2.2.2.2 Acid-catalyzed ring opening polymerization of hydroxyls.

Pahovnik *et al.* provided an alternative strategy to the organocatalysis of aminoalcohols by exploring the dichotomy between initiation and propagation with simple initiators as well as

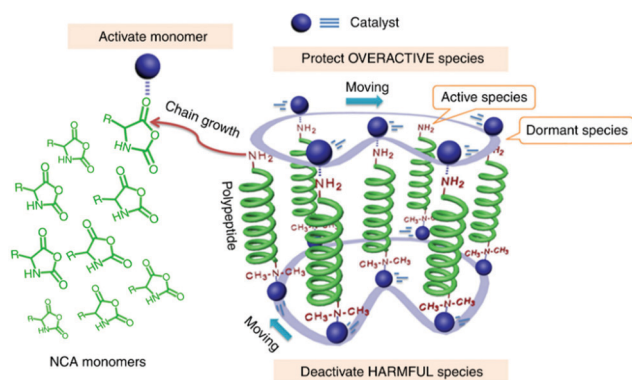


Fig. 10 Schematic representation of the proposed role of 1,3-Bis-HFAB fluorinated alcohol catalyst in the ring opening polymerization of BLG, ZLL and alkynyl NCAs. Adapted from ref. 52. Copyright 2019 Springer Nature.

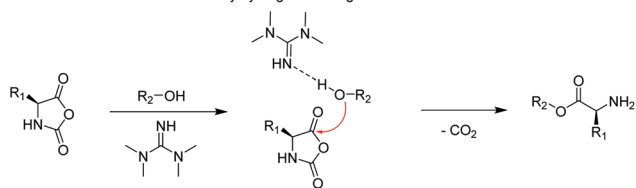
macroinitiators (Fig. 3C(iv)).^{77,78} In this instance the authors exploited the acid-catalyzed alcoholysis of NCAs to first obtain an inactive amino acid ester with 'dormant' amine functionality. Methanesulfonic acid (MSA) in chlorinated solvents (3 eq. with respect to initiator, 40 °C) exhibited attractive catalytic properties for the ring opening of NCA monomer by 3-phenyl-1-propanol.⁷⁷ Other acids tested (HCl/Et₂O, diphenyl phosphate, trifluoroacetic acid, trifluoromethanesulfonic acid) could not match the effectiveness of MSA, either due to inefficient conversions of alcohol to ester or in the case of the latter acid (which did provide fast rate), unacceptable cleavage of protecting groups. Importantly, MSA also acts as a suppressing agent for propagation due to its protonation of the N-terminal amino group. The protonation largely reduces the propagating terminal's propensity to attack a further NCA monomer and aids in maintaining a low PDI. After complete initiation at 24 h, addition of *N*-ethyldiisopropylamine could kickstart the propagation. Despite the potential of this method for accessing block and dense architectures with reduced synthetic burden—demonstrated by the author's subsequent one-pot synthesis of polyester/polycarbonate-*b*-polypeptides⁷⁸—several drawbacks may need to be considered. The polymerization requires several days, and it may not be suitable for the ring opening of NCA monomers protected by acid-labile groups.

2.2.2.3 Base-catalyzed ring opening polymerization of hydroxyls.

A further strategy to polymerize from alcohols involves 1,1,3,3-tetramethylguanidine (TMG), a strong base organocatalyst which activates the hydroxyl groups. It has been utilized for the synthesis of polypeptoids⁷⁹ and also has been reported in the ROP of conventional NCAs to obtain polypeptide stars from multifunctional alcohol cores with moderate PDIs.^{80–82} Recently, TMG has also been applied to the synthesis of poly(ethylene glycol) (PEG)-poly(amino acid) diblock copolymers in one-pot, as the organobase can first catalyze the ROP of ethylene oxide and upon full conversion, addition of NCA as well as a fresh dosage of TMG leads to block copolymers of similar characteristics as those initiated by classic amine initiators.⁸³

The studies involving conventional α -amino acid NCAs have not examined the mechanism of the polymerization, however based on the investigations by Zhang and co-workers using *N*-butyl NCA,⁷⁹ two mechanistic pathways are possible for the initiating step in the ROP of NCAs (Fig. 11). The first pathway proposes the formation of hydrogen bonding complexes between TMG and the alcohol which aid the nucleophilic attack at CO-5. This hypothesis is supported by ¹H NMR analyses of equimolar mixtures of varied alcohols and TMG, where a disappearance of the hydroxyl and imine protons was observed, replaced by a new broad peak downfield at 4.56–5.78 ppm which can be attributed to the dynamic exchange of these two protons in the hydrogen-bonded complex. The second less likely pathway may arise due to the fact that TMG is itself a good nucleophile. Thus it is possible that a zwitterionic intermediate is formed and subsequently displaced by the alcohol to form the initiating amino ester species shown in Fig. 11. While the role of TMG in the polymerization of *N*-substituted NCAs is

Mechanism 1. Initiation mediated by hydrogen bonding



Mechanism 2. Initiation via guanidinium intermediate formation

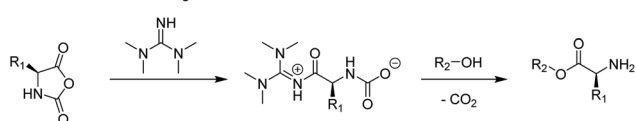


Fig. 11 Possible mechanisms of initiation of alcohols in the presence of TMG organocatalyst.

limited to facilitating the nucleophilic attack by the alcohol—the propagating species is a secondary amine and TMG has negligible interactions with it based on unappreciable increases in the rate of propagation of control kinetic experiments—the organobase may very likely interact with the primary amino end group in the normal polymerization of NCAs. Cabral and co-workers noticed accelerated rates of reaction in their diblock copolymer syntheses, an effect they attributed to TMG's ability to keep the growing primary ω -end group deprotonated.⁸³ In utilizing the TMG organocatalyst special consideration should be given to catalyst concentration, in order to pre-empt the double initiation by TMG and alcohol and ensure a close agreement between theoretical and experimental molecular weights. Additionally, the identity of the alcohol is important; sterically hindered secondary and tertiary alcohols, aromatic alcohols as well as primary alcohols with electron withdrawing groups may not realize a controlled polymerization if they initiate the polymerization at all.

2.2.3 ROP from organosilicons and their salts. As already described, silazane derivatives offer an alternative to classic amine initiators. HMDS and varied TMS amines can mediate a controlled polymerization through a pathway that differs from the NAM. In 2016 Lu and co-workers reported a new initiator within this class: phenyl trimethylsilyl sulfide.⁸⁴ This initiator can promote a well-controlled ROP and furthermore, leads to faster chain initiation than HDMS due to the more nucleophilic nature of sulphur, and the increased reactivity of the S–Si bond. Interestingly, the polymerization mechanism remains unchanged based on high resolution electrospray ionization (ESI) and ¹³C NMR experiments. The results confirmed fragmentation patterns in close agreement to an intermediate molecule bearing a phenyl thioester C-terminal end group and a TMS carbamate N-terminus. Similarly, characteristic thioester and TMS carbamate carbonyl peaks were observed by carbon NMR. Combined these analyses provide strong evidence to suggest the polymerization undergoes a TMS mechanism where initiation occurs by cleavage of S–Si bond and subsequent generation of a thiol group *in situ* which attacks CO-5 (Fig. 2E). Meanwhile the TMS group migrates to O-1 forming a carbamate, later transferring from the N-terminal chain end onto an incoming NCA monomer to continue propagation in this concerted manner. Phenyl trimethylsilyl sulfide

was shown to mediate the polymerization of a varied range of NCAs, furnishing polypeptides with dispersities below 1.10 and molecular weights in close agreement with theoretical values in most of the monomer systems studied, except for γ -chloropropyl-L-glutamate NCA. The thiol-reactivity of this monomer may pose disadvantageous interactions with the initiator. Notably, it is worth highlighting the usefulness of obtaining polypeptides with a reactive thioester end group at the C-terminal, as this group can readily react *via* chemoselective native chemical ligation.

Polymerizations involving silazanes can be challenging due to the inherent instability of the NH–Si bond, which readily cleaves upon exposure to moisture or acidic conditions. This proclivity implies that the polymerization must be carefully excluded from moisture and carried out employing highly purified solvents in order to ensure the polypeptides are formed through the TMS pathway. Recently, however Wu *et al.* reported that lithium hexamethyldisilazide (LiHMDS) – a lithium disilazide complex stable to air and moisture—affords a fast polymerization of BLG and *N*_ε-*tert*-butoxycarbonyl-D,L-lysine NCAs in THF both in the glovebox and surprisingly, in open vessel conditions.⁵⁷ The proposed mechanism for this novel NCA initiation is depicted in Fig. 3B. As a non-nucleophilic base, LiHMDS deprotonates the NH-3 position of the NCA in a similar manner to other strong bases. Following initiation, the formulated propagation mechanism (supported by HRESI-MS and MALDI-TOF observations) suggests N-terminal lithium carbamates attack C-5 carbonyl instead of undergoing a proton transfer and deprotonation of another NCA monomer as is typical of the AMM mechanism. This is due to the weak basicity of the N-terminal lithium carbamate. The anionic character of the polymerization explains the rapid propagation rate, with the polymerizations being completed in under 15 minutes for $[M]:[I] = 20$. The study also examined DPs of up to 500, reporting polypeptides with moderate dispersity ($D = 1.15$ – 1.25) even if the resultant molecular weight was in some cases much higher than targeted. This phenomenon has been previously observed for polymerizations carried out in THF.^{51,56} Based on control experiments, LiHMDS outperformed polymerizations from hexylamine and HMDS initiators at the maximum DP examined of 500, where the latter two initiating molecules exhibited a conversion ceiling at low initiator to monomer ratios. Similar to bipyNi(COD), LiHMDS could afford polypeptides with high molecular weight. Most importantly, NCA ROP could be performed satisfactorily in open atmosphere with untreated THF at 60% relative humidity.

2.2.4 NCA ROP mediated by other novel Lewis pairs. In earlier sections, common strategies to regulate the chain propagation species in NCA ROP through a Brønsted or Lewis acid agent have been described. Further systems include a novel initiator reported by Yuan *et al.*, who employed trimethyl stannyl phenyl sulfide (PhS–SnMe₃) (Fig. 12A).⁵⁶ This Lewis acid can withstand exposure to air and moisture, but is more easily cleaved than the reported TMS-sulfide previously examined by the same group.⁸⁴ As with other initiating systems, the kinetics exhibited solvent-dependent features. In DMF, the ROP of *Z* lysine NCA exhibited two stage-character, with a large proportion of the monomer being consumed rapidly in the first

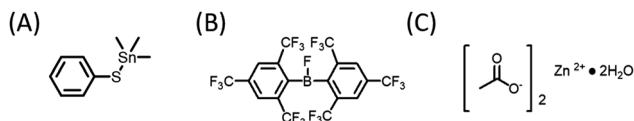


Fig. 12 (A) PhS-TMS mediates NCA ROP. Well-controlled BLG NCA ring opening polymerization investigated by the Yang group using^{85,86} (B) FMe₂BF and (C) Zn(OAc)₂·2H₂O.

stage (67% in 7 min) but a much slower second stage. Interestingly this observation is vastly different to the helico-genic systems previously discussed, where the second stage of the polymerization has an apparent rate constant orders of magnitude higher than the initial stage.

In addition to this, toward the late stages of the polymerization chain termination was observed in DMF. The experiments conducted in THF, a solvent of lower polarity that may likely stabilize the S-Sn bond, led to less active initiating sites and polymers of high molecular weight instead. The THF polymerizations exhibited first order kinetic character with respect to monomer concentration but were significantly slower. Based on these observations, a cosolvent mixture with different ratios of DMF and THF was investigated, affording linear polypeptides of varying molecular weights, some as high as 150 kDa, while maintaining high polymerization speeds (~4× faster than the similar phenyl TMS sulfide system and completed within a few hours).

A different approach proposed by the Yang group involves the use of frustrated Lewis pairs: systems containing a Lewis acid and base that do not form classical adducts due to steric hindrance. Their study examined the interaction between aromatic amines and bulky borane Lewis acids and led to the conclusion that in DCM, the frustrated Lewis pairs could offer greater control over the traditional polymerization employing said amine initiators alone, albeit with longer polymerization times (Fig. 12B).⁸⁵ A follow-up publication from the same group investigated how zinc acetate can have a beneficial effect on the controlled polymerization of polypeptides grown from anilines (Fig. 12C).⁸⁶

2.2.5 Post-polymerization modifications. The modification of polypeptide side chains is often desired to introduce responsive, structural, or functional properties into the polymer. Examples of desired properties achieved through side chain modifications, whether at the NCA synthesis stage or post-polymerization, include thermoresponsive behaviour,^{87–89} incorporation of sugars,^{90–92} and modulation of secondary structure.^{93,94} These conjugations are most commonly accomplished *via* click reactions, although other efficient techniques such as nucleophilic substitution and oxidation reactions have also been employed.²⁰

A 2016 review by Deming thoroughly described side chain modified polypeptides, which can be prepared *via* two different synthetic approaches; (1) polymerization of functionalized NCA monomers or (2) post-polymerization modification.⁷ In some cases, amino acid side chains can be modified directly while in others incorporation of a reactive handle is necessary, thereby requiring multi-step reactions. For example, the thiol group of cysteine is readily modified *via* radical and nucleophilic thiol-X

chemistries, which have been comprehensively reviewed by Brosnan and Schlaad.⁹⁵ Meanwhile, the thioether side chain of methionine is susceptible to oxidation and alkylation, allowing for facile modification.⁹⁶ Native chemical ligation is also a powerful post-polymerization reaction which allows polypeptides with thioester bioorthogonal functionality to be conjugated with proteins containing a N-terminal cysteine.⁹⁷ Other very common strategies include the incorporation of clickable handles such as azide, alkene, and alkyne groups onto amino acid side chains.⁹⁸ This approach was first reported by Hammond and coworkers, who functionalized the side chain of glutamic acid residues with alkyne groups and then “clicked” PEG-azide moieties *via* Huisgen cycloaddition post-polymerization.⁹⁹ Glutamic acid side chains are commonly used as the acid group is compatible with several conjugation chemistries, while the amine side chain of lysine can also be readily modified. In addition, numerous amino acids with clickable azide, alkyne, and alkene groups are commercially available. Readers interested in further reviews on polypeptide side chain modifications are directed to reviews from the Cheng,²⁰ Schlaad,⁹⁵ Deming,^{7,96} and Hammond⁹⁸ groups.

2.2.6 Secondary structuring. Polypeptides are intriguing materials compared to traditional polymers due to their folding into secondary conformations which mirror the fundamental motifs of proteins. Ordered secondary structures arise due to the nature of the polypeptide’s side chain, increasing degrees of polymerization and environmental influences, with stabilization of the varied secondary conformations being achieved by intra- and intermolecular hydrogen bonding as well as hydrophobic and electrostatic interactions.¹⁰⁰ The secondary structuring in polypeptides is an important determinant of physico-chemical properties as it strongly impacts on the biomaterial features^{101,102} chemistry,^{99,103} and self-assembly^{104,105} of these compounds. The propensity of certain amino acid residues to adopt α -helical or β -sheet conformations is widely exploited for several applications as the establishment of stable or switchable secondary structures offers marked differences in the packing, solubility and interactions of polypeptides with biological targets, amongst others.¹⁴ For instance, the inclusion of hydrophobic segments in polypeptide block design can induce a non-reversible temperature-dependent association of β -sheets, a conformational transition routinely employed to create polypeptide hydrogels through direct gelation, as reviewed in Section 3.4.

2.2.6.1 Water soluble polypeptides with stable secondary structure. The structuring of polypeptides made from canonical amino acid residues into ordered secondary conformations (*e.g.* α -helix or β -structures) leads to a reduction in hydrophilicity and solubility in aqueous media, however good water solubility is an important prerequisite in many biomedical applications. Ionic polypeptides such as poly(L-glutamic acid) (PLGlu) and poly(L-lysine) (PLLys) are widely used as polyelectrolytes since they offer good water solubility in their random coil state. They can also undergo pH-dependent coil-to-helix transitions which greatly diminish their solubility. Other polypeptides such as water soluble poly(carboxymethyl-L-cysteine) undergo pH-responsive

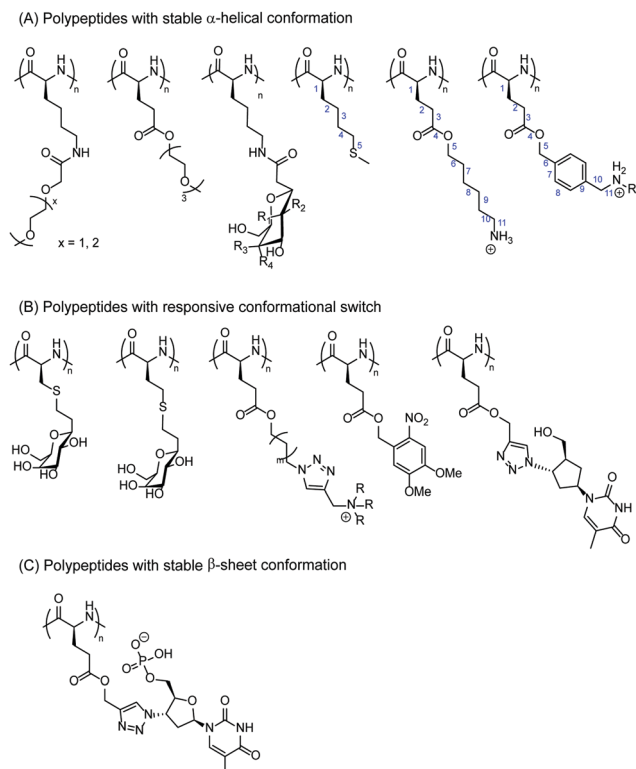


Fig. 13 Examples of polypeptides that undergo secondary structuring.

β -sheet-to-coil switches.¹⁰⁶ The elucidation of side chain structures able to confer optimal water solubility and guide the formation of stable secondary structure has been a subject of intense study as demonstrated by the several strategies described in the literature and eloquently summarized in excellent reviews.^{14,16,100} This article highlights some noteworthy examples (Fig. 13). A first approach involves the synthesis of neutral α -helical polypeptides by modifying amino acid residues with helical propensity. Deming and co-workers developed purely α -helical polypeptides with insensitivity toward salt and a wide range of pH variations more than twenty years ago from lysine NCAs containing one or two pendant ethylene glycol (EG) units.¹⁰⁷ Li *et al.* synthesized poly(L-EG₃-glutamate) that also remained α -helical over a wide temperature range.⁸⁷ Kramer and Deming demonstrated glycosylated poly(lysines) containing β -D-glucose, mannose or galactose exhibited nearly 100% helical conformation in deionized water at 20 °C; their helicity could be disrupted by increasing the temperature.¹⁰⁸ Recently, sulfoxide containing polypeptides have been shown to contain high α -helical content in water (*circa* 58%) when the carbon spacer length separating the polypeptide backbone from the sulfoxide moiety is at least three units long.¹⁰⁹ A second approach to establish water-soluble polypeptides with stable α -helices involves the use of cationic polypeptides with elongated hydrophobic side chains to achieve optimal separation between the polypeptide backbone and the positively charged cations. In 2011, Cheng and co-workers reported the first cationic and remarkably stable α -helical polypeptide based on a side chain design containing at least 11 Sigma bonds between the charged

moiety and the backbone, thus reducing side charge repulsion.¹¹⁰ The use of poly(γ -(4-vinyl-benzyl)-L-glutamate) (PVBLG) allowed for facile ozonolysis and hydroamination to install charged secondary amines. PVBLG could also be functionalized using thiol-ene chemistry.¹¹¹ Additional studies by the Cheng group have ring opened γ -(6-chlorohexyl)-L-glutamate NCA monomer, as this polypeptide enables facile post-polymerization amination to introduce the desired cation.^{112,113} In 2018, Bonduelle and co-workers reported a remarkable ionic polypeptide that adopts stable β -sheet conformation whilst retaining complete water solubility, in contrast to other materials where the emergence of β -conformation leads to water insoluble amyloid aggregates.¹¹⁴ The successful polypeptide design employed poly(γ -propargyl-L-glutamate) (PPLG), which allows for the grafting of the nucleotide azidodeoxythymidine monophosphate *via* copper-catalyzed azide alkyne cycloaddition (CuAAC). The resulting polypeptide contains multiple phosphate moieties which impart water solubility and most likely stabilize the β -sheet conformation through intermolecular interactions between side chain nucleobases belonging to amino acids two units apart.

2.2.6.2 Water soluble polypeptides with responsive conformations.

In addition to the synthesis of water soluble and structured polypeptides, there has been great interest in developing polypeptides which mimic biological adaptive systems, *i.e.* able to undergo triggered conformational switches in response to one or various stimuli. Recent developments in this field now make it possible to design smart polypeptides with a responsive secondary structure transition based on temperature,¹⁰⁸ pH,^{94,115} salt concentrations,¹¹⁶ light,^{117–119} redox changes,^{109,120,121} metal coordination,^{122,123} DNA binding,¹²⁴ enzymatic presence,¹²⁵ *etc.* Glycopolypeptides are particularly promising since the sugar moieties offer improved water solubility in the conformers but can also aid in binding to specific proteins.^{126,127} Addition of just a few units can also enhance the α -helical content, as demonstrated by Krannig and Schlaad.⁹⁰ The manipulation of secondary structure has important implications for the material properties of polypeptides, as secondary structuring can direct the assembly of polypeptides into nanoscale structures,^{105,128} its unravelling,^{93,129} and furthermore modulate bioactivity^{113,130} (see Applications for more discussion of bioactivity).

2.2.6.3 Cooperative polymerization.

The emergence of helical secondary structure, unlike β -sheets² can also be exploited as an autocatalytic feature in polymerization. Kinetic investigations in the 1950s by Doty and Lundberg, and Ballard and Bamford, amongst other key researchers, revealed two-stage kinetics exist for various NCAs, initiators and solvents.¹³¹ The initial phase is considerably slower than the “growth” stage, where the rate increases 4–6 fold. From these early investigations three hypotheses were formulated to explain the phenomenon: (1) the helical chain growth,¹³² (2) the association mechanism involving microphase separation which would promote a higher concentration of NCAs in the peptide phase and which does not necessarily require α -helices^{133,134} and lastly, (3) the least supported theory postulating a change

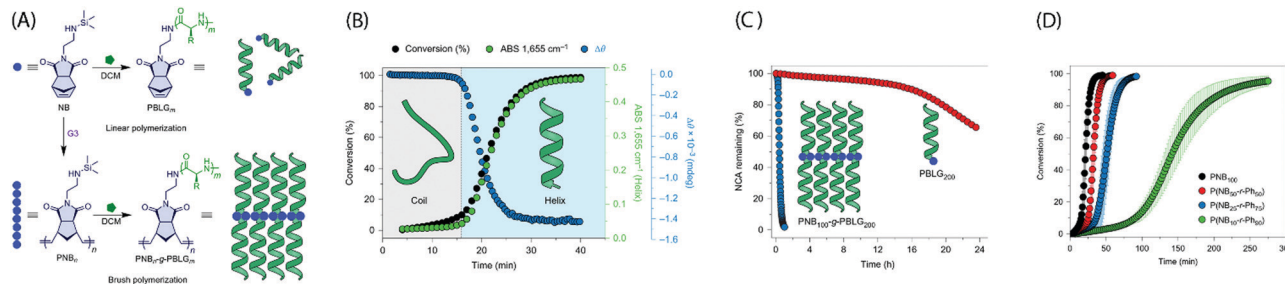


Fig. 14 Cooperative polymerization of α -helices as demonstrated by (A) linear and brush macroinitiators. (B) How acceleration of the rate of ROP coincides with a shift in secondary conformation from random coil to α -helix based on the increase in absorbance at 1655 cm^{-1} and the change in ellipticity measured by CD. (C) Time-conversion plots depicting faster ROP from macromolecular initiators than linear ones or (D) increasingly separated macroinitiating sites. Adapted from ref. 33. Copyright 2017 Springer Nature.

in mechanism. We refer the reader to Kricheldorf's book chapter for an in-depth discussion on these early works.¹³¹ Improvements in characterization techniques and computational advancements make it possible to probe these early theories further. In 2017, Baumgartner *et al.* reported the cooperative behaviour of the grafting-from polymerizations of BLG, γ -ethyl-L-glutamate (ELG) and EG₂-L-lysine NCAs (all NCAs with helical propensity), initiated by poly(norbornene-TMS) macroinitiators.³³ Fourier-transform infrared spectroscopy (FTIR) and circular dichroism (CD) characterizations revealed the polymerization rate greatly accelerated in macroinitiators containing a high density of TMS amines side by side (Fig. 14A). This phenomenon was convincingly tied to the folding of the growing polypeptide side chains into helices (Fig. 14B). The conformational switch from random coil at very low degrees of polymerization to the "growth" stage occurred upon attainment of a critical length of 8–12 repeat units, as previously reported.¹³¹ It was proposed that the α -helix promotes the close parallel alignment of the macrodipoles in proximity to the active polymerization site and that a high grafting density can further aid in the structuring. A reduction in the proximity of the macroinitiating sites or use of a linear initiator led to polymerization rate diminishments which could be explained by the reduction in templating effects (Fig. 14C and D). It is important to note that autoacceleration in the brush formation was only observed in solvents of relatively low polarity such as chloroform and DCM. Said cooperativity was not present when the polymerization was conducted in DMF – a hydrogen bonding solvent known to favor random coil conformations in the growing polypeptide side chains – nor when racemic mixtures of NCAs were employed since these disfavor secondary structuring.

Further recent studies into the cooperative polymerization promoted by α -helices have provided important insights into the minimal macromolecular complexity that may exhibit this desirable property. Chen *et al.* examined aliphatic diamine initiators with linker lengths from $n = 2$ to 12, discovering that the establishment of just a double helix motif, termed hinged polypeptide, could aid in significantly accelerating the reaction in helicogenic solvents (Fig. 15).⁵⁰ 1,6-Diaminohexane, compared to its linear analogue, could achieve complete conversion in 40 min (target DP 50, $[\text{NCA}]_0 = 0.1\text{ M}$), by which time point only 10% monomer consumption was recorded for the linear

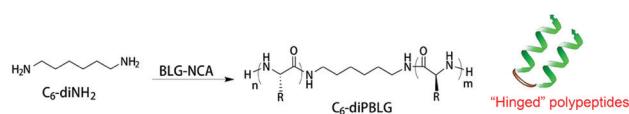


Fig. 15 Synthesis and schematic representation of a "hinged" polypeptide. Adapted with permission from *J. Am. Chem. Soc.*, 2019, **141**(22), 8680. Copyright 2019 American Chemical Society.

polymerization sample. Additionally, the kinetic acceleration effects were most pronounced in initiators with shorter carbon spacing, with the effect rapidly diluted at carbon linker lengths of ten units or above.

The important role of hydrogen bonding interactions on the autoaccelerated polymerization of helices has later been examined by Song *et al.*¹⁰³ The four unbound N-H groups at the N terminus of linear polypeptides, not engaged in the intramolecular hydrogen bonding which affords the helical conformation, have reduced rotational freedom which may explain their alignment with the direction of the macrodipole of the helix. Incoming NCAs, which contain three hydrogen bond acceptors, can reversibly bind to the four hydrogen-bond donor sites; this association then enables their ring opening at an accelerated rate, in a manner reminiscent of enzymes, which contain signature pockets onto which substrates dock. This hypothesis was supported by saturation transfer NMR and molecular dynamics. Furthermore, control experiments examining the polymerization of sarcosine further strengthened the hypothesis, as these revealed the second stage of the polymerization proceeded much slower than the polymerization of α -amino acid NCAs, owing to the lack of hydrogen bonds at the N terminus.

2.2.7 Miniemulsion ROP of NCAs and ROPISA. The use of emulsions is normally undertaken for the reaction of monomers with initiators that are not miscible in the same solvent. Typically this is done with the emulsification of two immiscible phases such as DCM and water, followed by addition of the initiator to the bulk phase. The high surface area of an emulsion allows for high reaction rates, while the amphiphilic environment allows for the removal of waste products during the reaction. Colloidal stability and polymerization control can be given to the emulsion with careful choice of surfactant to stabilize the microemulsion.^{135,136}

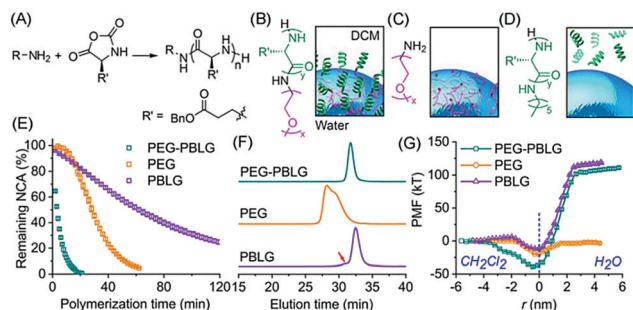


Fig. 16 SIMPLE polymerization depicting (A) schematic of the ROP of BLG NCA. Proposed interfacial anchoring behavior of macroinitiators (B) PEG-PBLG, (C) PEG, and (D) PBLG. (E) Conversion of purified BLG NCA in a w/o emulsion in the presence of three macroinitiators. Polymerization condition: $[M]_0 = 50$ mM, $[I]_0 = 0.5$ mM, water : DCM = 1 : 100 (wt/wt). (F) Normalized GPC-light scattering traces of resulting polymers. The red arrow indicates the high molecular weight shoulder peak from PBLG-initiated polymerization. (G) PMF profiles of PEG-PBLG, PEG, and PBLG in a DCM/water biphasic system computed using molecular dynamics simulation. Error bars correspond to estimated SD using the bootstrap method. Reproduced with permission from *Proc. Natl. Acad. Sci. U. S. A.*, 2019, **116**(22), 10658.

NCA ROP has not been traditionally carried out in emulsion, owing to the highly sensitive nature of NCA monomers to moisture. Very recently however, the Cheng and Heise groups have reported two very different systems to perform NCA ROP under miniemulsion conditions.^{29,137} The system first reported by Song *et al.* employed a water-in-oil emulsion to demonstrate the kinetic and population distribution differences in poly(γ -benzyl-L-glutamate) (PBLG) block formation based on macroinitiator properties.²⁹ Incidentally, the macroinitiators introduced in this study acted as surfactants for the emulsion, and emulsification was achieved *via* sonication of a 1 : 50 (wt/wt) aqueous buffer : DCM mixture. NCA monomer dissolved in DCM was added to the emulsion such that final conditions of 1 : 100 (wt/wt) buffer : DCM at the appropriate monomer ($I_0 = 50$ mM) to initiator concentrations were achieved for the desired DP. As shown in Fig. 16, the ROP of BLG was initiated by three different macroinitiators: amine-terminated PEG_{5k}, a diblock copolymer consisting of PEG_{5k}-PBLG_{9.8k} and thirdly, a PBLG polypeptide. PEG, being mostly hydrophilic, only had the terminal free amine groups at the boundary between the two phases, and the resulting polymer lacked control as the initial rate of the reaction was slow (Fig. 16E and F). At the other end of the spectrum, a PBLG block with α -helical secondary structure was too hydrophobic to thermodynamically favor positioning at the interface of the emulsion, and dispersed in the bulk of the DCM phase. In this instance, the resulting block copolymer was well controlled though the kinetics of the reaction were slower due to the distance between initiators. Thoughtful introduction of a helical initiating molecule containing the right balance of hydrophobic and hydrophilic components (PEG-PBLG) was crucial to stabilize the emulsion and favor tight alignment at the interface. This macroinitiator afforded a well-controlled polypeptide extension and exhibited remarkable kinetics—the polymerization rate was enhanced 100-fold compared to conventional polymerization in DCM—presumably due to the α -helicity of the macroinitiator,

but also very likely due the accelerating rate effects afforded by the increased surface area of the emulsion. Importantly, the authors discovered the chlorinated impurities present in crude NCA monomers could be eliminated *in situ* as these partition towards the aqueous phase. This methodology, termed SIMPLE, may provide an attractive alternative for the synthesis of large-scale polypeptides given that a rigorous purification of NCA monomers can be bypassed. The high rates of reaction under the right conditions ensure that the polymerization outpaces NCA hydrolysis.

The work recently reported by the collaborative team of Lecommandoux and Heise¹³⁷ makes use of more traditional emulsion polymerization conditions in comparison to the previous study. The miniemulsion ROP of *S*-(*o*-nitrobenzyl)-L-cysteine (NBCys) was studied, yielding poly(NBLCys) (PNBLCys) nanoparticles that could be cross-linked *via* photochemistry upon degradation of the UV-sensitive protecting group. In this study the fast anionic character of AMM was exploited to obtain monomodal polypeptides. Moreover, the authors found the order in which the materials were added is very important in determining the successful outcome of the polymerization. A glycosylated block polypeptide acting as a surfactant was first dissolved in deionized water (bulk solvent) at 0 °C, followed by addition of the NBC monomer dissolved in DCM under sonication. TEA was added immediately after the formation of a stable emulsion ($P = 13$ W, $c = 100$, $A = 70\%$). The droplets/micelles containing encapsulated NBC monomer were stabilized by the surfactant and effectively functioned as microreactors. The latexes were crosslinked into stable nanoparticles by irradiation with UV light after 24 h of reaction at room temperature, affording nanoparticles of approximately 200 nm in diameter.

Another area of expansion for NCA ROP is the polymerization induced self-assembly (PISA) field. PISA, particularly mediated by RAFT polymerization, has been extensively studied and can be carried out in emulsion or dispersion.^{138,139} In PISA processes the solid content, length of the macroinitiating block and degree of polymerization are important factors which impact the morphology of attainable particles.¹⁴⁰ Du and co-workers were the first to apply this valuable polymerization method toward the ring opening of L-phenylalanine NCA in THF at 10 °C (Fig. 17A).¹⁴¹ This development is important as it allows the formation of nano-assemblies from amphiphilic hybrid copolymers in one-pot, foregoing the nanoprecipitation step and providing advantageous properties such as high reproducibility and high concentrations compared to conventional methods of self-assembly. Self-assembled polypeptides are particularly relevant to the potency and utility of polypeptides in various applications, as will be further discussed in later portions of this review. Du *et al.* carried out the reaction at a considerably high solid content of 20% utilizing PEG_{2k}-NH₂ macroinitiator, under which conditions vesicles of average hydrodynamic radius (D_h) of 600 nm were obtained (Fig. 17B and C). By further examining targeted DP of the poly(L-phenylalanine) (PLPhe) hydrophobic block and solid content, the authors could demonstrate that at reduced solid content of 10% and short target DP of 10 or 15 solid nanoparticles of average size 100–150 nm were obtained. A higher solid content

NCA - PISA without nanoprecipitation step

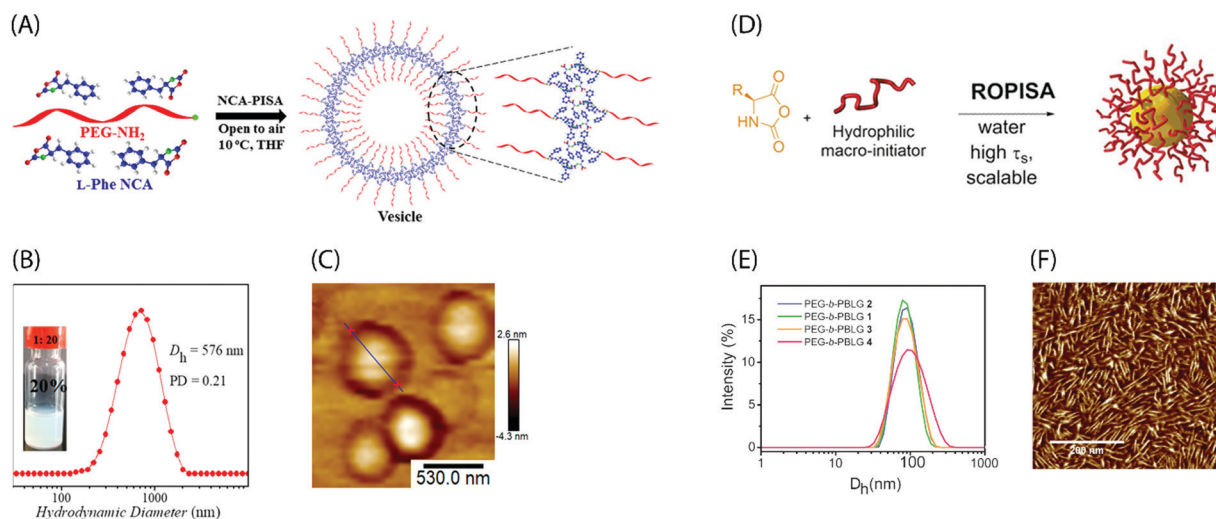


Fig. 17 NCA PISA carried out in (A) cooled THF allowing the obtaining of (B) ~600 nm vesicles measured by DLS. (C) AFM image of the vesicles.¹⁴¹ Adapted with permission from ACS Macro Lett., 2019, 8(10), 1216. Copyright 2019 American Chemical Society. (D) ROPISA carried out in aqueous solution. (E) DLS analysis of nanoparticles. (F) Needle-like morphology observed by AFM upon drying. Adapted with permission from ref. 30 Copyright 2020 Jon Wiley and Sons.

(20%) and elongation of the hydrophobic block (DP 20) favoured the formation of hollow vesicles.

Very recently the Lecommandoux and Bonduelle team has also unveiled the water-based ring opening polymerization induced self-assembly (ROPISA) of NCAs (Fig. 17D). Similarly to the work carried by the Du research team, this approach foregoes a nanoprecipitation step. PEG_{5k}-NH₂ and PEG_{10k}-NH₂ were successfully employed as hydrophilic macroinitiators for the ring opening polymerization of BLG and BLL NCAs in aqueous sodium bicarbonate buffer (pH 8.5) at 4 °C.³⁰ At solid contents ranging from 4 to 13%, block copolymers and nanostructures with needle and worm morphologies were formed (Fig. 17F). Higher solid contents resulted in soft gels. Interestingly the synthesis of well-controlled copolymers was dependent on the macroinitiator type and its concentration. The authors reported that PEG_{5k}-OH and ethanolamine were not capable of forming narrowly defined block copolymers. The hypothesis for the success of this surprising polymerization is a rapid polymerization/self-assembly process heavily influenced by the secondary structure of the peptidic block, the speed of the reaction may explain why hydrolysis of the monomer was not a major disturbance in the study. The secondary structure of the polypeptide block may also direct nanoparticle morphology, as the authors found a large proportion of β-sheet and α-helix in their lyophilized samples.

3. Biomedically-relevant architectures obtained through NCA ROP or in combination with other chemistries

3.1 Hybrid block copolymers

Block copolypeptide segments with specific functionality are highly desirable due to their ability to form higher order

assemblies and architectures. The chemistries presented in Section 2 allow for the synthesis of linear homopolypeptides, but can also be applied toward block copolypeptides due to their living features and suppression of side reactions.^{52,142–156} Block copolypeptide segments are well suited to different applications due to their favourable biointeractions (see Section 4 Applications). Their synthesis is relatively straightforward and easily performed in one-pot by sequential addition of NCA monomers. A previous review on the generation and application of block copolypeptides has been published by Thornton *et al.*,¹⁵⁷ while Savin *et al.*¹⁵⁸ recently reviewed the advances, self-assembly and application of polypeptide amphiphiles.

Hybrid block copolymers containing polypeptides are formed using one of three broad strategies: employment of a macroinitiator with suitable initiating functionality for NCA ROP,^{29,30,77,78,159–187} polymerization of the non-polypeptide block from a synthetic polypeptide,^{188–192} and conjugation of two independent block segments, often *via* click chemistry.^{130,193–200} A summary of recently investigated macroinitiators is shown in Fig. 18. We refer the reader to an excellent review by Barz *et al.* which describes the strategies available to introduce functional end groups in synthetic polypeptides.²⁰¹

The macroinitiator approach is the most commonly reported method for the synthesis of hybrid block copolymers, with the use of amine-terminated PEG and PEG derivatives particularly widespread.^{29,30,77,159–177} PEG has a high hydration capacity, resulting in a water “shell” that reduces protein adsorption.^{202,203} This is particularly important for biomedical applications where protein adsorption can cause deterioration of the material or stimulate an immune response. Additionally, PEG’s naturally hydrophilic character has been exploited in combination with more hydrophobic polypeptide blocks to produce polypeptide micelles and vesicles well-suited to biomedical

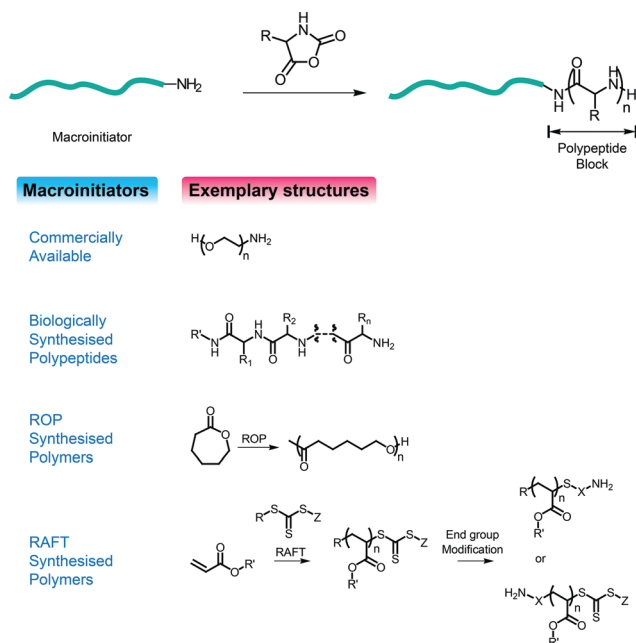


Fig. 18 Schematic representation of macroinitiated NCA ROP.

applications, as these amphiphilic copolymers readily self-assemble in aqueous solutions.^{29,77,159–164,193}

Despite the wide utility of PEG segments, hydrophilic designer polymers or molecules are desirable in instances where more specific block functionality is desired for an application. Biologically synthesized proteins are viable macroinitiators for NCA ROP *via* polymerization from their N-terminal.¹⁸⁶ It is important to note that when proteins are used as macroinitiators, the presence of certain exposed amino acid residues within the macromolecule (*e.g.* lysine or cysteine) initiates ROP. Thus, to achieve the linear grafting of a polypeptide from a protein, recombinant precursors must not contain any of these unprotected residues to prevent dendritic by-products. An elastin-like polypeptide (ELP) macroinitiator was used by Le Fer *et al.* to extend a PBLG block as shown in Fig. 19.¹⁸⁶ In this study the polymerization was carried out at 5 °C which enabled very narrow molecular weight distributions ($D < 1.04$), maintaining a high degree of control even at high molecular weights (overall molecular weight of 56.2 kDa, $DP = 180$).

Poly(ϵ -caprolactone),⁷⁸ poly(lactic acid),²⁰⁴ poly(pentadecalactone)²⁰⁵ and poly(lactic-co-glycolic acid)¹⁷⁸ obtained through acid-catalyzed ROP represent another group of well-known macroinitiators. Chemical modification of the propagating hydroxyl end group of the non-polypeptide block to install an amine is a standard practice for producing hybrid polymers.^{178,205} Xu *et al.* modified PEG-poly(lactic-co-glycolic acid) post-polymerization *via* conjugation of a molecule bearing an exposed carboxylic acid functional group and a Boc-protected amine.¹⁷⁸ Coupling was performed using Steglich esterification. Deprotection of the amine was then performed and subsequent NCA ROP of γ -benzyl-L-glutamate NCA provided the desired hybrid block copolymer. Recent synthetic advances in the ROP of NCAs may make post-polymerization modification redundant.

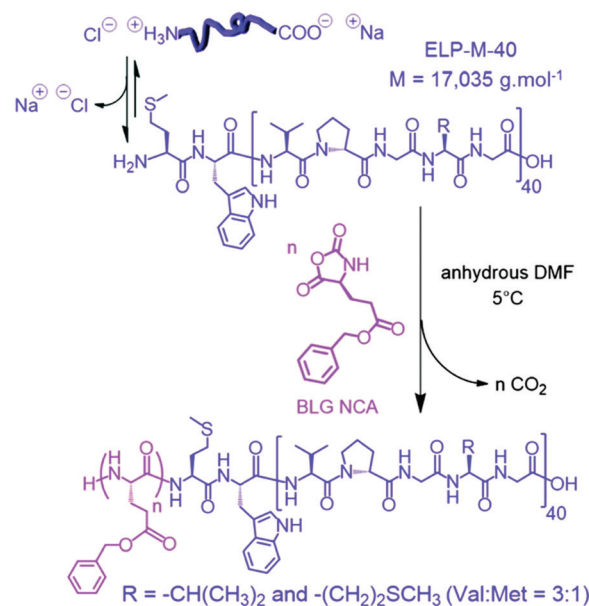


Fig. 19 Synthetic scheme of elastin-like polypeptide (ELP) initiated NCA ROP, yielding ELP-PBLG conjugates. Reproduced from ref. 186 with permission from The Royal Society of Chemistry. Copyright 2017.

Gradišar *et al.* studied unmodified hydroxyl-terminated macroinitiators obtained from the ROP of ϵ -caprolactone and trimethylene carbonate in MSA.^{77,78} Subsequent addition of base resulted in controlled initiation and propagation of poly(β -benzyl-L-aspartic acid).

With recent advances in RAFT polymerization, RAFT polymer macroinitiators have gained popularity.^{181–183} Because primary amines are known to cleave RAFT chain transfer agents (CTAs) *via* nucleophilic attack on the thiocarbonylthio group,²⁰⁶ CTAs require either the R or Z group to possess a protected amine¹⁸¹ or a functional group suitable for subsequent coupling of an initiating molecule for NCA ROP.^{182,183} Jacobs *et al.* developed a bifunctional initiator for both RAFT and NCA ROP, using a CTA with a phthalimide protecting group which can be easily deprotected using excess hydrazine to expose an amine functional group after RAFT polymerization.¹⁸¹ Wang and Ling instead took advantage of the existing thiol groups in the CTA and used thiol-ene click and thiol-methanethiosulfonate chemistries to insert an amine.¹⁸² The latter method, though more synthetically involved, has the added benefit of introducing easily cleavable disulfide bonds for *in vivo* applications. Overall, recent advances and ongoing discoveries of novel macroinitiators for NCA ROP will enable simplified steps in the formation of hybrid block copolymers, allowing for a wider arsenal of hybrid copolymers to be synthesized.

The second overarching category of hybrid copolypeptide synthetic techniques focuses on polymerization from polypeptides grown by NCA ROP.^{187–189} Shi *et al.* described the sequential living polymerization of two separate blocks *via* one single initiator.¹⁸⁸ A polypeptide obtained *via* transition metal catalysis with a nickelacycle active species remained living for the polymerization of a second poly(phenyl isocyanide) (PPI)

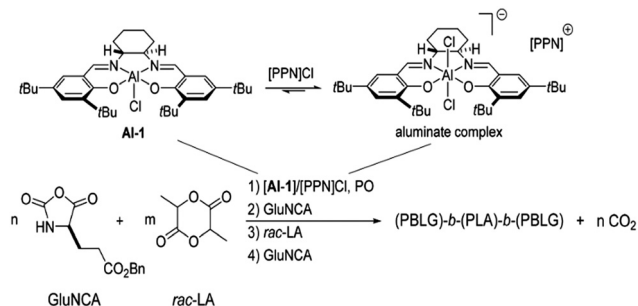


Fig. 20 Schematic representation of the one-pot synthesis of poly-peptide/polyester block copolymers mediated by a H_2 -salen aluminium catalyst. Adapted with permission from ref. 187. Copyright 2019 John Wiley and Sons.

block, allowing for one-pot synthesis of the block copolymer. Recently Thomas and co-workers have also reported the one-pot copolymerization of BLG NCA and lactide monomers *via* the use of a H_2 -salen aluminium catalyst activated by bis(triphenylphosphoranylidene)iminium chloride (PPNCl) in propylene oxide, yielding di- and tri-block polypeptide/polyester copolymers (Fig. 20).¹⁸⁷ The terminal amine of NCA ROP polypeptides also allows for the coupling of an initiator suitable for other polymerization techniques. Tappertzhofen *et al.* coupled a RAFT chain-transfer agent to the N-terminus of poly(lysine), allowing for the synthesis of a poly((2-hydroxypropyl)-methacrylamide) block *via* RAFT, though this coupling reaction prevented the synthesis from being one-pot.¹⁸⁹

The coupling of an NCA ROP-synthesized polypeptide to other polymers has also been investigated.^{130,193–200} The Lu group further investigated proteins coupled with synthetic polypeptides *via* native chemical ligation.^{130,195,196} PLGlu-based polypeptides with a terminal phenyl thioester were reacted with the thiol side chain of cysteine terminated proteins (human growth hormone and interferon) (Fig. 21).¹³⁰ Interestingly, coupling was found to maintain similar efficiency regardless of the stereochemistry of the building blocks, which are known to direct secondary structure. Johnson *et al.* have reported the

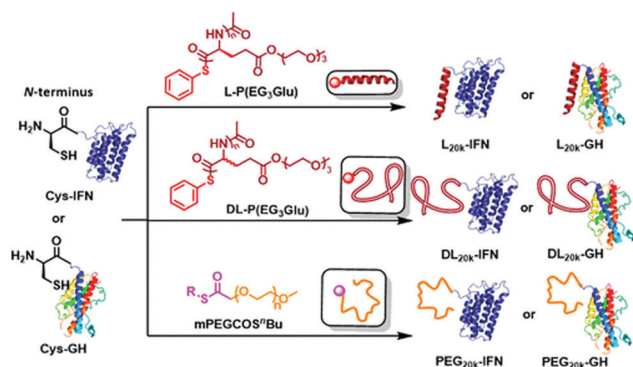


Fig. 21 Conjugation of poly(glutamic acid) with side chains modified with oligo(ethylene glycol) and interferon (INF) and human growth hormone (GH) *via* native chemical ligation using different stereoisomers to control helix formation. Reproduced with permission from¹³⁰ *ACS Cent. Sci.*, 2019, 5(2), 229. Copyright 2019 American Chemical Society.

coupling of poly(ethylene glycol) methyl ether acrylate to poly(lysine) *via* the use of CuAAC.¹⁹⁷ The use of azide-alkyne cycloaddition chemistry is especially popular when designing hybrids with biological polymer blocks such as glycoproteins.

3.2 Stars

The strategies described for the initiation of linear polymers *via* NCA ROP can also be utilized for the synthesis of more complex architectures such as star polymers, which are most often synthesized with drug and gene delivery in mind. Star polymers consist of three or more linear arms radiating outwards from a central core, which results in distinct core, arm, and peripheral regions that each offer unique opportunities for functionalization. In addition, star polymers are generally more compact and have lower solution viscosities than linear polymers of the same molecular weight. Star polymer synthesis is versatile with three approaches possible, known as the core-first, arm-first, and grafting-to approaches.²⁰⁷ This section will focus on the synthetic approaches to star polymers *via* NCA ROP and highlight important works published in recent years. Meanwhile, the uses of star polymers will be covered in the Applications section.

3.2.1 Core-first approach. The core-first approach has been the most commonly used strategy in the synthesis of star polymers *via* NCA ROP (Fig. 22 and 23). The core-first approach requires a multifunctional initiator from which arms can be polymerized, most commonly a terminal amine for NCA ROP. Although the number of arms and core size are limited, the core-first approach gives high yields and the star polymers are easily purified.²⁰⁷ Commercially available dendrimers such as poly(amidoamine) (PAMAM),^{208–210} poly(ethyleneimine) (PEI),²¹¹ and poly(propyleneimine)^{212,213} have been used as cores due to their widespread availability and the variety of generations available, enabling facile synthesis of star polymers with the desired number of arms. Groups have also synthesized dendrimers and hyperbranched structures, typically *via* click chemistry, to use as cores for star polymer synthesis.^{214,215} The use of less conventional cores has also been investigated.^{216–220} The Moretto group used carbon quantum dots (CQD) containing amine groups to initiate the polymerization of PBLG, resulting in a star-shaped CDQ-polypeptide hybrid.²¹⁶ A CDQ shell was then formed *via* functionalization of the star arm terminal amines with CQD, which induced aggregation of the star polymers. Interestingly, the aggregates maintained the characteristic emission properties of the unmodified CDQs. In a separate

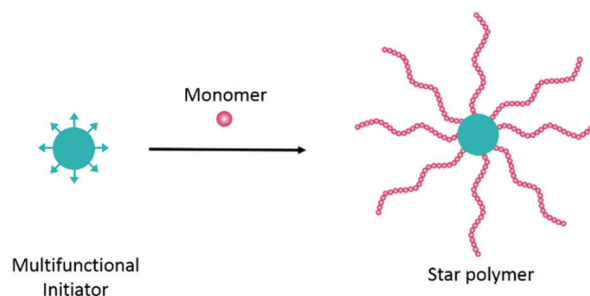


Fig. 22 Synthesis of star polymers *via* the core-first approach.

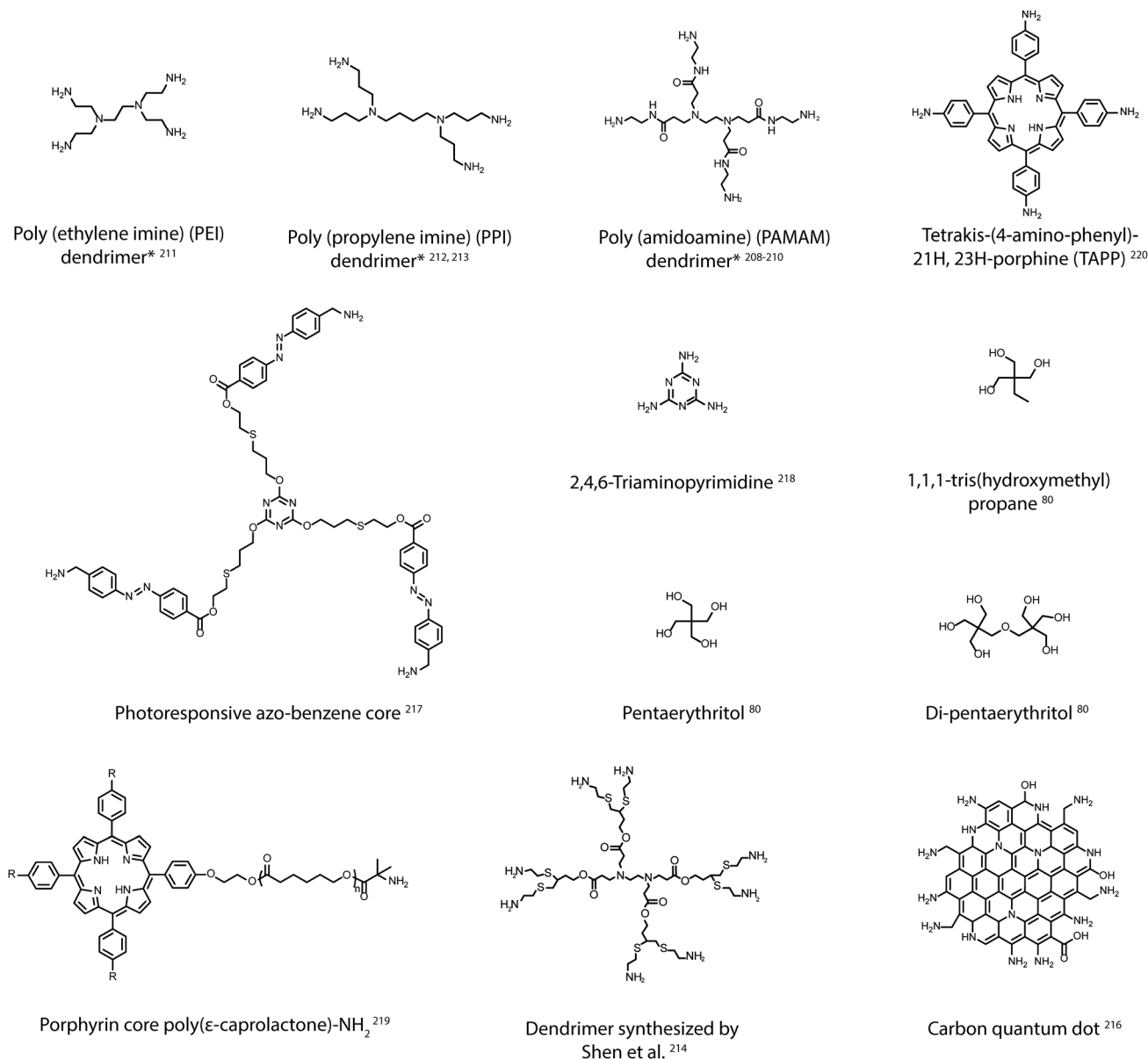


Fig. 23 Multifunctional initiators used for the synthesis of star polymers *via* the core-first approach using NCA ROP. *Dendrimers of varying generations and with different number of arms have been used. For simplicity, only the 4 arm versions are shown.

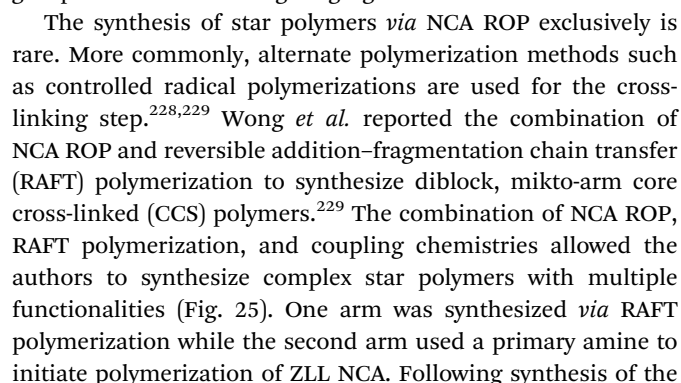
report, the Moretto group synthesized photoresponsive linear and star-shaped polymers by combining the photo-induced *cis/trans* conformational changes of azobenzene groups with polypeptides arms.²¹⁷

Star polymers with block copolymer arms can be synthesized by using multiple NCAs as well as by combining NCA ROP and other polymerization methods. Jia *et al.* synthesized a star with block co-polymers by combining ROP and NCA ROP, first polymerizing ε-caprolactone from a porphyrin core.²¹⁹ The resulting terminal hydroxyl groups were converted to bromide, then azide, and finally amino groups, which were used to initiate NCA ROP of a poly(ε-carbobenzyloxy-L-lysine) (PZLL) block.

Recent advancements in NCA ROP from hydroxyl initiators have reduced the need for end group modifications and

expanded the pool of possible initiators.^{77,79} Building on work by the Zhang group using 1,1,3,3-trimethylguanidine to promote NCA ROP from hydroxyl groups,⁷⁹ Shen *et al.* reported the synthesis of polypeptide stars with 3, 4, and 6 arms from alcohol groups using 1,1,1-tris(hydroxymethyl)propane, pentaerythritol, dipentaerythritol respectively, as cores.⁸⁰

One disadvantage of the core-first approach unique to stars synthesized *via* NCA ROP is cross-chain termination, or “backbiting”. Cross-chain termination occurs when the propagating amine attacks the benzylester protecting group of an amino acid instead of the CO-5 of an NCA, and results in chain termination.^{221,222} Stars are particularly susceptible due to the dense nature of star polymers. This phenomenon has most commonly been seen during polymerization of PBLG, and



polypeptide block, a trithiocarbonate (TTC) RAFT agent was conjugated to the terminal amine and a second block was prepared using RAFT polymerization, resulting in a block copolymer with a terminal TTC group. Star synthesis was achieved through RAFT polymerization of both diblock arm species and di-functional crosslinker *N,N'*-methylenebis(acrylamide) (MBA).

Recently the Heise group investigated the use of both RAFT and free radical polymerization (FRP) for the crosslinking step of polypeptide-based star polymers.²³⁰ ROP of BLG NCA was initiated by 4-vinyl-benzylamine, ensuring a vinyl end group was present on every polymer chain. Crosslinking agent divinylbenzene was used with and without RAFT agent to study star formation *via* RAFT polymerization and FRP, respectively. Star polymers synthesized *via* RAFT polymerization showed narrower molecular weight distributions than those synthesized *via* FRP, although both methods suffered from low conversion of linear polymers to star polymers.

Star polymers can also be synthesized through the self-assembly of linear arms into micelles which are then cross-linked. In this method, crosslinking generally proceeds *via* coupling chemistries as opposed to polymerization. The arms are often block copolymers containing a block with crosslinkable pendant groups. Following self-assembly of the block copolymers into micelles, the crosslinkable blocks are reacted to form a star polymer, also frequently called core-crosslinked micelles or nanogels. Self-assembly prior to crosslinking offers some advantages for star polymer synthesis. Crosslinking is promoted because the arms are already correctly oriented and aligned. As self-assembly is a thermodynamic process, the micelles have near uniform size and a low size distribution. Finally, star-star coupling is reduced because the crosslinkable units are all situated in the core of the micelle.²⁰⁷ Ding *et al.* used the self-assembly route to synthesize star polymers with a sheddable PEG corona and a disulfide crosslinked core.²³¹ The authors synthesized PEG-*b*-PCys arms that were dissolved in DMF and self-assembled to form micelles following the addition of water. The nanoparticle solution was oxidized by the addition of H₂O₂, crosslinking the pendant thiol groups of the poly(cysteine) (PCys) block into disulfide bridges to form a CCS polymer. Dynamic light scattering (DLS) indicated the star polymers had hydrodynamic radii of 135 ± 6 nm and 182 ± 7 nm when synthesized from linear arms with 21 and 79 cysteine repeat units respectively, highlighting the narrow size distributions that can be achieved with the self-assembly route.

3.2.3 Grafting onto. The grafting-onto approach can be considered a combination of the core-first and arm-first approaches as pre-synthesized linear arms are attached to a multifunctional core (Fig. 26).^{207,232} Although the least commonly used synthetic approach, grafting-onto offers the best characterized star polymers because the arm and core can be analysed separately prior to conjugation.²⁰⁷ Because stars are synthesized *via* coupling instead of polymerization reactions, star-star coupling is unlikely. The use of a multifunctional core with a set number of conjugation sites ensures that the maximum number of possible arms is known, however 100% conjugation is difficult, especially as the core becomes congested.²³³ As a

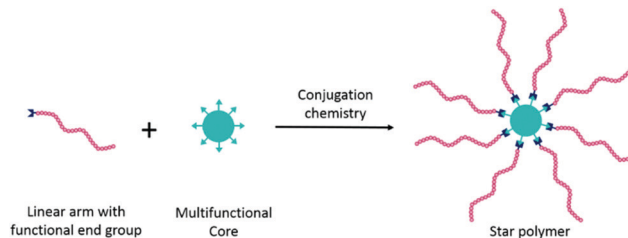


Fig. 26 An illustration of star polymer synthesis *via* the grafting-onto approach. Linear arms with a functional end group can be synthesized using a functional initiator or functionalized post-polymerization. Arms are then conjugated to a multifunctional core, often using click chemistry.

result, star polymers synthesized *via* the grafting-onto approach generally have a low number of arms. As with the arm-first approach, purification can be difficult if unreacted arms are present.

The grafting-onto approach often capitalizes on the quick and near-quantitative reactions of click chemistry.²⁰⁷ Chang *et al.* reported the synthesis of linear PZLL with a disulfide centre from a cystamine initiator.²³⁴ The two terminal amines were converted to alkyne groups, to each of which two PEG-SH polymers were conjugated using UV-initiated thiol-yne click chemistry. The result was 4 PEG arms attached to a disulfide-centred PZLL core. Pu *et al.* reported the synthesis of a diblock star polymer with a polyhedral oligomeric silsesquioxanes (POSS) core (Fig. 27).²³⁵ POSS are inorganic-organic hybrids composed of alternating silicon and oxygen atoms that form cage-like structures used in a wide variety of applications.²³⁶ An amine-terminated PEG was used to initiate the ROP of β -benzyl-L-aspartate (BLA) NCA, yielding a diblock polymer. Diblock polymers were then conjugated to the carboxylic acid group of the POSS using the terminal amine of the polypeptide block. Due to their hydrophobic cores and hydrophilic shells, the star polymers self-assembled to form micelles. Pranantyo *et al.* synthesized miktoarm stars using a combination of the core-first and grafting-onto approaches with a modified tetra-azide core.²³⁷ Two azide groups were converted to propionyl bromide groups which served as initiating sites for ATRP of vinyl monomers containing pendant carbohydrate moieties. Linear PZLL polymers initiated from propargylamine were then conjugated to the two remaining azide groups *via* copper-mediated azide-alkyne cycloaddition.

The grafting-onto approach has also been used in conjunction with the core-first approach, with linear polymers being “clicked” onto the ends of the star polymer ends. Lin *et al.* described the core-first ATRP of styrene from a tri-bromo-functionalized core.²³⁸ Post-purification, the bromine end groups were reacted with sodium azide to yield terminal azide groups. PBLG synthesized from a propargylamine initiator was then conjugated to the three-arm star polymer by copper-catalyzed azide-alkyne cycloaddition, yielding di-block star polymers.

3.3 Molecular polymer brushes

Polypeptide-bearing macromolecular brushes are desirable architectures due to the dense concentration of active sites they exhibit compared to linear products, in addition to the

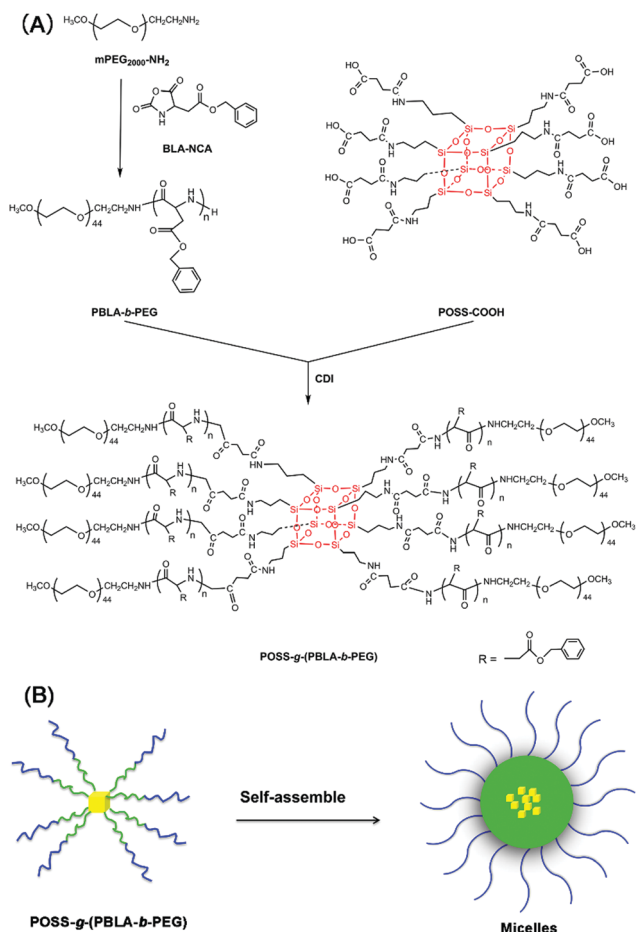


Fig. 27 Synthesis of star polymers with polyhedral oligomeric silsesquioxane (POSS) core and PEG-*b*-PBLA arms via the grafting-onto approach. The resulting stars self-assembled to form micelles with a hydrophobic POSS core and hydrophilic PEG corona. Reproduced with permission from ref. 235. Copyright 2013 John Wiley and Sons.

ability to design and control their large hydrodynamic radii and elongated cylindrical features. However, their synthesis poses diverse challenges. As with the star architecture, bottle-brushes can be obtained in one of three ways: by grafting-from, grafting-through and grafting onto.²³⁹ Grafting-from requires a macroinitiator backbone, whilst grafting through takes advantage of linear polymers bearing a polymerizable terminal group (*i.e.* macromonomers (MMs)). Grafting onto employs coupling chemistries to covalently bond polymeric side chains onto the backbone.

NCA ROP grafted from naturally-derived polymers has been explored and various reports of polypeptide brushes grafted from the primary amine sites of chitosan are published.^{240–243} Additionally, Du's group explored the grafting onto methodology by coupling an acid-functionalized chitosan biopolymer with linear polypeptide copolymers modified with a reactive isocyanate end group.²⁴⁴ In the majority of these publications however, the grafted products tend to exhibit either poor control or low grafting densities as the NCA polymerization initiated by primary amines can go through both normal amine and activated monomer mechanisms simultaneously.^{240,241,245}

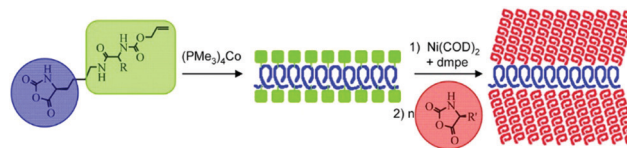


Fig. 28 One-pot synthesis of peptidic brushes through tandem catalysis with cobalt and nickel initiators. Reproduced with permission from ref. 252. Copyright 2012 American Chemical Society.

Additionally, solubility issues associated with a large presence of free primary amines or amine salts as well as termination by-products have greatly impacted the pursuit of well-defined macromolecular brushes from varied backbones.

The synthesis of entirely polypeptide-based brushes is also particularly challenging as steric congestion may adversely affect the efficiency of the grafting process.^{246,247} Dendrigrafts have been obtained through sequential ROP and deprotection of NCAs, yet these materials require intermediate isolation or purification steps that make their syntheses tedious.^{248–251} Moreover the morphology achievable is closer to globules than dense cylinders. In contrast, Rhodes and Deming in 2012 reported a successful methodology based on tandem cobalt and nickel catalysis for the production of densely grafted, fully peptidic brushes (Fig. 28).²⁵² In this one-pot procedure lysine NCAs bearing alloc- α -aminoamide groups were first polymerized by $\text{P}(\text{Me}_3)_4\text{Co}$ in THF, obtaining backbones of up to 300 residues long. Upon full conversion of N_ϵ -(alloc-L-methionyl)-L-lysine NCA, the backbones were concentrated and dissolved in DMF to avoid nickelacycle aggregation. DMF, unlike THF, affords quantitative initiation efficiency during the *in situ* activation of the nickelacycle complex (Fig. 2C(ii)). Side chains of PBLG were grafted with DPs of up to 25, although the authors envisaged this strategy could be used to obtain new brush copolypeptide architectures of longer side chains if desired. Recently, Liu *et al.* reported alpha-beta peptidic molecular brushes with a backbone made up of β -copolypeptides.²⁵³ The backbone was obtained *via* base-catalyzed anionic ROP of two β -lactams, with one of the monomers affording pendant amine groups along the β -backbone which were subsequently employed as initiating sites for the ROP of Boc lysine NCA.

Despite the dearth of exclusively peptidic brush examples in the literature span covered by this review, multiple studies have focused on the synthesis or properties of hybrid polypeptide brushes.^{33,254–256} Within these studies, ring opening metathesis polymerization (ROMP) has emerged as the polymerization technique of choice given that it affords the researcher some of the best results in terms of brush backbone length and narrow polydispersity.²⁵⁷ ROMP is also amenable to a wide range of chemical functionalities, although free amines are not well tolerated by the Grubb's catalyst.²⁵⁸ The incompatibility can be by-passed through initial polymerization of the unsaturated backbone, followed by NCA ROP. Grafting-from ROP to introduce polypeptide side chains onto a norbornene backbone was reported in 2009 by the Cheng group (Fig. 29).²⁵⁹ The key discoveries into the controlled ROP of NCAs mediated by NHTMS initiators enabled the controlled syntheses of polypeptide brushes with

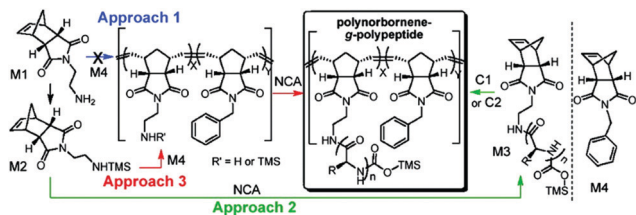


Fig. 29 Synthetic strategy followed by Lu *et al.* to produce synthetic brushes with polypeptides as side chains in a one-pot approach. Adapted with permission from *J. Am. Chem. Soc.*, 2009, **131**(38), 13582. Copyright 2009 American Chemical Society.

remarkably low molecular weight distributions. Attempts to polymerize the backbone from M1 as shown in Fig. 29, containing a primary amine, proved unsuccessful as the amine coordinated to the Grubb's catalyst, deactivating it. The introduction of TMS into the monomeric unit decreased these unfavourable interactions, producing backbones with increased solubility in the aprotic solvents necessary for NCA ROP and providing an alternative mechanism for the polymerization as discussed in the earlier synthetic section. Recently, as already discussed in Section 2, this same approach has been revisited to demonstrate how careful choice of solvent for the ring opening polymerization and choice of monomer can be utilized to create polypeptide brushes in shortened time with long polypeptide side chains, wherein blocks may be established, as well as polypeptide bottlebrushes with differing grafting densities.³³

Wooley's group reported an alternative method toward the synthesis of polypeptide-brushes utilizing graft-through ROMP of norbornene-polypeptide MMs.²⁵⁵ The graft-through polymerization of ROMP-amenable macromonomers functionalized with short peptide segments synthesized through SPPS has been previously reported,^{260–263} however this was the first instance where graft-through ROMP of polypeptide-bearing MMs obtained through NCA ROP was optimized. Carrying out the ROMP reaction in a cosolvent mixture of DCM and ionic liquid afforded well-controlled PBLA brushes. This approach may be pursued by interested researchers to produce polypeptide brushes of moderate side-chain length, as increasing the macromonomer length leads to considerable steric hindrance at the site of polymerization, leading to lower conversions and the necessity to remove unreacted macromonomer from the bottlebrush product. MMs with DP 10 reacted almost to full conversion but with DP 20, 80% conversion was achieved for $[M]_0/[C]_0 = 100$.

In addition to ROMP-based brushes, other hybrid polymer brushes have been produced through free radical polymerization of methacrylamide polypeptide-macromonomers,²⁶⁴ combinations of NCA ROP with ATRP^{265,266} or RAFT,^{267–270} and the use of CuACC click or thiol-yne chemistries.^{99,271–273} Some of these allow the formation of densely grafted macromolecular brushes with polypeptide backbones,^{99,265–267,269,271–273} while other examples cited have the polypeptide segments located on the side chain of the brushes.^{264,268,270}

3.4 Soft polymeric networks

3.4.1 Hydrogels. Polypeptide-based hydrogels, a subset of soft polymeric networks, are highly valuable materials for

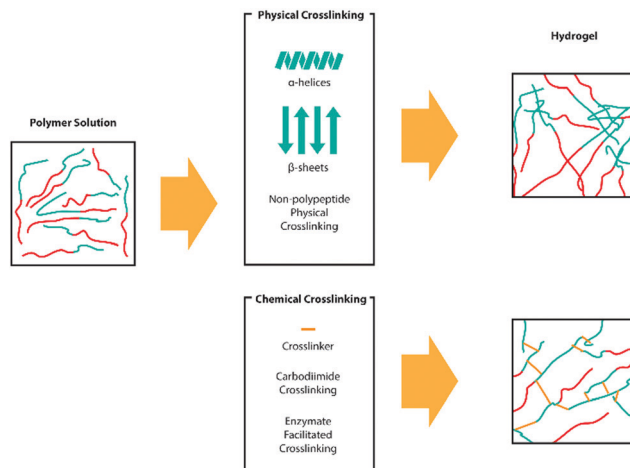
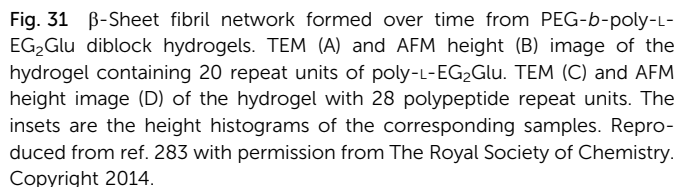


Fig. 30 Schematic of hydrogel formation via different polypeptide-based techniques.

biomaterials applications owing to several desirable properties (highly porous structures, biomimetic mechanical properties, inherent biocompatibility and biodegradability, hydrophilicity) which allow for the delivery of a variety of cargos as well as sustainment of cell functions.^{12,274} Comprehensive reviews by Deming,²⁷⁵ Wooley,¹² and Iatrou²⁷⁶ provide thorough insight into this class of materials, hence a brief overview is provided in this article. Generally, hydrogels are formed using either physical or chemical crosslinking, with different crosslinking methods pertaining to polypeptide-based hydrogels (Fig. 30).

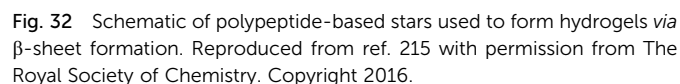
3.4.1.1 Physically crosslinked hydrogels. Physically crosslinked hydrogels are the result of non-covalent bonds (e.g. hydrogen bonding, van der Waals forces) between polymer chains. Strong physical interactions are exploited in synthetic polypeptide-based hydrogels primarily through environmentally-triggered secondary structure formation.^{62,214,277–313} Environmental changes in pH,^{62,277–282} temperature,^{281–306} solvent exchange/concentration,^{214,307–310,312} or introduction of ions/ionic polypeptides^{309–311,313} have all been shown to induce hydrogelation of polypeptide solutions. Furthermore, star and branched polymers have been used to enhance the ability of polymers to gelate in aqueous media.^{214,215,256,314,315} Alternatively, a wide array of side chain modification allows for coupling molecules which can physically crosslink using different chemistries such as DNA hybridization^{316–318} and host-guest chemistry.^{319,320}

3.4.1.1.1 Popular amino acids in polypeptide hydrogel design. Employment of homopolypeptides and homopolypeptide blocks with well-known secondary conformations allows for hydrogel design with predictable characteristics. Glutamic acid-based polypeptides possess α -helical propensity and undergo facile modification. Side chain modification of glutamic acid affords the modulation of secondary conformation, trigger-responsiveness and spontaneous formation of hydrogels.^{214,280,282–285} For instance, Li and colleagues reported that PEG-*b*-poly(L-EG₂-Glu) diblocks exhibited length-dependent β -sheet secondary structure; longer polypeptide DP correlated to a higher concentration



Poly(L-alanine) (PLAla) segments are also popular gelating blocks in polypeptide hydrogels,^{292–295,297–306} inspired by the pivotal role this amino acid residue plays in the formation of β -sheet nanocrystals in natural spider silk.³²¹ In general, β -sheet forming blocks are difficult to handle due to their hydrophobic character and subsequent predisposition to form ill-defined and poorly soluble aggregates.^{322,323} PALa blocks are however able to fold into both α -helical and β -sheet conformations in response to specific environmental conditions (*i.e.* pH and temperature). This ability, in addition to the modulating effects of block length and stereochemistry,³²⁴ further amplifies the value of this polypeptide. Several studies highlight the need to include hydrophilic polymeric blocks to diminish the uncontrolled β -sheet aggregation of the polymers. Lin *et al.* synthesized a pentablock sequence consisting of poly(L-lysine)–poly(L-alanine)–poloxamer–poly(L-alanine)–poly(L-lysine) which did not readily form β -sheets in solution at reduced temperatures due to the inclusion of the hydrophilic poly(L-lysine) chains.³⁰⁰ Circular dichroism revealed approximately 60% of secondary structures were α -helical, while only 2–4% of secondary structures were β -sheets under these conditions. Despite this, gelation was still achieved by reaching the threshold temperature of 16 °C, where β -turns transition into β -sheet conformations. The thermal transition of PALa-containing polypeptides is well-established in a range of works^{300–302} and indicates that establishment of β -sheet secondary structure is crucial in the design of stable hydrogels.

3.4.1.1.3 Hydrogels obtained using non-linear polypeptides. The use of non-linear polypeptides increases the gelation potential of polypeptides due to an increased amount of intermolecular forces occurring between each molecule. Star-based polypeptides are useful in the design of hydrogels alongside the aforementioned linear blocks, providing a greater number of arms with ordered secondary structures.^{214,215,314,315} The use of glutamic acid- and alanine-based polypeptide segments continues to be popular in the reported star hydrogels. A study from the Heise Group sought to expand the range of amino acid residues used for gelation within star-based systems and elucidate their gelation ability (Fig. 32).²¹⁵ In all cases, an 8-arm polypropylene imine core was used to initiate ROP to form PZLL inner blocks, followed by further ROP of hydrophobic NCA monomers (L-tyrosine, L-phenylalanine, L-valine, L-alanine). Amphiphilic stars that formed hydrogels possessed a critical



gelation concentration (CGC) of approximately 2 wt%. Only the stars containing PLAla blocks were unable to form a hydrogel due to the proclivity for random coils at such a low concentration (as revealed by circular dichroism measurements), implicating the importance of stable β -structure formation in the synthesis of star-based hydrogels.

3.4.1.1.4 Physical hydrogels obtained through DNA hybridization crosslinking. Besides acting as physical crosslinks, polypeptides can also be designed as a versatile polymer backbone with functional side chains onto which molecules can be coupled for alternative crosslinking strategies.^{316–320,325,326} The implementation of sticky-ended DNA strands as crosslinker molecules – DNA strands with sequences that exclusively bond to each other – is one such example.^{316–318} This property was cleverly employed by Li *et al.*, who synthesised the first 3D bioprinted synthetic polypeptide–DNA hydrogel with live cells (Fig. 33).³¹⁷ DNA hybridization was utilized as a crosslinking strategy by grafting multiple single-stranded DNAs onto a poly(L-glutamic acid₂₄₀-co- γ -propargyl-L-glutamate₂₀) backbone using carbodiimide chemistry (labelled Bio-ink A in Fig. 33). By simultaneously depositing this solution with another solution containing a complementary sequence to that expressed in Bio-ink A, gelation could be achieved within 30 seconds. Importantly, degradation of the hydrogel was possible *via* two pathways: protease degradation of the backbone through the action of endoproteinase Glu-C, or nuclease cleavage of DNA linkers.

3.4.1.1.5 Physical hydrogels obtained through host–guest crosslinking. Host–guest chemistry provides another crosslinking method for polypeptide hydrogels where cyclodextrin acts as the host molecule, and hydrogen bonding with the guest within the hydrophobic interior results in crosslinking.^{319,320,325,326} Cyclodextrins consist of 6–8 glucose subunits joined by glycosidic bonds in a macrocyclic ring, with pendant hydroxyl groups which can be used for coupling.³²⁷ However, commercial amine-modified cyclodextrin can also be employed, as exemplified by Li *et al.* (Fig. 34).³¹⁹ In this work β -cyclodextrin was grafted onto the

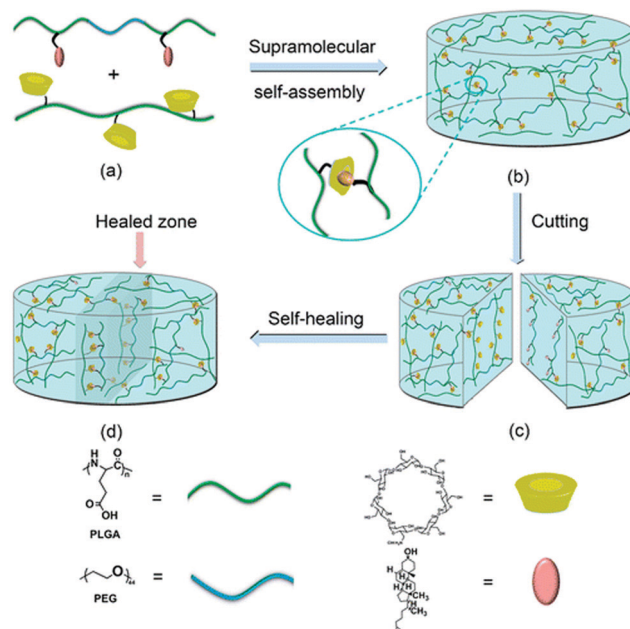


Fig. 34 Schematic of poly(glutamic acid)-based hydrogel crosslinked using guest–host chemistry. The “host” in this type of chemistry is commonly cyclodextrin while cholesterol is used in this particular case as the “guest”. Reprinted with permission from *Biomacromolecules*, 2015, 16(11), 3508. Copyright 2015 American Chemical Society.

side chain of PLGlu *via* carbodiimide coupling, whilst Steglich esterification was employed to couple the guest molecule cholesterol onto a PLGlu-*b*-PEG-*b*-PLGlu triblock, resulting in self-healing hydrogels upon formation of the host–guest linkage. In another study, Hou *et al.* used an alkyl initiator to form poly(L-threonine), which enabled the alkyl end group to be used directly as the guest molecule.³²⁰ It should be noted that the study performed by Hou *et al.* found that supramolecular host–guest chemistry played a stronger role in the gelation of such systems than ordered secondary structuring. As such, host–guest crosslinking is an attractive strategy which imparts self-healing capabilities and tunable mechanical properties, but can disrupt the usual secondary structure conformations afforded by the polypeptides present in the hydrogel.

3.4.1.2 Chemically crosslinked hydrogels. Lastly, polypeptide hydrogels obtained through chemical covalent crosslinking are well-established,^{69,312,328–341} as this crosslinking strategy generally results in more robust hydrogels than physical crosslinks. In the span of time covered by this review article, chemically crosslinked polypeptide networks are less commonly reported than physically crosslinked networks due to the biocompatibility concerns associated with the use of external chemical crosslinkers for biological applications.

Chemically crosslinked hydrogels often take advantage of both the secondary structure formation from polypeptides and the stabilization of the hydrogel macrostructure using chemical crosslinking.^{312,339} Schlaad and co-workers proposed an interesting organogel-to-hydrogel design exploiting the α -helical propensity of PBLG copolypeptides and chemical crosslinks.³³⁹

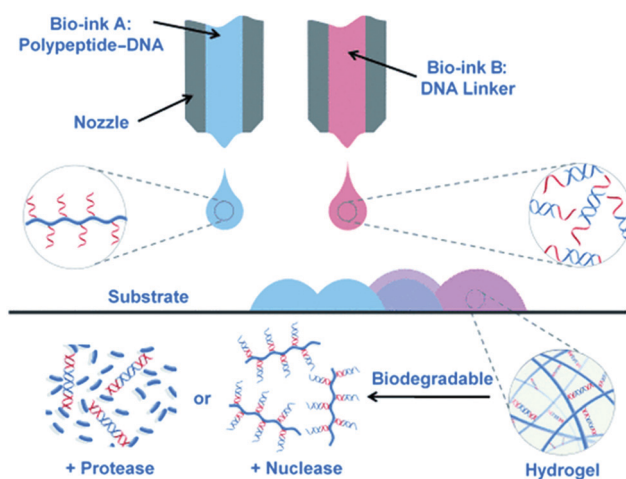


Fig. 33 The potential degradation of DNA-linked hydrogels. Reproduced from ref. 317. Copyright 2015 John Wiley and Sons.

This strategy employed P(BLG-*co*-allylglycine) in helicogenic dioxane to drive the aggregation of the polypeptide into α -helices in dilute conditions. The allylglycine groups were subsequently photocrosslinked *via* thiol-ene chemistry to enable the fixation of the porous, fibrillar organogel. Finally, deprotection of the benzyl group yielded pH-sensitive PGLu hydrogels upon return to aqueous solution. The hydrogels displayed a high swelling ratio and the ability to reversibly shrink upon changes in pH, due to coil-to-helix transitions. The Qiao group has uniquely employed both chemical and physical crosslinking recently, using a chemically crosslinked network to spatially control the formation of β -sheets.³¹² Free radical polymerization was used to form a PEG-based hydrogel with pendant amine side chains. These sites were used to graft long-chain P(LVal-*co*-glycine) polypeptides and subsequently induce β -sheet formation. This approach resulted in materials with a high density of β -sheets and a complete change of properties, including an increase in toughness of three-orders of magnitude.

Other crosslinking strategies rely on the covalent linking of peptide side chains, thereby removing concerns associated with the use of external crosslinkers.^{69,328–341} PLGLu^{328–333,338,339} and PLLys^{325,326,330,331,334,335} are the most popular polypeptides studied due to the facile modification of their side chains *via* carbodiimide chemistry. The Qiao and Chen groups both studied the statistical copolymer P(LLys-*co*-LGlu) to directly crosslink lysine and glutamic acid side chains, and create hydrogels.^{330,331} Cryogelation was possible using the technique (Fig. 35).³³⁰ Enzymatically-mediated crosslinking of phenol-bearing polypeptides can also be pursued, allowing for site specific gelation to occur.^{69,328,329,334,337,338} For example, Xu *et al.* used poly(L-glutamic acid) with side chains modified through the coupling of phloretic acid.³²⁹ The phenol side chains were then crosslinked using horseradish peroxidase, yielding a polymer that could undergo hydrogelation *in vivo*.

As with other techniques, the polymerization of monomers with difunctional groups (*i.e.* able to undergo polymerization at two sites simultaneously) is a relatively straightforward method of forming a chemically crosslinked polymeric network.^{340,341} In 2018, Raftery *et al.* demonstrated gelation using a range of novel sulfur-containing non-canonical di-NCAs depicted in Fig. 36, also employing cystine NCA for comparison.³⁴⁰

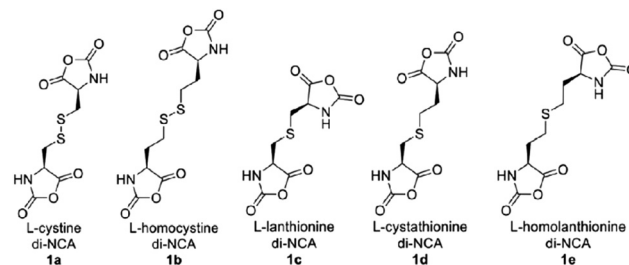


Fig. 36 Structures of di-NCAs which can serve as a crosslinker for hydrogelation. Reproduced from ref. 340. Copyright 2015 John Wiley and Sons.

Copolymerization of 2.5 mol% of di-NCA with *tert*-butyl-L-glutamate NCA led to self-supporting organogels and hydrogels (once deprotection and dialysis were carried out) with the exception of L-homolanthionine di-NCA, which did not achieve full monomer conversion. The non-canonical amino acid derivatives formed elastic hydrogels with significantly higher storage moduli than the cystine-crosslinked samples (~ 5 kPa for L-homocystine and L-lanthionine), whilst L-cystathionine produced the stiffest hydrogels (storage modulus of ~ 10 kPa). This study thus sheds light onto the possible fine tuning of hydrogel stiffness that can be achieved based on the tether length and chemical identity of the crosslinker. The introduction of disulfide or thioether linkages as part of the crosslinker adds a further dimension of control, as stimuli-responsive hydrogel degradation can be triggered by oxidation or reduction. Interestingly, the use of difunctional NCAs for crosslinking is relatively rare, with the majority of reports employing L-cysteine di-NCA for nanogel synthesis, as discussed next.

3.4.2 Nanogels. Discrete hydrogel nanoparticles, known as nanogels, define an intersection between soft polymeric networks and nanoparticles (Fig. 37).³⁴² They are desirable as a platform for targeted drug delivery. A large proportion of nanogels follow a core-shell design crosslinked in a similar manner to hydrogels. Such nanogels all contain a crosslinked core to hold the nanogel components together, and a shell made of long chain hydrophilic polymers which sterically

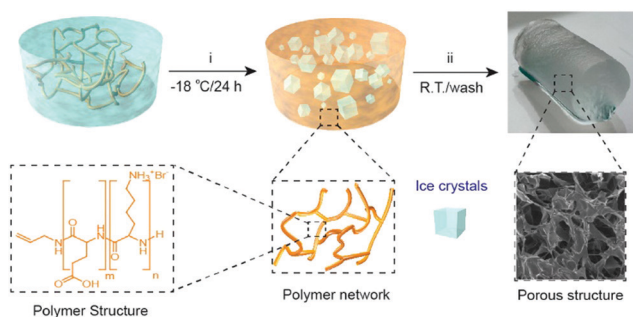


Fig. 35 Cryogelation using carbodiimide crosslinking with poly(lysine-*co*-glutamic acid) polypeptides. Adapted with permission from *Biomacromolecules*, 2016, 17(9), 2981. Copyright 2016 American Chemical Society.

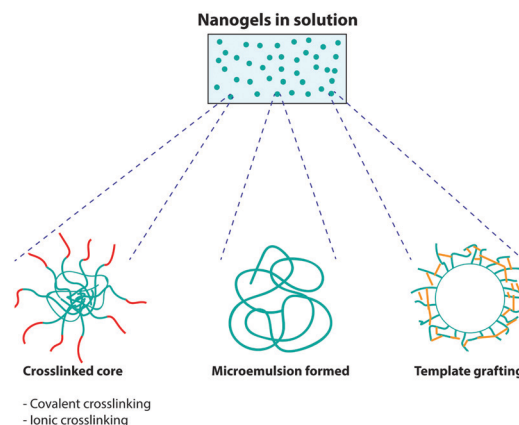


Fig. 37 Schematic of techniques used to form nanogels.

hinder crosslinking between nanogel particles, thus keeping them discrete. Synthetic polypeptides can be made into core-shell nanogels using two different methods of crosslinking: chemical crosslinking with cystine NCA^{343–354} or amphiphilic self-assembly followed by core crosslinking.^{149,355–361} To obtain a nanogel without a core-shell design, two alternative strategies have been used to synthesize nanogels using synthetic polypeptides: the use of microemulsions where the discrete phase is used to isolate regions where crosslinking can occur,^{137,362–366} and the use of pre-existing particle templates to support nanogel formation with template removal performed post-gelation.^{367–369} We refer the reader to a review from the Chen group on stimuli responsive polypeptide nanogels for more information on this subject.³⁴²

One of the most common methods for the creation of core-shell nanogels is the use of cystine NCA as a crosslinker within the hydrophobic core, allowing for reduction-responsive nanogels. Hydrophilic macroinitiators can be used to initiate ROP of the di-NCA to give a dense, crosslinked core,^{352–354} or the cystine residues can be copolymerized with other residues to modify swelling characteristics.^{343–351} This yields a cystine crosslinked core with the hydrophilic macroinitiator forming the shell.^{343–354} In some cases, PLLys-based hydrophilic blocks are used,^{351–353} yet PEG remains one of the most popular materials for the hydrophilic block as it is less susceptible to crosslinking with other particles.^{343–350,354}

Self-assembly of amphiphile polypeptides, followed by physical or covalent crosslinking is an alternative strategy to create discrete core-shell nanogels.^{149,355–361} Ionic complexation has been employed in conjunction with this self-assembly technique by both the Jeong and the Jan groups.^{355,356} The Jeong group designed PEG-*b*-PLLys-*b*-Pala amphiphiles with Pala blocks forming the hydrophobic core. Ionic complexation with the cationic amines on the lysine residues and subsequent gelation occurs upon introduction of anionic hyaluronic acid.³⁵⁵ This leads to the integration of PLLys as a second layer of the core, and only the PEG remaining as the outer shell. The Jan group used a cationic PLLys-based polymer with grafts of lipoic acid and lactobionolactone along with anionic poly(acrylic acid).³⁵⁶ This structure required further stabilization, achieved *via* cleavage of disulfide bonds within lipoic acid side chains and formation of new disulfide linkages with the freshly deprotected thiol groups of other lipoic acid side chains. Both of these cases required multiple forms of nanogel stabilization, suggesting nanogel formation *via* ionic complexation must be carefully designed. A separate study from the Jan group used a unique amphiphile with a random conformation instead of the blocks stated above.¹⁴⁹ The use of PLLys-co-P(LTyr) still resulted in discrete nanoparticles upon dissolution in phosphate buffer saline (PBS). The nanoparticles were then stabilized *via* UV-activated crosslinking of phenolic tyrosine side chains, and could then be embedded with gold nanoparticles within the nanogel networks.

In contrast, the use of microemulsions is a simple, well controlled approach to form nanoparticles.^{137,362–366} Due to the discrete nature of emulsion droplets, they allow for individual crosslinking of nanogels, while avoiding the formation of bulk

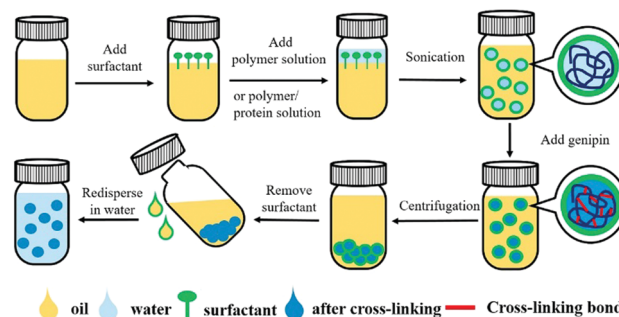


Fig. 38 Nanogel formation via microemulsion crosslinking. Reproduced with permission from ref. 362. Copyright 2017 Elsevier.

hydrogels. Microemulsions are formed by breaking the discrete phase into smaller droplets using ultrasound sonication to shear these droplets and through the use of surfactants to stabilize the droplets (Fig. 38). This results in a system where nanogel size can be controlled *via* the time of sonication and ratio of the discrete to continuous phases. As such, the chemistry used to form these nanogels can remain uncomplicated, allowing well-established crosslinking methods such as genipin-based crosslinking of PLLys side chain amines to be pursued.^{362,363}

The Heise group, as already discussed in Section 2, recently employed microemulsion polymerization to form nanoparticles, which based on their size and softness may be classified as nanogels. The miniemulsion ROP of NBCys was studied, yielding poly(NBCys).¹³⁷ To achieve this result, an amphiphilic block copolypeptide (P(LPhe-*b*-PLLys)) partially randomly glycosylated with lactobionic acid was used as a surfactant to stabilize the DCM-in-water microemulsion. NBCys monomer was added to the microemulsion under sonication, and it dispersed into the preformed particle seeds for the polymerization. Addition of TEA could then rapidly ring open the NCA within the microdroplets leading to nanoparticles of average diameter 210 nm. The photolabile protecting groups of Cys were subsequently cleaved under UV irradiation, affording disulfide bridge-crosslinked nanoparticles with denser cores and reduced hydrodynamic diameters (~186 nm after 60 min).

Alternatively, template grafting can be pursued to obtain nanogels.^{367–369} The common strategy is to use aminated silica templates particles to grow polypeptides *via* surface initiated NCA ROP, stabilize a gel layer *via* crosslinking and then remove the templates leaving a hollow nanogel with a controlled cavity size and shape. Yan *et al.* first grafted PLGLu from the silica templates, followed by insertion of chitosan, which acted as a crosslinker to strengthen the polypeptide layer (Fig. 39).³⁶⁷ In contrast, the Heise group first grew a random copolypeptide of L-tryptophan and D,L-Phe NCA, followed by a chain extension with a polysarcosine (PSar) polypeptoid block.³⁶⁸ The nanogels were formed by the crosslinking of hexamethylene-bis-1,2,4-triazoline-3,5-dione with L-tryptophan residues *via* indole-ene click reaction. The particles were finally dissolved in strong hydrofluoric acid to etch the silica template and reveal the nanogel. The Qiao group similarly grafted polypeptide chains from aminated silica to form their porous shells, but instead

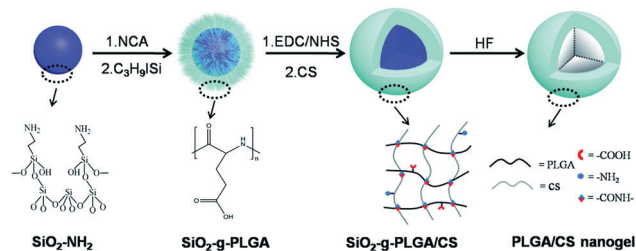


Fig. 39 Nanogel formation via the templating approach.³⁶⁷ [RSC Adv., 2017, 7(24), 14888] – published by The Royal Society of Chemistry.

polymerized PLVal chains which are well known for forming β -sheets.³⁶⁹ The PVal shells could be further functionalized by grating of other amino acids or by PEGylation to provide amphiphilic shells. With this approach the stabilization of the gel layer using chemical crosslinking could be bypassed, providing a strategy to directly control the nanogel dimensions *via* judicious choice of the template nanoparticle. Moreover, the thickness of the shells can be easily controlled *via* adjustment of the polymerization time or crosslinking chemistry. Nonetheless, this templating approach is not as established other methods, perhaps due to the harsh conditions necessary to remove the silica particle templates.

4. Applications

As nature's building blocks, amino acids are uniquely suited for *in vivo* applications. Polypeptides are degraded by intracellular enzymes into biocompatible oligomers and monomers. The 20 canonical amino acids have a wide range of side chain groups and functionalities to suit most applications and modified amino acids are easily synthesized.³⁷⁰ Interested readers are encouraged to refer to Deming's review on this topic.⁷ With this given variety, polypeptides have been investigated as suitable drug and gene carriers, but are also increasingly emerging as antibacterial agents in their own right or as part of conjugates. Their ability to adopt stable secondary structures has also led to their exploitation in the field of tissue engineering, as scaffolds. In this section, the most recent work is discussed based on the field of application, from antibacterial agents to biosensors and bioadhesives.

4.1 Polypeptides as antibacterial agents

Antibiotics are primarily sourced from the natural products isolated from microorganisms, although they can be synthetic. A major concern in the antibiotic field is the development of antimicrobial resistance and by 2050 it is predicted that more people will die from bacterial infections than cancer.³⁷¹ This prediction is based on the continuing rise in multi-drug resistant bacterial infections that are not being controlled by traditional antibiotics. A simple glance at the antibiotics marketed in the past five decades reveals a languishing trend in the number of promising discoveries; 90 antibiotics were marketed in the years 1982–2002,³⁷² while in the years since only 30 antibiotics have been approved by the FDA, most of which have been variations of existing classes.^{373–376} The dearth

of new antibiotic discoveries in the past few decades is owed in part to reduced funding and return on investment for the pharmaceutical industry sector, leading to downsizing or abandonment of research efforts altogether. Additionally, the reduction in clinical development of antibiotics is due in part to the difficulty in finding new natural products, as well as the lead-time in developing these for clinical use. Antimicrobial resistance is considered as 'one of our most serious health threats' and there is now unanimous agreement that new, potent and selective antimicrobial agents that do not induce resistance are urgently required.³⁷⁷ Recent incentives launched by governmental bodies and regulatory relaxation by the FDA hold the promise of a change in this bleak picture.

Polypeptides obtained from NCA ROP are promising but underutilized candidates for antibacterial applications. Polypeptides are desirable as antimicrobial agents due to rapid syntheses and the fact that their secondary structures can modulate selectivity. They are also biocompatible and biodegradable, and most importantly exert a non-specific mode of action against bacteria. Amphiphilic polypeptides containing cationic and hydrophobic amino residues interact with the bacteria's negatively charged phospholipid surface through electrostatic interactions. This mode of action is a welcome alternative to the very specific intracellular or membrane targets favoured by antibiotics, which inhibit for example bacterial cell wall, protein or DNA/RNA synthesis.³⁷⁸ The highly specific and common single mode of action of antibiotics has meant that bacteria, through mutations, are able to circumvent these destructive interactions and become resistant even towards our last-line of defence antibiotics, the polymyxins.³⁷⁹ The clinically-available forms of polymyxin, polymyxin B and E (also known as colistin), bind to the bacteria's lipopolysaccharides (LPS) and perturb the outer membranes of Gram-negative pathogens through displacement of Mg^{2+} and Ca^{2+} cations, but have nephrotoxic side-effects, leading to kidney failure in some patients, and to a lesser extent neurotoxicity.³⁸⁰ Needless to say, these side effects have limited their use. In most studies investigating mechanisms of resistance for colistin, modifications to the LPS are reported, as well as high expression and mutations in the *pmrA* and *pmrB* genes that regulate LPS modifications.³⁸⁰ The introduction of polypeptide-based materials as antibacterials has shown great promise as the investigations so far carried out into the synthetic polypeptides' electrostatic interactions with the bacterial cell envelope have shown non-specific but multimodal activity such as extended membrane depolarisation and leakage of cytoplasmic contents which greatly diminish the chances of resistance build-up and cast a promising outlook on this class of antimicrobials.^{209,381}

Publications preceding 2014^{242,244,382–384} have mostly focused on the antimicrobial potency of linear architectures,^{383,384} or polypeptide-grafted chitosan^{242,244} exhibiting both cationic amino acid residues (predominantly lysine), and hydrophobic segments (where alanine, leucine and phenylalanine were most extensively studied) *in vitro*. More recent publications covered by this review have built upon the knowledge generated from these early studies and examined higher order architectures. Table 2 summarizes the NCA ROP polypeptide systems reported in the

Table 2 Summary of antibacterial polypeptides reported from 2014–time of publication. In publications where several tested polypeptides are screened, MIC values are reported for the most potent and viable formulation and this one can be found highlighted in red on the first column of the table. Depending on the study, MIC values are reported in terms of molarity or $\mu\text{g mL}^{-1}$ (in parenthesis)

	Architecture	Active against	MIC μM ($\mu\text{g mL}^{-1}$)	HC ₅₀ μM ($\mu\text{g mL}^{-1}$)	Therapeutic index (HC ₅₀ /MIC or HC ₅₀ /MBC)	In vivo	Ref.
P(Glyco-ethylmethacrylate)-PLLys	4-Arm star	<i>E. coli</i>	1.6 (18)	(> 8192)	> 455	No	237
P(Mannose-ethylmethacrylate)₁₀-PLLys		<i>P. aeruginosa</i>	5.5 (64)		> 128		
		<i>S. aureus</i>	2.8 (32)		> 256		
		<i>S. epidermidis</i>	2.8 (32)		> 256		
PAMAM₁₆-g-P(LLys₂₀-co-DLVal₁₀)	Star	<i>E. coli</i>	0.72 (MBC)	> 45	> 63	Yes	209
		<i>P. aeruginosa</i>	1.42 (MBC)		> 32		
PAMAM₃₂-g-P(LL₂₀-co-DLVal₁₀)		<i>K. Pneumoniae</i>	1.54 (MBC)		> 29		
		<i>A. baumannii</i>	0.85 (MBC)		> 53		
		CMDR <i>P. aeruginosa</i>	1.38 (MBC)		> 33		
		CMDR <i>A. baumannii</i>	1.61 (MBC)		> 28		
PAMAM_{4,8 or 16}-g-P(LLys-co-DLVal) PAMAM₁₆-g-P(LL₁₂-co-DLVal₆)	Star	ATCC25922 (Gram-)	62 (3.3)	—	—	Yes	210
x(PLLys₁₅-b-PHEAm₅₀) + y(PGSA₃₀-b-PHEAm₅₀) + MBA 25 mol % glycopolymer	CCS star	<i>E. coli</i> ATCC8739 (> 512)		> 10 000	> 208	No	229
		<i>P. aeruginosa</i> ATCC27853	(> 512)				
		<i>S. aureus</i> ATCC29213	(32)				
		<i>S. aureus</i> ATCC25923	(64)				
		MRSA	(32)				
		<i>E. faecalis</i> OG1RF	(16)				
		<i>E. faecalis</i> V583	(64)				
Arm compositions							
HEAm = N-(2-hydroxyethyl)acrylamide PGSA = poly(glucosamine)							

Table 2 (continued)

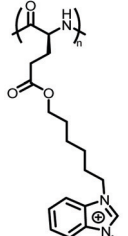
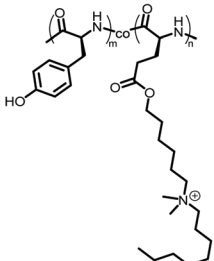
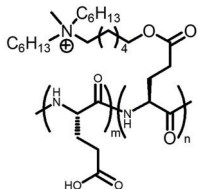
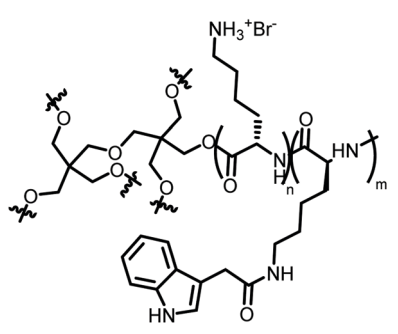
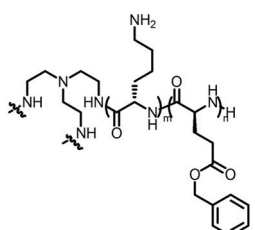
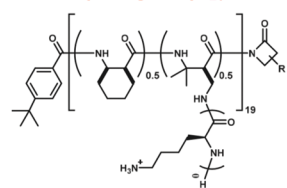
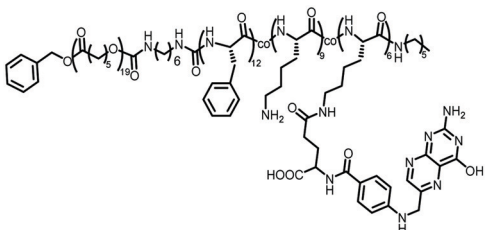
	Architecture	Active against	MIC μM ($\mu\text{g mL}^{-1}$)	HC ₅₀ μM ($\mu\text{g mL}^{-1}$)	Therapeutic index (HC ₅₀ /MIC or HC ₅₀ /MBC)	<i>In vivo</i>	Ref.
PHLG-BIm₄₀ PHDLG-BIm₄₀ PHLG-BIm₂₈ 	Radially amphiphilic	DH5 α (Gram $-$)	3.3	> 104.6	> 32	No	112
	Linear	MG1655 (Gram $-$)	26.1		> 4		
		ATCC12608 (Gram $+$)	13.1		> 8		
		ATCC1178 (Gram $+$)	13.1		> 16–70		
		Clinical <i>H. pylori</i>	1.5–6.6		> 70		
		MRSA	1.5				
PHLG-BIm = poly(γ -6- <i>N</i> -(1-methylbenzimidazole)hexyl- <i>L</i> -glutamate)							
PHOT-1 PHOPT-1 PHOT-2 PHOPT-2 DL-PHOT-2 DL-PHOPT-2 	Helix- transitionable	ATCC12608 (Gram $+$)	0.5		> 33	No	125
	Random coil $\rightarrow$ α -helical	ATCC11778 (Gram $+$)	0.5		> 33		
		MRSA (NRS384)	0.5		> 33		
PHOT = poly((γ -6-(<i>N,N</i> -dimethyl- <i>N</i> -octylamino)hexyl- <i>L</i> -glutamate)) _{<i>m</i>} - <i>co</i> -(<i>L</i> -tyrosine) _{<i>n</i>} PHOT-1 contains 20 mol% Tyr residues PHOT-2 contains 10% Tyr residues PHOPT = poly((γ -6-(<i>N,N</i> -dimethyl- <i>N</i> -octylamino)hexyl- <i>L</i> -glutamate)) _{<i>m</i>} - <i>co</i> -(<i>L</i> -phosphotyrosine) _{<i>n</i>}							
P(LGlu₁₈-<i>co</i>-HLG-MHH₂₀) (PHLG-MHH)₂₀ P(DLGlu₂₀-<i>co</i>-HDLG-MHH₂₅) 	Helix- transitionable	DH5 α (Gram $-$)	pH dependent	> 70		Yes	113
	α -Helical $\rightarrow$ random coil	MG1655 (Gram $-$) <i>H. pylori</i>					
HLG-MHH = γ -6- <i>N</i> -(methyldihexylammonium)hexyl- <i>L</i> -glutamate							

Table 2 (continued)

	Architecture	Active against	MIC μM ($\mu\text{g mL}^{-1}$)	HC ₅₀ μM ($\mu\text{g mL}^{-1}$)	Therapeutic index (HC ₅₀ /MIC or HC ₅₀ /MBC)	In vivo	Ref.
PLLys₂₀	Star	<i>K. pneumoniae</i>	70	—	—	Yes	81
PLLys₂₀-g-indole_{0.2}		EHEC	7.5				
3-arm PLLys₂₀		<i>P. aeruginosa</i>	70				
3-arm PLLys₂₀-g-indole_{0.2}		<i>S. sonnei</i>	70				
6-arm PLLys₂₀		<i>S. typhimurium</i>	8.75				
6-arm PLLys₂₀-g-indole_{0.2}		<i>S. aureus</i>	90				
							
Aniline-P(LLys₂₄-co-BLG₇)	Linear	<i>E. coli</i>	1.62	(>4000)	>500	No	70
Ethylenediamine-P(LLys₅₃-co-BLG₁₅)	Hinged	<i>B. subtilis</i>	3.23		>250		
TREN-P(LLys₇₈-co-BLG₁₆)	Star						
IHC-P(LLys₂₇-co-BLG₆)	Cyclic						
							
IHC = imidazolium hydrogen carbonate							
PβAA-g-PLLys₁₀	Molecular brush	MRSA USA300	0.26	—	—	No	253
PβAA-g-PLLys₂₀		MRSA 300 Lac	0.26				
		MRSA Newman	0.26				
		MRSA Mu50	0.26				
		MRSA USA400	0.26				
PCL₁₉-b-P(LPhe₁₂-co-LLys₉-co-LLys₆-g-FA)	Vesicles	ATCC35218 (Gram-)	(16 MIC ₅₀)	—	—	No	385
P(LPhe₁₂-co-LLys₁₅)		ATCC29213 (Gram+)	(16 MIC ₅₀)				
							

FA = folic acid

Table 2 (continued)

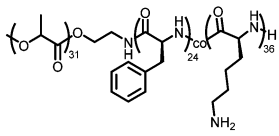
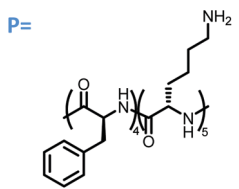
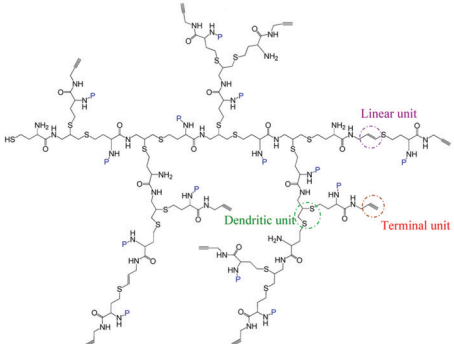
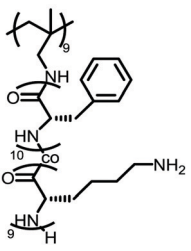
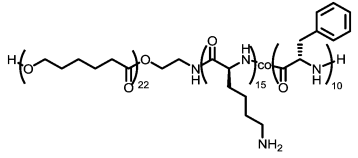
	Architecture	Active against	MIC μM ($\mu\text{g mL}^{-1}$)	HC ₅₀ μM ($\mu\text{g mL}^{-1}$)	Therapeutic index (HC ₅₀ /MIC or HC ₅₀ /MBC)	In vivo	Ref.
PLLA₃₁-P(LPhe₂₄-co-LLys₃₆) 	Micelle	ATCC35218 (Gram-)	(40 MIC ₅₀)	—	—	No	386
		ATCC29213 (Gram+)	(40 MIC ₅₀)				
P(LPhe₄-co-LLys₅)-g-Hyperbranched polymer P= 	Nanosheet	ATCC35218 (Gram-)	(16 MIC ₅₀)	~400	~25	No	387
		ATCC29213 (Gram+)	(16 MIC ₅₀)		~25		
							
Adapted with permission from <i>Biomacromolecules</i> , 2016, 17(6), 2080. Copyright 2016 American Chemical Society.							
P(2-MAA)-b-P(LLys₉-co-Phe₁₀) P(2-MAA)-b-P(LLeu₉-co-LLys₈) MAA-g-P(LLys₉-co-Phe₁₀) 	Nanoparticle	<i>E. coli</i> (BL21)	(16)	—	—	No	381
		<i>E. coli</i> (NDM-1)	(16)				
		<i>E. coli</i> (ImiS)	(16)				
		<i>S. aureus</i> ATCC29213	(16)				
		MRSA (ATCC43300)	(16)				
		VRE <i>K. pneumoniae</i> (ATCC70060)	(16)				
		<i>P. aeruginosa</i>	(16)				
PCL₂₂-b-P(LLys₁₅-co-LPhe₁₀) 	Dual corona vesicle	<i>E. coli</i>	(128)	—	—	Yes	388
		<i>S. aureus</i>	(128)				

Table 2 (continued)

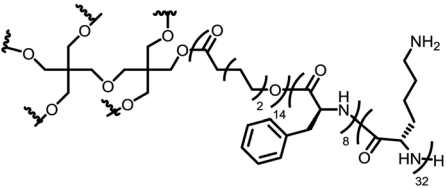
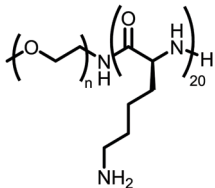
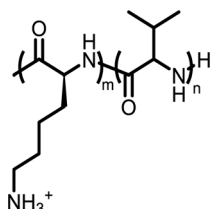
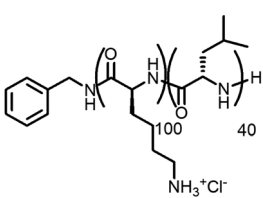
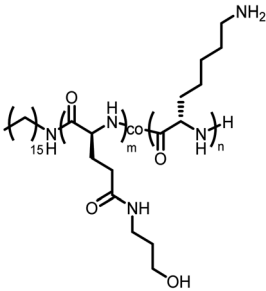
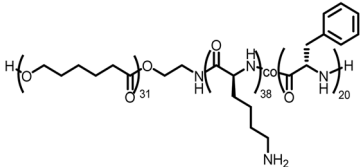
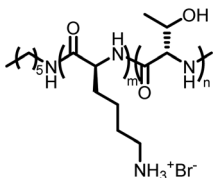
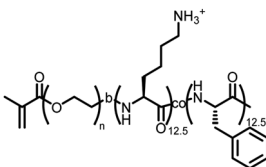
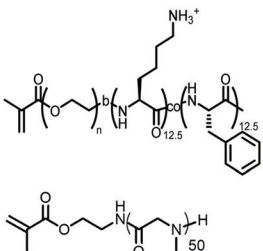
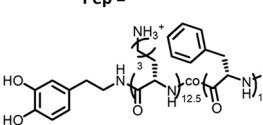
	Architecture	Active against	MIC μM ($\mu\text{g mL}^{-1}$)	HC ₅₀ μM ($\mu\text{g mL}^{-1}$)	Therapeutic index (HC ₅₀ /MIC or HC ₅₀ /MBC)	In vivo	Ref.
Ag@PCL-<i>b</i>-AMP AMP = 6s-PCL₁₄-<i>b</i>-P(LPhe₈-co-LLys₃₂) 	Ag nanocompo- site particle	<i>E. coli</i> (BL21)	(4)	—	—	Yes	82
		<i>S. aureus</i> ATCC29213	(2)				
		<i>P. aeruginosa</i>	(8)				
		MRSA (ATCC43300)	(4)				
		VRE	(4)				
		<i>K. pneumoniae</i> (ATCC700603)	(8)				
PEG-<i>b</i>-PLLys + EDTA PLLys-EDTA 1 : 1 	Nanofiber	<i>E. coli</i> ATCC8739	(10.9)	—	—	No	389
		<i>S. aureus</i> ATCC29213	(21.9)				
		<i>P. aeruginosa</i> ATCC27853	(43.8)				
PLLys-<i>b</i>-PDLVal crosslinked with glutaraldehyde 	Cryogel	<i>E. coli</i>	95.6% (CFU reduction)	—	—	No	336
PLLys₁₀₀-<i>b</i>-PLLeu₄₀ 	Solutions and hydrogel	<i>S. aureus</i>	% CFU red. 99.99	—	—	Yes	390
		MRSA	99.97				
		VRE	99.94				
		<i>Streptococcus</i> <i>pyogenes</i>	99.35				
		<i>A. Baumannii</i>	100				
		MDR <i>A.</i> <i>Baumannii</i>	100				
		ESBL <i>E. coli</i>	100				
		<i>K. pneumoniae</i>	100				
		EBSL, KPC <i>K. pneumoniae</i>	100				
		<i>P. aeruginosa</i>	100				
		MDR <i>P. aeruginosa</i>	100				

Table 2 (continued)

Architecture	Active against	MIC μM ($\mu\text{g mL}^{-1}$)	HC ₅₀ μM ($\mu\text{g mL}^{-1}$)	Therapeutic index (HC ₅₀ /MIC or HC ₅₀ /MBC)		In vivo	Ref.
C₁₆-P(BLG(aminolysed)₄-co-LLys₁) crosslinked with sodium alginate 	MDR <i>Bacteroides fragilis</i>	99.77					
	<i>C. albicans</i>	100					
	Fluconazole-resistant <i>C. albicans</i>	100					
	<i>E. coli</i>	—	—	—		No	391
PCL₄₇-b-P(LLys₃₈-co-LPhe₂₀) polymersome loaded with Penicillin G grafted into chitosan hydrogel crosslinked by DF-PEG 	<i>E. coli</i>	(64)	—	—		No	392
	<i>S. aureus</i>	(32)					
TRAIL-crosslinked P(LLys-co-LThr) 	<i>K. pneumoniae</i>	—	—	—		Yes	366
MA-P(LLys_{12.5}-co-LPhe_{12.5}) MA-PEG₁₀-b-P(LLys_{12.5}-co-LPhe_{12.5}) MA-PEG₄₅-b-P(LLys_{12.5}-co-LPhe_{12.5}) MA-PEG₉₀-b-P(LLys_{12.5}-co-LPhe_{12.5}) And their brushes 	Peptide in solution	<i>E. coli</i>	17 (125)	268 (2000)	16	—	393
		<i>S. aureus</i>	17 (125)				
		<i>P. aeruginosa</i>	17 (125)				
	Bottlebrush surface coating	<i>E. coli</i>	92–100% kill	—	—	Yes	
		<i>S. aureus</i>					
		<i>P. aeruginosa</i>					

MA = methacrylate

Table 2 (continued)

	Architecture	Active against	MIC μM ($\mu\text{g mL}^{-1}$)	HC ₅₀ μM ($\mu\text{g mL}^{-1}$)	Therapeutic index (HC ₅₀ /MIC or HC ₅₀ /MBC)	<i>In vivo</i>	Ref.
Poly(PPep) Poly(PPep/PSar) Macromonomers incorporated 	Bottlebrush surface coating	<i>E. coli</i>	% contact kill 99.7	—	—	Yes	394
		<i>S. aureus</i>	97.6				
		<i>P. aeruginosa</i>	94.6				
		<i>C. albicans</i>	95.6				
Titanium nanospike peptide coating Pep = 	Pep in solution	<i>E. coli</i>	11.1/31	—	—	Yes	395
		<i>S. aureus</i>	11.1/31				
		<i>P. aeruginosa</i>	11.1/31				
	Coating	<i>E. coli</i>	> 99.9% contact kill				
		<i>S. aureus</i>					

literature from 2014 until the time of publication, along with their minimum inhibitory concentration (MIC) values, HC₅₀ (if reported) and an indication of whether *in vivo* experiments were carried out. It is important to note that a direct comparison of these minimum inhibitory concentrations is not possible due to the different methodologies and assay conditions utilized to determine MIC value. Thus, the reader is directed to the specific articles referenced if interested in finding a detailed description of the experimental methods.

4.1.1 Antibacterial polypeptides in solution. A large number of antimicrobial peptides (AMPs) under investigation contain mixtures of cationic and hydrophobic amino acid residues which segregate into facially amphiphilic conformations. As a point of difference, Cheng and co-workers in 2015 reported a new class of antimicrobial polypeptides, termed radially amphiphilic (RA) (Fig. 40).¹¹² These single stranded polypeptides were constructed

from the ring opening polymerization of γ -(6-chlorohexyl)-L-glutamate (CHLG) NCA followed by amination with 1-benzimidazole. The resultant cationic polypeptides adopted a stable α -helical conformation and said helicity was driven by the careful molecular design with 11 σ bonds separating backbone from the charged moiety. The α -helical polypeptides were active against both Gram-positive and Gram-negative bacteria and stable in serum and plasma. Stereochemistry of the amino acid building blocks was found to affect antibacterial activity, as polypeptides consisting of D,L residues were unable to form stable α -helices and had reduced efficacy. The length of the polypeptide was also of importance, as a DP of 40 proved more active than 28 repeat units.

Spurred by this promising antibacterial design but limited by the inherent cytotoxicity of helical cationic polypeptides, the Cheng and Lu groups have since extensively investigated the design and synthesis of RA polypeptides able to transition from random coil to α -helix in response to a stimulus.^{113,116,125} Xiong *et al.* were the first to report increased antibacterial activity in response to modulation of secondary conformation, by exploiting the action of bacterial phosphatase, an enzyme which is overexpressed at infection sites.¹²⁵ The transition in secondary structure has wide ramifications for the synthesis of novel AMPs with tunable selectivity. The authors demonstrated that statistical copolymers of quaternary ammonium-bearing glutamate and phosphorylated tyrosine are effective against Gram-positive *S. aureus* and *Bacillus cereus* (MICs ranging from 14.9 to 2.1 μM) but even more so once bacterial phosphatase dephosphorylates the tyrosine moiety. In the phosphorylated form, the polypeptides caused virtually no

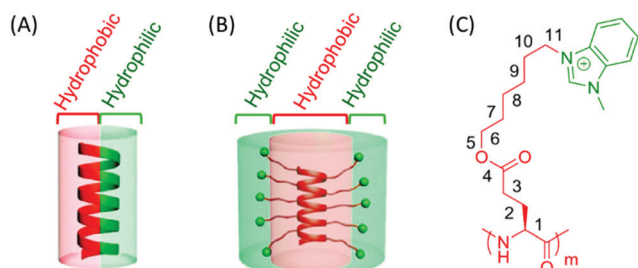


Fig. 40 Schematic representation of antimicrobial peptides and polypeptides with (A) facial amphiphilicity or (B) radial amphiphilicity. (C) Chemical structure of RA polypeptide. Reproduced with permission from ref. 112. *Proc. Natl. Acad. Sci. U. S. A.*, 2015, **112**(43), 13155.

haemolysis up to concentrations of 70 μM , and they also exhibited reduced mammalian cell toxicity compared to their helical non-phosphorylated counterparts, as determined by MTT assay. Circular dichroism experiments provided evidence that activity and selectivity were due to a switch from random coil to α -helix secondary structure, as demonstrated by the appearance of the characteristic double minima at 208 and 222 nm. As the phosphatase cleaved the phosphate groups, the electrostatic interaction between anionic phosphate groups and quaternary ammonium groups was disrupted. In the dephosphorylated and therefore helical form, the same polypeptides had significantly improved MIC values in the range of 3.9 to 0.5 μM . This study thus shows how careful design can indeed modulate membrane-disruptive activity. It is however important to note that in this work dephosphorylation was achieved by alkaline phosphatase purified from *E. coli* prior to incubation with the bacteria strains of interest. Additional experiments investigating the degradation of the polypeptide in the growth medium of *S. aureus* revealed much slower dephosphorylation rates, therefore, it would be of interest to investigate to what extent helicity is induced during the *in vitro* testing of *S. aureus* or *B. cereus* and what MIC values are obtained under these experimental conditions.

Another study by the Cheng group further investigated how to tailor helix-transitionable RA design for the treatment of *Helicobacter pylori* infections, pathogens which are responsible for gastric ulcers and carcinomas.¹¹³ In this work Xiong *et al.* implemented a pH-responsive polypeptide design based on the copolymerization of *L*-tert-butyl-Glu and CHLG NCAs, which enabled the killing of *H. pylori* in the stomach without damaging the commensal bacteria located further along the digestive track (Fig. 41). The inclusion of PGlu allowed for carboxylic acid protonation/deprotonation as a function of pH, ensuring the polypeptide's ability to transition from helix (active) to random coil (inactive) in the intestine environment. At physiological pH the carboxylate anion interactions with the cationic charges installed on PCHLG rapidly destabilized the α -helix, leading to its collapse. Corroborating earlier *in vitro* observations of improved cytotoxic profiles, *in vivo* experiments using mice showed no elevated inflammatory markers, a stable body weight, and stable electrolyte levels in blood for the mice

that underwent treatment, demonstrating the excellent biocompatibility of the helix-transitionable synthetic polypeptides.

Moving away from RA investigations, other researchers have instead probed the hypothesis that a dense architectural design may result in elevated local concentrations of cationic charges and in turn potentiate the antimicrobial activity of amphiphilic polypeptides. The collaborative team of Qiao and O'Brien-Simpson investigated the remarkable antibacterial efficacy of structurally nanoengineered antimicrobial peptide polymers (SNAPPs) (Fig. 42A).^{209,210} These star-shaped peptide polymers are polymerized from PAMAM cores using the grafting-from approach and are composed of statistical mixtures of *L*-lysine and *D,L*-valine amino acid residues. Importantly, they were found to be effective against a wide range of Gram-negative bacteria at nano-molar concentrations. Co-incubation of SNAPP₁₆ (a star polymer with 16 peptide arms) with different Gram-negative strains in minimal essential medium (MEM) yielded minimum bactericidal concentration (MBC) values ranging between 0.05–0.19 μM , even for colistin and multi-drug resistant (CMDR) *P. aeruginosa* and *A. baumannii*. Interestingly, a linear analogue with similar peptide composition to the star polypeptide exerted much weaker activity (MBC 40-fold higher), accentuating the importance of charge density and possibly architectural design in the quest for effective antibacterial agents. While the *in vitro* results were promising, the successful story of the SNAPPs lies in their *in vivo* anti-infective efficacy against established CMDR *A. baumannii* infections. This study provided the first report of a synthetic polypeptide with high efficacy in a mice model challenged with a CMDR Gram-negative bacterial infection. The mice treated with SNAPP₁₆ had significantly decreased bacteria counts in the blood, spleen and at the initial site of injection, the peritoneal cavity. Additionally, higher numbers of neutrophils were detected in the peritoneal cavity compared with the imipenem and control groups. Mechanistic studies to determine the SNAPP's mode of action suggested that the SNAPPs first interact with the negative charges of lipopolysaccharides located at the outer bacterial membrane through electrostatic interactions, followed by transition to the inner membrane and internalization (Fig. 42B and C). These processes elicit loss of cytoplasmic membrane stability, with concomitant unregulated ion movement and membrane potential dissipation, ultimately enabling cell lysis. At dosages equalling the MBC, but also sub-MBC concentrations, the SNAPPs also generate reactive oxygen species (ROS), one of the markers for apoptotic-like death. The combination of these multiple mechanisms as mode of action aids in preventing the on-set of resistance and provides a much needed alternative to treat colistin and multi-drug resistant infections. Passaging of *A. baumannii* cells exposed to SNAPPs at sub-MBC concentrations (1/10 of MBC) for 600 generations revealed no increase in the MBC value, suggesting no acquisition of resistant traits.

A subsequent study into the architectural features necessary for potent SNAPPs has further elucidated the effects that arm length and number of arms play on the antibacterial activity against *E. coli* cultures.²¹⁰ Here, it was shown that increased degree of polymerization and PAMAM cores of higher generations

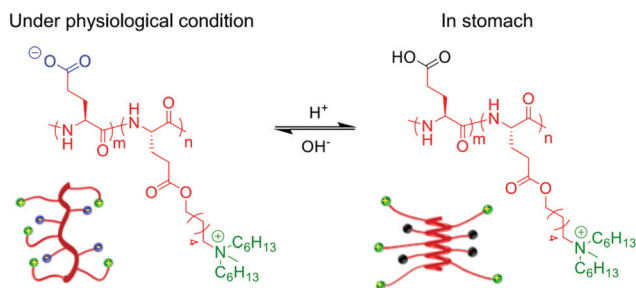


Fig. 41 pH-Responsive radially amphiphilic design of an antimicrobial polypeptide effective against *H. pylori*, a likely culprit in the development of gastric ulcers and carcinoma. Reproduced with permission from ref. 113. *Proc. Natl. Acad. Sci. U. S. A.*, 2017, **114**(48), 12675.

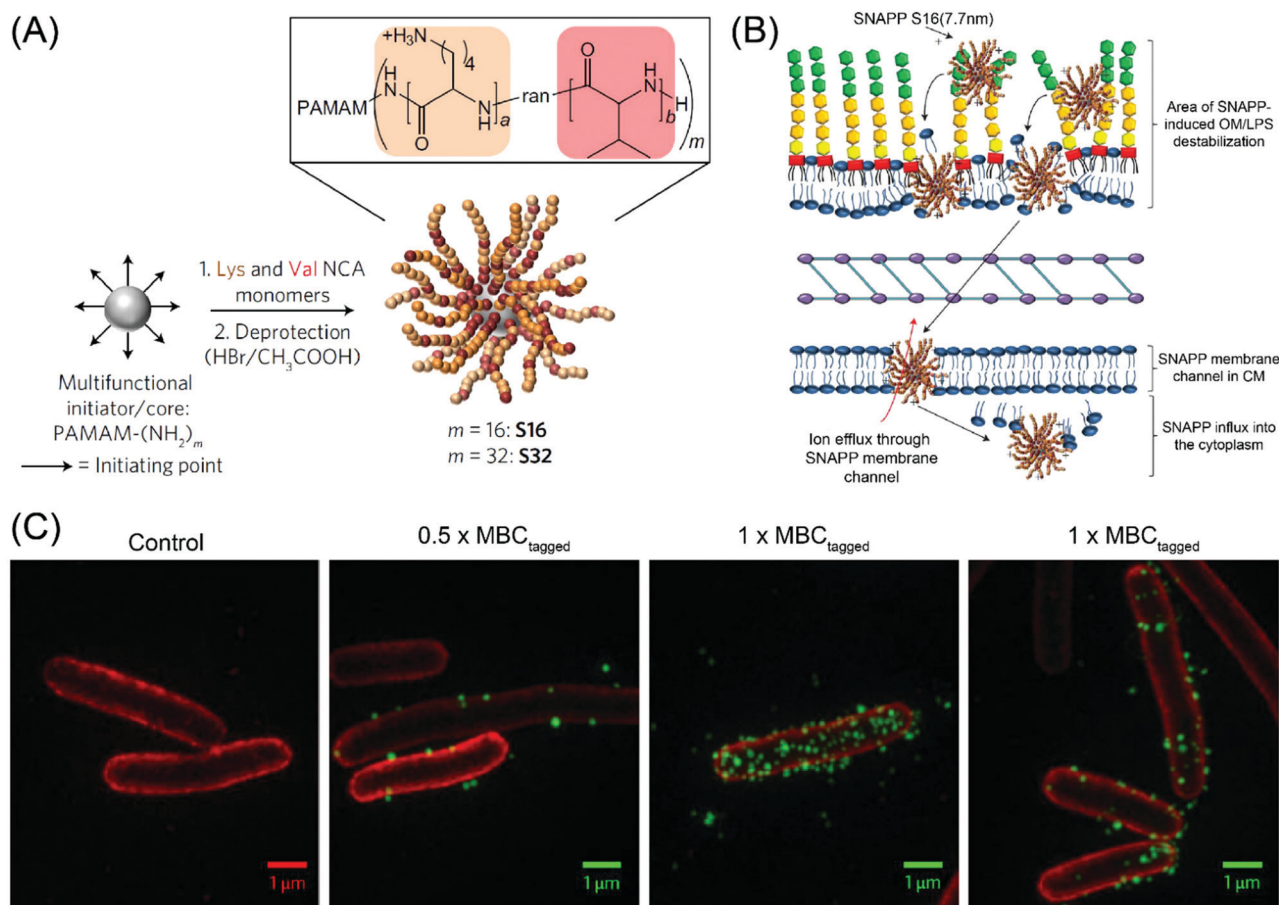


Fig. 42 Schematic representation of SNAPPs obtained through (A) the random ROP of ZLL and D,L-valine NCAs and (B) their proposed mechanism of action against Gram-negative species. (C) Fluorescence microscopy images of *E. coli* before and after treatment with fluorescently labelled AF488-SNAPP₁₆ at different dosages. Adapted from ref. 209. Copyright 2016 Springer Nature.

led to increased antibacterial efficacies (lower MIC and MBC values), albeit with a concomitant increase in toxicity towards mammalian cells. Longer arm DP translated to higher α -helical content in environments mimicking the lipid bilayer, which has been shown by others to be a strong indicator of antibacterial efficacy. Independently, Zhang *et al.* carried out coarse-grained molecular dynamic studies to further elucidate the mechanism of action of SNAPPs and why they might display such optimal antibacterial activity and selectivity.³⁹⁶ Simulations were carried out on a SNAPP analogue with arm sequence (K₂V₁)₅ and a model AMP with sequence KWKLFGIGAVLKVL-NH₂ (CM-15) at varying levels of grafting density, in order to examine the interactions that each of these two molecules experience when loaded onto prokaryotic-mimicking bilayers (PE/PG) or eukaryotic models composed of dipalmitoyl phosphatidylcholine (DPPC). Interestingly, the molecular dynamic simulations revealed that the SNAPP analogue overall exerts stronger electrostatic interactions with the PE/PG bilayer, leading to decreased diffusion of lipid components and phase separation of the lipid domains. The strong sequestering and clustering of PG by the SNAPP star caused phase boundary defects which in turn led to destabilization and disruption of the membrane and its eventual permeabilization. In the simulations involving the DPPC

bilayer, the CM-15 star interacted and inserted into the hydrophobic domains of the membrane, but surprisingly the star SNAPP was unable to do so, instead floating in the aqueous space above the bilayer. Even though the simulation conditions are not exactly the same as the system we reported in our studies, taken together these results emphasize the importance of tuning hydrophobicity carefully if selectivity between mammalian and bacterial cells is to be achieved.

A publication by Chen *et al.* also probed the importance of both architecture and amphiphilic tuning.⁸¹ A library of PLLys-modified stars identified indole as a potent functional group to tune the amphiphilicity of the hydrophilic stars, with said materials displaying improved antibacterial activity against a range of Gram-negative bacteria. This study exploited a clever strategy for the production of amphiphilic antibacterial polypeptides since a facile homopolymerization was first undertaken, followed by EDC-NHS conjugation of the desired hydrophobic moiety. With this strategy copolymerization of several NCAs is avoided, as it can sometimes lead to bimodal polypeptides and heterogeneity in the final products, especially if the NCA monomers have different reactivity. A recent study by Kim and co-workers further emphasized the critical role of architectural design for potent antibacterial activity.⁷⁰

Copolymers of Lys and BLG with linear, hinged, star or cyclic architecture and an average DP of 33 were tested against *B. subtilis* and *E. coli*. At similar Lys to BLG ratios, the star polypeptides displayed the most potent activities, followed by cyclics, which outperformed the linear samples. With increasing benzyl glutamate content, more helical secondary conformation was detected by circular dichroism, however high therapeutic indexes were maintained in all cases examined. Liu and co-workers recently investigated the antibacterial properties of $\alpha\beta$ chimeric polypeptide brushes.²⁵³ To this end, a base-catalyzed anionic ROP of two β -lactams was first undertaken, followed by the grafting from polymerization of Boc-L-lysine NCA to install the hydrophilic side chains. Brushes with side chain DP of 10 and 20 were tested against multiple strains of methicillin-resistant *S. aureus* (MRSA) with reported MIC values at 0.38 and 0.26 μM respectively. The brushes were found to be more active than their linear counterparts, however in all cases they were also highly cytotoxic. The authors hypothesize this may be due to the cytotoxic character of poly(lysine), however it would be of interest to explore further whether architecture also plays a factor in the low cell viability. Put together, the results summarized by this review present strong evidence regarding the attractive and superior properties of star architecture over other topologies. Nonetheless, it is not yet fully understood why this architecture behaves so remarkably, and more investigations are warranted.

Other researchers have instead focused on the design of self-assembled polypeptide nanostructures with enhanced antibacterial properties compared to their unimeric counterparts. The studies herein summarized highlight the favourable properties to be harnessed from supramolecular assembly. Du's research group has investigated self-assembled systems by exploring designs incorporating PLLys and PLPhe to respectively facilitate electrostatic interactions with the bacteria cell envelope and promote insertion into the lipophilic domains of the membranes.^{244,385} In 2016, the group reported PLLA₃₁-poly(Phe₂₄-co-Lys₃₆) polypeptide systems that self-assembled into micellar structures of average size 70 nm.³⁸⁶ These had modest MIC₅₀ values of 40 $\mu\text{g mL}^{-1}$ against *E. coli* and *S. aureus*, a concentration above the critical micelle concentration (CMC) of 13.2 $\mu\text{g mL}^{-1}$, but could be hydrolysed by lipase and did not exhibit cytotoxicity up to concentrations of 50 $\mu\text{g mL}^{-1}$. The ζ -potential of the polypeptide was +51.8 mV and linear polymers of the same composition displayed much higher MIC and MBC values, in good agreement with other studies reviewed. In another study, Gao *et al.* synthesized weakly positively charged polypeptide-ended nanosheets which had MIC values of 16 $\mu\text{g mL}^{-1}$ against both *E. coli* and *S. aureus*.³⁸⁷ The CMC was reported at 14.8 $\mu\text{g mL}^{-1}$, indicating the importance of nanoaggregation for the inhibition of bacteria growth. Very recently, Du and co-workers also investigated the use of dual corona vesicles for the treatment of periodontitis.³⁸⁸ Said disease is caused by the build-up of plaque biofilm, and triggers inflammation of the gums and supporting teeth structures. The design of polymersomes containing a PEG corona and amphiphilic polypeptide could aid the delivery of ciprofloxacin hydrochloride. The dual corona vesicles enabled insertion into the biofilm and

allowed for a 50% reduction in the dosage of antibiotic required for complete biofilm eradication, which may help to prevent antibiotic resistance. A recent study by the Yang group further reinforces the increased ability of nano-sized particles to kill pathogenic bacteria.³⁸¹ Self-assembled particles ranging in size from 150–200 nm showed broad antimicrobial activity against Gram-positive and Gram-negative bacteria, including clinically relevant MRSA, *E. coli* BL21(D3) and strains expressing NDM-1 and ImiS resistant traits. The nanostructures were obtained after amphiphilic poly(Phe-co-LLys) or poly(Leu-co-LLys) macromonomers underwent free radical polymerization from 2-methylallylamine, followed by self-assembly. Polypeptide designs containing Phe were more potent than those with Leu. Several mechanistic studies provided evidence to suggest a non-specific mode of action; examination of the electrical conductivity revealed a sharp increase in electrical conductivity upon treatment with the nanoparticle, indicative of leakage of cytoplasmic contents upon membrane damage. Importantly, the nanoparticles did not elicit resistant trait development in *S. aureus*, *E. coli*, MRSA or vancomycin-resistant *Enterococcus* (VRE) after 14 serial passages (unlike the gentamicin control). They also elicited little cytotoxicity in normal mouse fibroblast cells up to 40 $\mu\text{g mL}^{-1}$. A subsequent study by the same group has revealed the synergistic antimicrobial activity of silver nanocomposites and 6-arm star-*g*-PCL₁₄-*b*-poly(Phe₈-co-Lys₃₂).⁸² Importantly, the antimicrobial nanoparticles exhibited low cell toxicity *in vitro* and *in vivo*, most likely due to the complexation of Ag with polypeptide, a property in remarkable contrast with pristine silver nanoparticles which on their own are known to be highly toxic.³⁹⁷ Interestingly, different stable nanocomposites could be elaborated based on the concentration of the polypeptide copolymer. Below the CMC, the silver nanocomposites obtained by *in situ* complexation reaction between the copolymer amine groups and AgNO₃ had a size of 500 ± 20 nm, as measured by scanning electron microscopy (SEM). If, however, the reduction of Ag⁺ was carried out after self-assembly of the polypeptides, spherical nanostructures ($D_h \sim 200$ nm) were obtained (Fig. 43). The nanocomposite particles were highly antibacterial against Gram-negative and Gram-positive bacteria strains, with MIC values ranging between 2–8 $\mu\text{g mL}^{-1}$; a slight selectivity for Gram-positive killing was observed based on the lower MBCs obtained for these species. In addition, time kill assays revealed nanocomposites with assembled structure (200 nm) killed faster than those constructs obtained at concentration below the CMC of the polypeptides, a find ascribed to these particle's potential increased ability for membrane disruption. In this study an increased antibacterial activity against *E. coli* (reduction in MIC from 43.8 to 10.9 $\mu\text{g mL}^{-1}$) was observed after complexation of a PEG-*b*-PLLys₂₀ copolymer with EDTA to form nanofibers. Another interesting example of complexation to potentiate antibacterial activity was provided by Pu *et al.*, who employed ethylenediaminetetraacetic acid (EDTA).³⁸⁹

Remarkably, this desirable synergy was not present for *S. aureus* or *P. aeruginosa*. EDTA facilitates the release of lipopolysaccharide from *E. coli*, which leads to an increase in cell membrane permeability^{398,399} and subsequently improves the

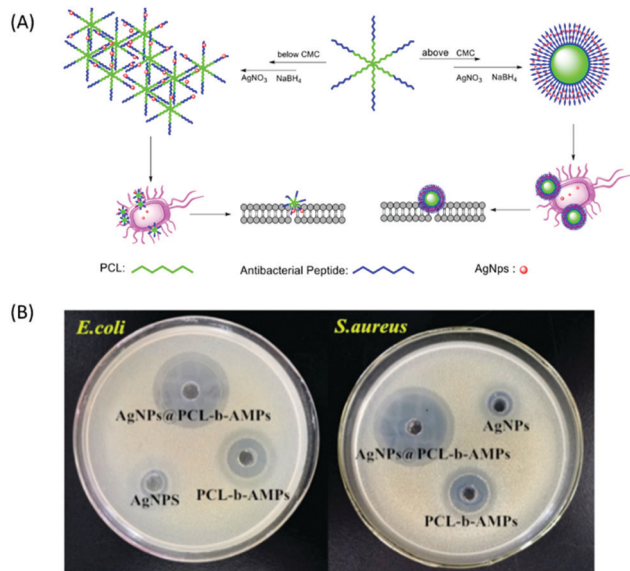


Fig. 43 Schematic representation of (A) methodologies to produce nanosized silver-decorated antibacterial agents. (B) Inhibition zone experiments showing the synergistic properties of silver nanoparticles and amphiphilic block copolymers against *E. coli* and *S. aureus*. Adapted with permission from *Bioconjugate Chem.*, 2020, **31**, 51. Copyright 2020 American Chemical Society.

membrane permeation capacity of the polypeptide. Additionally, complexation of the polypeptide attenuated the cytotoxicity of PLLys by decreasing the overall charge density; the reported zeta potential (+8.7 mV) is similar to the nanosheets recently published by the Du group where the authors also noticed improved biocompatibility based on reduced overall cationic charges.³⁸⁷

A further strategy this review wishes to highlight is the combination of polypeptides with glycopolymers in order to attenuate their cytotoxicity; Chan-Park's group has published several studies in this area.^{229,237} Wong *et al.* initially combined linear PLLys arms with glucosamine arms to form miktoarm stars of increasing glycopolymer content through an arm first photoRAFT polymerization.²²⁹ It was shown that incorporation of glucosamine could aid in improving cytocompatibility toward human aortic smooth muscle cells and to selectively target Gram-positive bacteria. In a follow-up study Pranantyo *et al.* conjugated linear PLLys to 4-arm glycosylated stars, creating a library of antibacterial compounds through a combination of ATRP, NCA ROP and CuACC (Fig. 44).²³⁷ Out of the carbohydrates tested, mannose displayed the best results, with the authors providing evidence that *E. coli* recognise these moieties and interact with them. Although the MIC and MBC values were higher for conjugated polypeptide products than for linear poly(lysine), cell viability was greatly enhanced.

4.1.2 Antibacterial hydrogels and cryogels. Hydrogels and cryogels (as their macroporous counterparts) are excellent candidates in wound healing applications as they offer good gas permeability, are swellable and thus able to retain water or adsorb wound exudates.⁴⁰⁰ They also offer the ability to deliver therapeutics. Antibacterial activity (either intrinsic or through

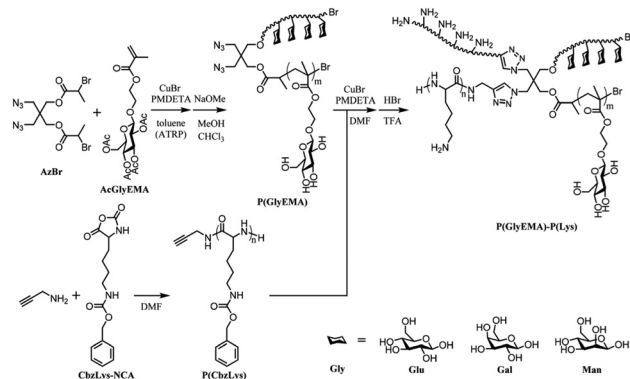


Fig. 44 Schematic synthesis of 4-arm star glycopolymers with enhanced cytocompatibility.²³⁷ Adapted from *Polym. Chem.*, 2017, **8**(21), 3364 – Published by the Royal Society of Chemistry.

the inclusion of inorganic nanoparticles or antibiotics) is a prerequisite property in such hydrogels as bacterial infections can greatly delay or prevent healing altogether, leading to increased clinical burden. Hydrogels bearing antimicrobial peptides (AMPs) or synthetic sequence-specific peptides which offer intrinsic antibacterial properties have been studied in the past years.^{401–403} NCA ROP has also been successfully utilized to produce hydrogels and cryogels with intrinsic antibacterial properties.^{336,390,391,404,405} For a recent review on antimicrobial hydrogels the reader may refer to Ding *et al.*⁴⁰⁶

Bevilacqua *et al.* reported the synthesis of a lysine-leucine block (PLLys₁₀₀-b-LLeu₄₀) biopolymer which could assemble into stable hydrogels based on hydrophobic interactions of the leucine segments at concentrations above 2 wt%.³⁹⁰ These amphiphilic hydrogels exhibited a broad antimicrobial activity against several multi-drug resistant and clinically-relevant isolates, displaying multi-log reductions in colony forming units (CFU) of *S. aureus* and *P. aeruginosa* in time-kill assays. Additionally, they prevented microbial contamination in porcine and rat *in vivo* models as well as porcine skin explants. Another example of an antibacterial cryogel was provided by Qiao and co-workers, who synthesized macroporous gels comprised of P(LLys-b-DLVal) crosslinked by glutaraldehyde (GA) with different pore sizes.³³⁶ The best performing cryogel had a pore size of 120–150 μm , and it was theorized that the 95.6% reduction in *E. coli* colony CFU was due to a trap and kill mechanism, whereby the bacterial cells enter and subsequently get trapped within the pores of the cryogel, where the cationic and hydrophobic residues of the amino acids perturb and damage the bacterial membranes.

Additionally, Hong *et al.* synthesized a polymersome-hydrogel composite with effective drug loading and sustained release of penicillin G, an antibiotic suitable for treating staphylococcus infections (Fig. 45).³⁹² The polypeptide, a random block copolymer of lysine and phenylalanine amino acid residues, made up the corona of the polymer vesicle. The polymersome was subsequently grafted to chitosan through Schiff base linkages, affording a nanocomposite hydrogel with embedded antibiotic. This is an interesting approach to produce an antibacterial hydrogel as it

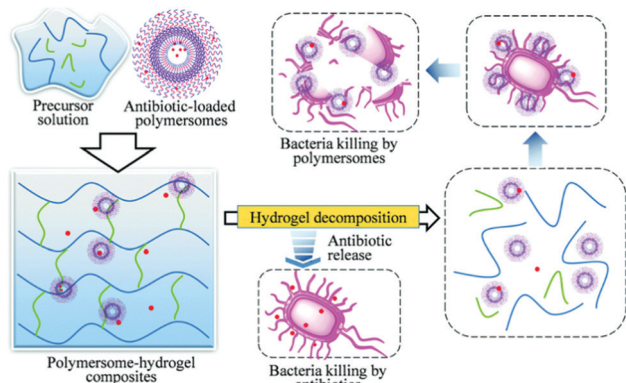


Fig. 45 Polymersome-hydrogel systems with drug loading capability. Adapted from ref. 392 with permission from The Royal Society of Chemistry. Copyright 2018.

enables the controlled drug release of Penicillin G in the first few hours, while the degradation of the hydrogel releases the remaining antibiotic and the intrinsically antibacterial polymersomes, achieving a dual antimicrobial effect.

Another example of effective encapsulation was provided by Jan and co-workers.³⁶⁶ In this work, TFN-Related Apoptosis-Inducing Ligand (TRAIL) was encapsulated within cationic poly(L-lysine)-*b*-poly(L-threonine) nanogels. The composite nanogels were antibacterial against *K. pneumoniae* *in vitro*, and importantly, showed remarkable activity in *K. pneumoniae*-induced and LPS-induced murine sepsis models, where the mice exhibited prolonged survival rates and reduced levels of LPS-induced glomerulonephritis, respectively. The authors also found that the TRAIL-nanogel could induce the apoptosis of immune cells *via* receptor-dependent activation of caspase-3 expression. This find has large implications for the treatment of sepsis, given that in this disease uncontrolled bacterial infection is compounded by extensive organ damage caused by overactive immune responses.

4.1.3 Antibacterial material surfaces. Antibacterial surfaces are particularly attractive in clinical devices and in the field of tissue engineering, where it is of utmost importance to

deliver implants which can resist biofilm formation. Ma's group has investigated several brush-like coatings from NCA ROP with antimicrobial and antifouling functions.^{393,394} In 2017, the synthesis of diblock polypeptide brushes composed of PEG and a random P(LL-*co*-Phe) polypeptide segment was reported, which showed increased cytocompatibility, reduced protein adsorption and platelet adhesions as well as excellent antimicrobial activities.³⁹³ The flexible backbone tether anchoring the brushes onto the silicone surface ensured enough conformational mobility for the brushes to still disrupt the bacterial cell membranes. In another work, Gao *et al.* also synthesized random polypeptide-polypeptoid brushes grown from a dopamine-rich surface which showed potent antimicrobial activity against clinically relevant *E. coli*, *S. aureus*, and *P. aeruginosa* strains.³⁹⁴ These brushes were obtained through the phototriggered polymerization of methacrylic macromonomers (statistical Lys-Phe content in ratio 1:1, DP 24) and polysarcosine methacrylic polypeptoids, given that free radicals are formed when dopamine is oxidized under UV irradiation. The PSar segments in the brush coatings conferred antifouling properties, allowing for the development of active brush surfaces that prevented the attachment of dead bacteria, debris, and proteins to a large extent. Overall, the surfaces containing both PSar and polypeptide could prevent biofilm formation, whereas polypeptide brushes alone were unable to prevent biofilms (Fig. 46).

Going one step further, Gao and co-workers very recently published an intriguing mechano-bactericidal TiO₂ nanospike peptide coating (TNPC) with excellent antibacterial and antibiofilm properties (Fig. 47).³⁹⁵ This type of coating holds great promise for advanced orthopaedic and dental implants since its design closely resembles natural antifouling insect structures with nanotopographies capable of rupturing bacterial cell membranes. TNC was obtained by alkali hydrothermal treatment of medical-grade titanium, followed by annealing treatment. The functional polypeptide coating was then obtained through simple mixing of TNC with the previously studied P(LLys-*co*-LPhe) polypeptide which exhibits potent and broad antibacterial spectrum in solution. Interestingly, the TNC surfaces

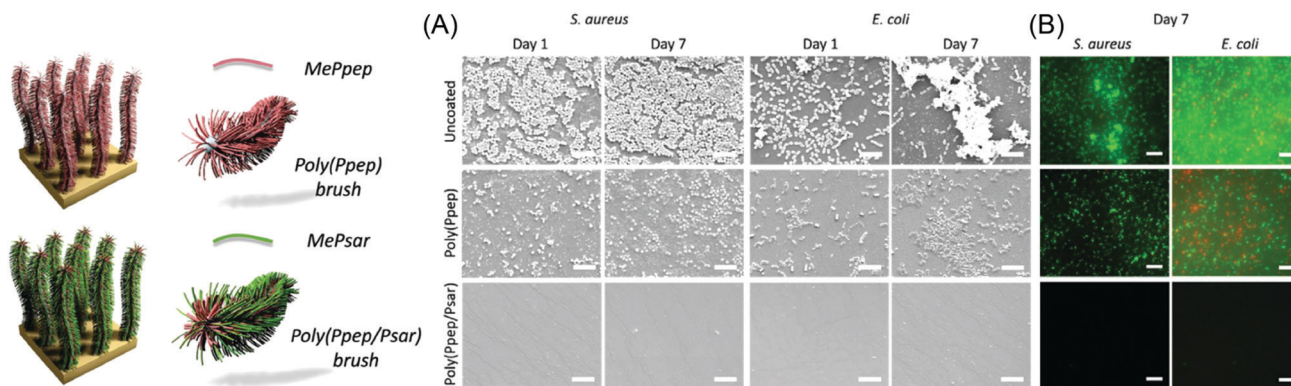


Fig. 46 Surface immobilized polypeptide-polypeptoid brushes. (A) Formation of biofilm formation by *S. aureus* and *E. coli* on uncoated, polypeptide-only and polypeptide-polypeptoid surfaces observed by FE-SEM (scale bar 10 μ m). (B) LIVE/DEAD staining of surfaces at day 7 (scale bar 10 μ m). Adapted from ref. 394 with permission from The Royal Society of Chemistry. Copyright 2017.

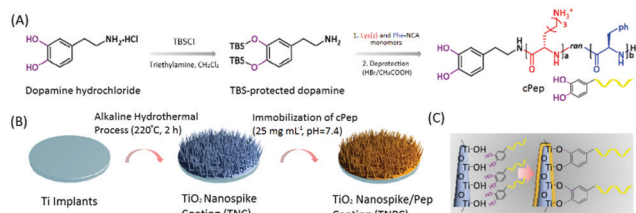


Fig. 47 Preparation of durable bactericidal TiO_2 coatings. (A) Synthetic strategy for the NCA ROP of catechol-functional polypeptides. (B) Methodology for the production of TiO_2 Nanospikes/Pep coating. (C) Coordinative interactions between catechol and TiO_2 . Reproduced from ref. 395 with permission from The Royal Society of Chemistry. Copyright 2020.

on their own displayed modest contact-killing activity afforded by the nanospikes, but inclusion of the peptide potentiated this property. More than 99.9% contact killing of *S. aureus* and *E. coli* was achieved despite high seeding concentrations (10^8 CFU per mL) and the potent activity persisted even after repeated challenges with fresh bacteria, also preventing biofilm formation up to 14 days. This is particularly desirable as titanium-based implants often suffer from detrimental bacterial adhesion. The TNPC coatings exhibited no toxicity to the growth of murine osteoblastic cells and could furthermore promote mineral deposition and *in situ* formation of hydroxyapatite, an integral component of bone and teeth. *In vivo* anti-infective examination also revealed these coatings had better healing performance than pristine titanium implants.

4.1.4 Future perspectives. Polypeptides, as highlighted here, are potent and versatile materials which display excellent antibacterial activities against a wide range of planktonic pathogens and in some cases, biofilms. The studies published in the past five years showcase the great interest in applying NCA ROP toward the synthesis of antibiotic replacements and antimicrobial agents with broad activity and reduced emergence of resistance. Evidently, creative polypeptide design and advances in this chemistry offer the ability to tune architectural features and side chain composition, parameters which are crucial in not only protecting mammalian cells but also in directing polymers to specific bacteria. Most studies reviewed have investigated amphiphilic compositions containing poly(lysine) and poly(phenylalanine) residues. However, ample opportunity remains to employ the extensive chemical toolbox available for peptide and combinatorial chemistries towards NCA ROP, in order to include different functional side chain groups which will enable specific targeting of bacteria or pathogenic species, for instance Gram-positive or Gram-negative bacteria. Additionally, side chain functionalization will offer extra protection for eukaryotic cells and an enhancement in half-life and pharmacokinetics. It is also necessary to explore the polypeptide's action against other microbes such as fungi and yeasts and to further investigate their effect in established biofilms, where decreased metabolic rates and presence of extracellular components hinder the activity of the current classes of antibiotics at our disposal. Achieving these goals would truly enable polypeptides to become a competitive class of antimicrobial agents.

4.2 Polypeptide-based drug delivery systems

Delivery of therapeutics is one of the most common applications of polymers synthesized using NCA ROP. As previously discussed, linear, brush, and star polymers are readily synthesized and can be used for the synthesis of micelles, vesicles, gels, and particles. While complex architectures can be readily achieved, the most common morphologies employed in polypeptide-based drug delivery systems (DDS) are relatively simple micelles and polymersomes, synthesized through self-assembly of multiblock linear polypeptides containing hydrophilic and hydrophobic blocks. The combination of well-defined architectures, human-compatibility, and wide range of functional side chain groups make polypeptides some of the most promising materials for DDS for a range of pathologies.^{276,370} These features of polypeptides have recently been explored to tackle the challenging hurdles in the successful delivery of therapeutics. While these challenges are outside the scope of this review, several excellent reviews have been published in recent years.^{407–409}

4.2.1 Polypeptide nanocarriers in cancer therapy. Polypeptide nanocarriers are most commonly developed with cancer therapy in mind and have gained popularity in recent decades due to improved efficacy of treatment while reducing toxicity compared to unbound/free chemotherapeutics.^{410–413} Polymeric nanocarriers also aim to exploit the enhanced permeability and retention (EPR) effect to improve drug delivery to tumours. Tumours are thought to possess a “leaky” vasculature, allowing nanocarriers between 10–800 nm in diameter to escape the vasculature and permeate into the tumour.⁴⁰⁹ It is hypothesized that nanocarriers can accumulate in the tumour while being too large for renal clearance or extravasate at the normal capillary bed.

Multiple clinical trials involving polypeptide-containing nanocarriers have been conducted during the past two decades, with the first clinical trial involving polypeptide-containing micelles beginning in 2001.⁴¹⁴ The Kataoka group pioneered the use of PEG-*b*-polypeptide diblock polymers to form micelles with a polypeptide core and PEG shell, most frequently containing poly(aspartic acid) or poly(glutamic acid) as the polypeptide component.⁴¹⁵ These micelles are capable of delivering payloads such as nucleic acids, proteins, photosensitizers, reporter agents, and hydrophobic drugs.^{415,416} In addition, Kataoka and others were able to tune the size of the micelles and investigated different mechanisms of drug loading.⁴¹⁷ Several formulations have reached clinical trials for cancer therapy, with the phase III trial of NK105, a PEG-*b*-poly(aspartic acid) micelle containing paclitaxel, reported as recently as February 2019.⁴¹⁸ Despite polypeptide-containing nanocarriers generally being well-tolerated by patients, many studies have not shown an improved therapeutic benefit of treatment with drug-loaded nanocarriers in comparison to free drug. This has been largely attributed to the lack of universally observed EPR effects in patients.^{419,420}

While the efficacy of relatively simple polypeptide-based formulations has been investigated in clinical trials, advances in NCA ROP and cancer biology have led to the development of

more complex systems. In recent years, targeted and/or stimuli-responsive nanocarriers have become a primary focus of the drug delivery field as they offer improved potential for successful drug delivery and improved therapeutic index. Although this section will only focus on polypeptide nanocarriers, readers interested in the current state of cancer nanomedicine are directed to recent review by the Farokhzad group.⁴²¹ Table 3 offers an overview of reported polypeptides used for drug delivery.

4.2.1.1 Targeted delivery. In recent years researchers have placed greater focus on the active targeting of drug nanocarriers to tumour cells. Cancer cells are known to overexpress certain surface molecules (biomarkers) that can be targeted by displaying a targeting ligand on the nanocarrier surface. The most commonly used targeting ligands are small molecules, antibodies, peptides, sugars, and aptamers.⁴²¹ Although the overexpression of biomarkers often varies between different cancers or even between individuals with the same cancer, some receptors such as folic acid and transferrin receptors are overexpressed in many forms of cancer.^{472,473} Readers interested in the use of targeting ligands for cancer therapy are directed to reviews focused on this topic.^{474,475}

Several groups have reported the synthesis of polypeptide-based nanocarriers with targeting ligands.^{423–427} The Qiao group developed a polypeptide-based vesicle for targeted delivery of cisplatin to cancer cells using folic acid.⁴²⁸ The authors synthesized a linear maleimide-PEG-PLGlu-FITC polymer as shown in Fig. 48. Interestingly, cisplatin conjugation to the glutamic acid side chain caused vesicle formation as shown by GPC and DLS analysis. Linear polymers did not self-assemble into vesicles in the absence of cisplatin, leading to the conclusion that intra-chain crosslinking of cisplatin induced vesicle formation. The impact of targeting ligand on internalization of the nanoconstructs into cells and cell viability was studied using FA-conjugated and unconjugated vesicles on healthy fibroblast cells and HeLa cancer cells that overexpress the folate receptor. Unsurprisingly, a dramatic increase in cell internalization of the FA-conjugated vesicles by HeLa cells was achieved. Cell internalization of both types of vesicles by fibroblast cells and unconjugated vesicles in HeLa cells were similar. In agreement with these findings, FA-conjugated vesicles were more toxic to HeLa cells than fibroblast cells, despite both cell types displaying similar IC₅₀ values for free cisplatin.

Glycopolymers also offer opportunities for targeted drug delivery due to their interactions with cell surface proteins.⁴⁷⁶ Hyaluronic acid (HA) is able to specifically interact with multiple HA-binding proteins, among which its interaction with cell surface adhesion receptor cluster of differentiation 44 (CD44) in particular has been widely studied due to the overexpression of CD44 in subpopulations of cancer cells. CD44 is also recognized as a molecular marker for cancer stem cells.^{477–479} The Lecommandoux group has reported extensively on the synthesis of glycopolypeptides, containing both glycopolymer and polypeptide moieties.^{429–431,480,481} The authors have demonstrated effective targeting to CD44-positive cancer cells *in vitro* using self-assembled nanoparticles synthesized from

PBLG-*b*-polysaccharide copolymers, and that nanoparticle size could be controlled by altering the ratio of PBLG to polysaccharide and the rate of nanoprecipitation.^{430,431}

Glyco-amino acids, which have both amino acids and carbohydrates in a single molecule and whose corresponding polymers can be synthesized *via* NCA ROP, have also been reported in recent years and are being investigated for potential applications in biomaterial and drug delivery applications.^{438,482,483} Gupta and co-workers reported the synthesis of miktoarm stars containing one hydrophilic poly(glycol-amino acid) arm and two hydrophobic poly(ϵ -caprolactone) (PCL) arms which could subsequently self-assemble.⁴³⁹ The glycol-amino acid consisted of a lysine residue with galactose or mannose conjugated to the lysine side chain amine. Morphologies including nanorods, vesicles, and micelles could be achieved by varying the lengths of the hydrophilic and hydrophobic arms, helicity of the glycopolypeptide arm, and the crystallinity of the PCL arms, although no difference was observed between mannose- and galactose-functionalized polymers. The authors also demonstrated the targeting of the mannose-functionalized polymersomes *in vitro* using the breast cancer cell line MDA-MB-231, which are known to overexpress certain mannose-specific receptors. Model hydrophobic drug rhodamine B octadecyl ester (RBOE) was encapsulated in galactose- and mannose-functionalized polymersomes and the mannose-functionalized polymersomes were shown to have nearly double the cell internalization of the analogous galactose-functionalized polymersomes.

In a more recent publication, Gupta and co-workers improved on the mannose-functionalized polymersomes by crosslinking the micelles at the interface of the hydrophobic and hydrophilic arms and incorporating stimuli-responsive drug release mechanisms (Fig. 49).⁴⁴⁰ Crosslinking was made possible by polymerizing a short alkyne-functionalized glycine block *via* NCA ROP followed by polymerization of the mannosylated lysine NCA. Redox-sensitive crosslinker biazide disulfide was clicked onto the glycine pendant alkyne groups to form interface crosslinked (ICL) micelles. ICL micelles were found to be more stable over 2–3 weeks than uncrosslinked (UCL) micelles. *In vitro* release studies also demonstrated the redox-responsiveness of the ICL micelles. DOX loaded UCL and ICL micelles displayed similar cytotoxicity in HepG2 hepatocellular carcinoma cells, release of DOX from the micelles differed, with ICL micelles taking 12–36 hours longer to release DOX inside the cell, indicating that ICL micelles have potential as slow/sustained release materials.

4.2.1.2 Stimuli-responsive delivery. The use of stimuli to release drug at the intended site is an important area of focus in the development of DDS. The stimuli can be internal (*e.g.*, pH, redox potential and enzymes) or external (*e.g.*, temperature, UV or infrared irradiation and magnetic fields).⁴⁸⁴ The use of stimuli-responsive materials promotes on-demand drug release, with payloads released at the desired time or location.

Polypeptide-based pH-responsive micelles and polymersomes (also known as polymeric vesicles) have been widely reported for applications in drug delivery.^{147,197,422,432–437,442–444} Linear di- and tri-block polymers containing hydrophilic and hydrophobic

Table 3 Polypeptide drug nanocarriers obtained through NCA ROP

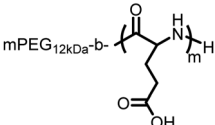
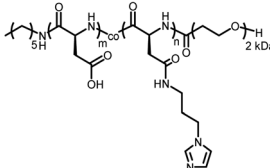
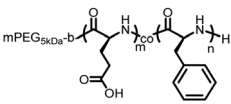
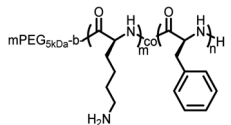
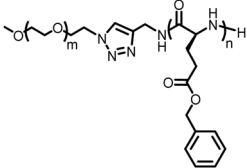
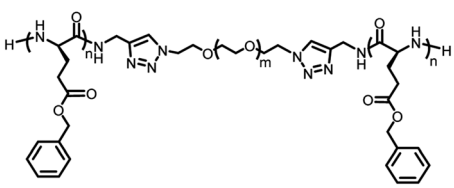
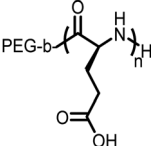
Polymer	Architecture	Drug/cargo	Cell/ <i>in vivo</i> testing	Targeting agent/stimuli	Ref.
PEG-<i>b</i>-PLGlu PEG-<i>b</i>-PDGlu PEG-<i>b</i>-PDLGlu 	Micelle	Cisplatin	BxPC3 tumour in BALB/c nu/nu mice	—	410
PLAsp-co-PLAsp-g-imidazole-<i>b</i>-PEG 	Micelle	DOX	MCF-7 <i>in vitro</i> ; MCF-7 tumour in female BALB/c nude mice	pH	422
PEG-<i>b</i>-P(LGlu-co-LPhe) 	Nanoparticle	DOX	HeLa and A549 <i>in vitro</i> ; A549 tumour in BALB/C nude mice	pH	411
PEG-<i>b</i>-P(LLys-co-LPhe) 					
PEG-<i>b</i>-PBLG 	Micelle	DOX	KB cells	pH	412
PBLG-<i>b</i>-PEG-<i>b</i>-PBLG 					
PEG-<i>b</i>-PLGlu 	Micelles	Oxaliplatin	BxPC3 tumour in BALB/c nu/nu mice	Anti-tissue factor anti-body fragment	423

Table 3 (continued)

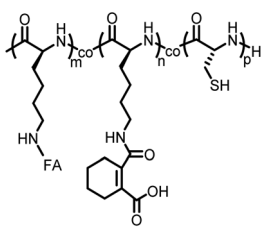
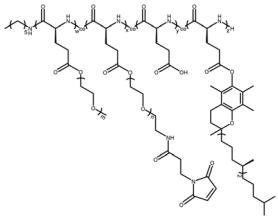
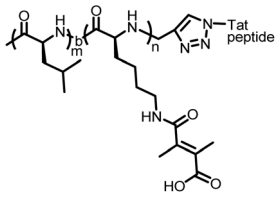
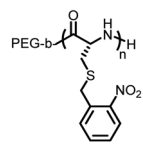
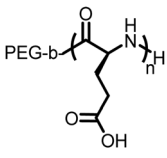
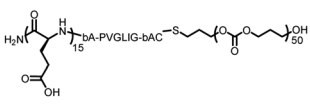
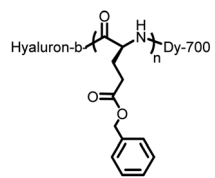
Polymer	Architecture	Drug/cargo	Cell/ <i>in vivo</i> testing	Targeting agent/stimuli	Ref.
P((LLys-g-FA)-co-(LLys-g-DCA)-co-LCys) 	Core-crosslinked micelle	DOX	HeLa and A549 <i>in vitro</i> ; HeLa tumour in nude mice	Folic acid (FA) targeting, reduction, pH	424
PLGlu-g-Ve/PEG 	Micelles	Cisplatin, DOX	HeLa and B16F1 <i>in vitro</i> ; B16F1 and B16F10 tumours in C57BL/6 mice	RGDfK	425
PLLeu-b-P(LLys-g-DMA)-Tat(SA) 	Micelles	DOX	HeLa <i>in vitro</i>	Tat peptide (nucleus targeting), pH	426
PEG-b-PNBLCys 	Composite nanoparticles	DOX	HeLa and HepG2 <i>in vitro</i>	Lactose, NIR	427
PEG-b-PLGlu 	Polymersome	Cisplatin	HeLa and NIH 3T3 <i>in vitro</i>	Folic acid	428
PLGlu-b-PVGLIG-b-PTMC 	Polymersome	—	—	pH, enzyme (MMP-2)	429
Hyaluronan-b-PBLG-Dy-700 	Micelle	—	H322, H358 and A549 <i>in vitro</i> ; H358-Luc tumour in NMRI nude mice and A549-Luc in BALB/c nude mice	Hyaluronan targeting to CD44	430

Table 3 (continued)

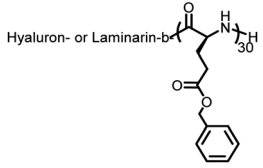
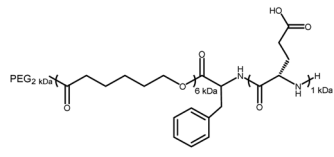
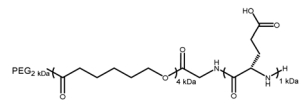
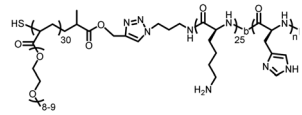
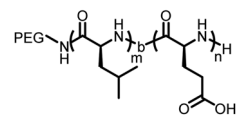
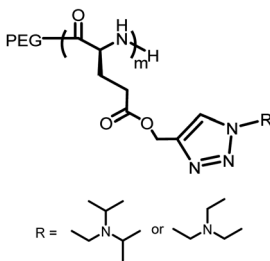
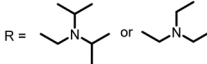
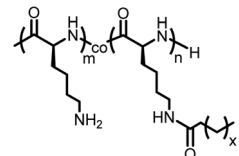
Polymer	Architecture	Drug/cargo	Cell/ <i>in vivo</i> testing	Targeting agent/stimuli	Ref.
Hyaluronan-<i>b</i>-PBLG and Laminarin-<i>b</i>-PBLG 	Nanoparticle	—	—	Hyaluronan and laminarin targeting to CD44 and Dectin-1	431
PEG-<i>b</i>-PCL-<i>b</i>-PLGlu 	Polymersome	DOX	A549 and MCF-7 <i>in vitro</i>	pH	432
PEG-<i>b</i>-PCL-<i>b</i>-PLGlu 	Polymersome	DOX, verapamil	MCF-7, MCF-7/ADR-resistant, and rabbit blood <i>in vitro</i>	pH	433
P(PEGA)-<i>b</i>-PLLys-<i>b</i>-PLHis 	Polymersome	DOX	NIH 3T3 and CT26 <i>in vitro</i>	pH	197
PEG-<i>b</i>-PLLys-<i>b</i>-PLGlu 	Polymersome	DOX	RAW 264.7, MCF-7/ADR, and L929 <i>in vitro</i>	pH	434
PEG-<i>b</i>-PPLG-<i>g</i>-alkylamine  R =  or 	Polymersome	DOX	MDA-MB-468 <i>in vitro</i> ; MDA-MB-468 tumours in BALB/c mice	pH	435
P(LLys-co-LLys-<i>g</i>-alkane) 	Polymersome	DOX	—	pH, Lac targeting to HepG2 cells	147

Table 3 (continued)

Polymer	Architecture	Drug/cargo	Cell/ <i>in vivo</i> testing	Targeting agent/stimuli	Ref.
PEG-<i>b</i>-PBLG-<i>b</i>-PLLys	Polymersome	DOX, paclitaxel	AsPC1 and BxPC-3 <i>in vitro</i> ; toxicity study in NOD.CB17-Prkdc ^{scid} /J	pH	436
PMPC-<i>b</i>-PLAsp	Micelle	DOX	NIH 3T3 and 4T1 <i>in vitro</i>	pH, reduction	437
P(LPhe₁₀-co-LLys₁₁-g-lactose)	Micelle	DOX	HepG2 <i>in vitro</i> ; H22 tumour in Kunming and BALB/c mice	Glycopeptide	438
(PCL)₂-<i>b</i>-P(LLys-<i>g</i>-sugar) Sugar = mannose, galactose	Nanorod, micelle, vesicle, sheets, tapes	Rhodamine B Octadecyl ester	MDA-MB-231 <i>in vitro</i>	Glycopeptide	439
(PCL)₂-<i>b</i>-P(propargyl DLGly₆)-<i>b</i>-P(LLys₄₀-<i>g</i>-Gal)	Crosslinked and uncrosslinked micelles	DOX	HepG2 <i>in vitro</i>	Glycopeptide, reduction, enzyme	440
PM-gala-<i>b</i>-PBLG	Micelle	DOX	HepG2 <i>in vitro</i>	Glycopeptide, reduction	441

Table 3 (continued)

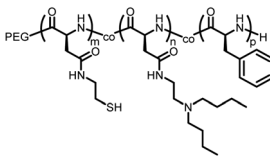
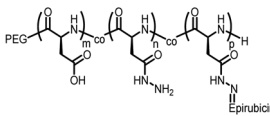
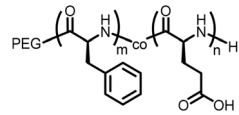
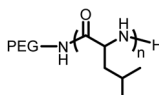
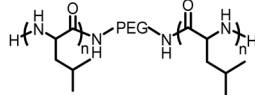
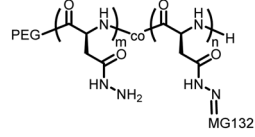
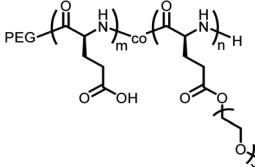
Polymer	Architecture	Drug/cargo	Cell/ <i>in vivo</i> testing	Targeting agent/stimuli	Ref.
PEG-<i>b</i>-P((Lasp-<i>g</i>-DBA)-<i>co</i>-(Lasp-<i>g</i>-MEA)-<i>co</i>-LPhe) 	Polymersome	DOX, gefitinib	N2a <i>in vitro</i> ; N2a tumour in BALB/c nude mice	pH, reduction	442
PEG-<i>b</i>-P(Lasp-<i>co</i>-(Lasp-<i>g</i>-hydrazide)-<i>co</i>-(Lasp-<i>g</i>-hydrazide-epirubicin)) 	Micelle	Staurosporine, epirubicin	MSTO-211H, MSTO-211H Epi-R, H226, and Meso-1 <i>in vitro</i> ; MSTO-211H-luc tumour in SCID and BALB/c nu/nu mice	pH	443
PEG-<i>b</i>-P(LPhe-<i>co</i>-LGlu) 	Nanoparticle	20(S)-Ginsenoside	SW480, SW620, CL40, CCD-18Co <i>in vitro</i> ; SW480 tumour in nude mice	pH	444
PEG-<i>b</i>-PDLeu PEG-<i>b</i>-PDLeu PEG-<i>b</i>-PLLeu 	Micelle	DOX	MDA-MB-231 <i>in vitro</i>	RGDfC for integrin targeting	445
PDLeu-<i>b</i>-PEG-<i>b</i>-PDLeu PLLeu-<i>b</i>-PEG-<i>b</i>-PLLeu 	Micelle	DOX	MG63 and Saos-2 <i>in vitro</i> ; Saos-2 tumour in BALB/c nude mice	—	173
PEG-<i>b</i>-P((Lasp-<i>g</i>-hydrazine)-<i>co</i>-(Lasp-<i>g</i>-hydrazinyl-aspartatinamide)) 	Micelle	MG132	MSTO-211H, MDA-MB-231, PC-3, HeLa-Luc <i>in vitro</i> ; HeLa-Luc tumour in BALB/c nu/nu mice	pH	446
P(LGlu-<i>co</i>-LGlu-<i>g</i>-mPEG) 	Nanoparticle	Fe ^{III} , tannic acid	MCF-7, HeLa, HepG2 <i>in vitro</i> ; MCF-7 tumour in BALB/c nude mice	—	447

Table 3 (continued)

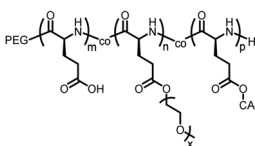
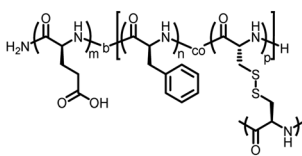
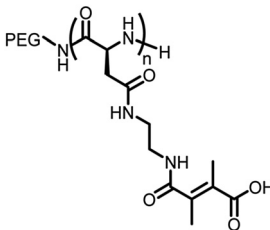
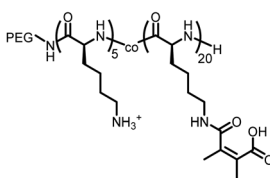
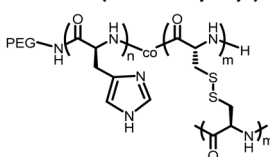
Polymer	Architecture	Drug/cargo	Cell/ <i>in vivo</i> testing	Targeting agent/stimuli	Ref.
PLGlu-co-(LGlu-g-mPEG)-co-(LGlu-g-CA4)	Nanoparticle	Combretastatin A4	C26 <i>in vitro</i> ; C26 tumour in BALB/c and BALB/c nude mice	—	448
	Nanoparticle	Combretastatin A4 sorafenib	HUVECs <i>in vitro</i> ; H22 tumour in BALB/c mice	pH	449
	Nanoparticle	Combretastatin A4, alone or co-administered with tirapazamine or imiquimod	44T1 <i>in vitro</i> ; 4T1 tumour in BALB/c mice	pH	450 and 451
PLGlu-b-P(LPhe-co-LCys-Cys)	Micelle	DOX	HeLa <i>in vitro</i>	pH and reduction	452
	Micelle	PAMAM-cisplatin	A549 and cisplatin-resistant A549 <i>in vitro</i>	pH and reduction	453
PEG-b-PLAsp-g-EDA-DM	Micelle	PAMAM-cisplatin	A549 and cisplatin-resistant A549 <i>in vitro</i>	pH and reduction	453
	Nanoparticle	DOX	A549 <i>in vitro</i> ; A549 tumour in BALB/c nude mice	pH and reduction	454
PEG-b-P(LLys-co-PLLys-g-DMMA) (shell)	Nanoparticle	DOX	A549 <i>in vitro</i> ; A549 tumour in BALB/c nude mice	pH and reduction	454
	Core-crosslinked micelle	Camptothecin	L929 and HeLa cells <i>in vitro</i>	pH and reduction	231
PLLys-b-P(LPhe-co-LCys-Cys) (core)	Core-crosslinked micelle	Camptothecin	L929 and HeLa cells <i>in vitro</i>	pH and reduction	231
PEG-b-PLCys	Core-crosslinked micelle	Camptothecin	L929 and HeLa cells <i>in vitro</i>	pH and reduction	231
PEG-b-P(LHis-co-LCys-Cys)	Nanogel	—	—	pH and reduction	346
	Nanogel	—	—	pH and reduction	346

Table 3 (continued)

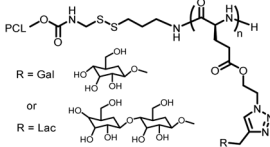
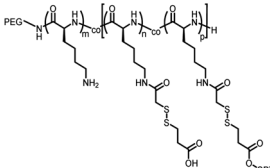
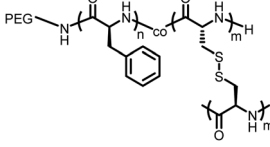
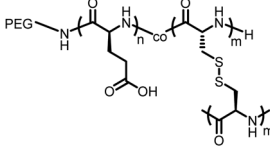
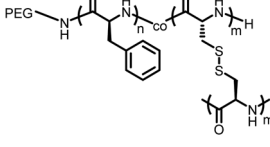
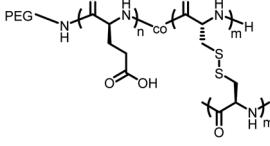
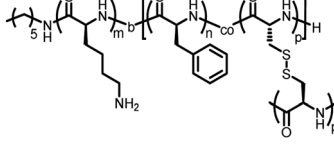
Polymer	Architecture	Drug/cargo	Cell/ <i>in vivo</i> testing	Targeting agent/stimuli	Ref.
PCL-<i>b</i>-PLGlu-<i>g</i>-sugar  R = Gal or R = Lac	Micelle	DOX, SPIO	HepG2 <i>in vitro</i>	Glycopolypeptide targeting to ASGP-R, reduction	455
PEG-<i>b</i>-P(LLys-<i>co</i>-(LLys-<i>g</i>-DP)-<i>co</i>-(LLys-<i>g</i>-CPT)) 	Micelle	Camptothecin	AY27 <i>in vitro</i> , AY27 tumour in nude mice	Reduction	456
PEG-<i>b</i>-P(LPhe-<i>co</i>-LCys-Cys) 	Nanogel	DOX	Lewis lung carcinoma cells	Reduction	457
PEG-<i>b</i>-P(LGlu-<i>co</i>-LCys-Cys) 					
PEG-<i>b</i>-P(LPhe-<i>co</i>-LCys-Cys) 	Nanogel	DOX	HepG2 <i>in vitro</i> ; HepG2 tumour in BALB/c nude mice	Reduction	344
	Nanogel	DOX	H22 tumour in BALB/c mice	Reduction	345
	Nanogel	DOX	RM-1 <i>in vitro</i> ; RM-1 tumour in C57BL/6 mice	Reduction	343
	Nanogel	Methotrexate	RAW 264.7 <i>in vitro</i> ; rheumatoid arthritis model in DBA/1J mice	Reduction	348
PEG-<i>b</i>-P(LGlu-<i>co</i>-LCys-Cys) 	Nanogel	DOX	HepG2 and H22 <i>in vitro</i> ; H22 tumour in Wistar rat	pH and reduction	347
PLLys-<i>b</i>-P(LPhe-<i>co</i>-LCys-Cys) 	Nanogel	HCPT	T24 <i>in vitro</i> ; BBN-induced bladder cancer in BALB/c mice	Reduction	351

Table 3 (continued)

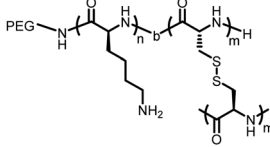
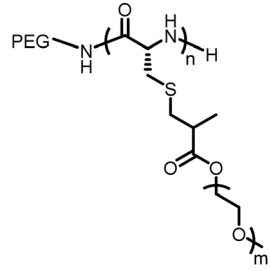
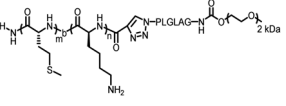
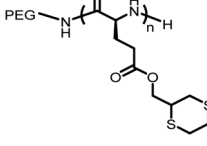
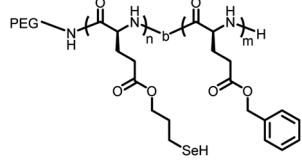
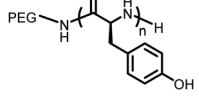
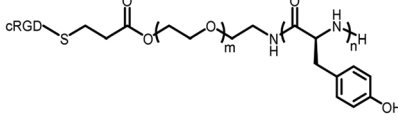
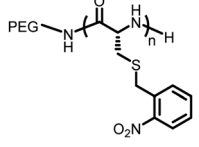
Polymer	Architecture	Drug/cargo	Cell/ <i>in vivo</i> testing	Targeting agent/stimuli	Ref.
PEG-<i>b</i>-PLLys-<i>b</i>-PLCys-Cys 	Nanogel	Br ₂ -IR808	HepG2 and MCF-7 <i>in vitro</i>	Reduction	458
PEG-<i>b</i>-PLCys-<i>g</i>-OEG 	Micelle	DOX	—	ROS	459
PLMet-<i>b</i>-PLLys-PLGLAG-PEG 	Micelle	DOX	NCI-H460 and HT-1080 <i>in vitro</i> ; NCI-H460 tumour in BALB/c nude mice	ROS, enzyme	460
PEG-<i>b</i>-PDTG 	Micelle	Nile Red	B16F10 and L929 <i>in vitro</i>	ROS	461
PEG-<i>b</i>-PLGSe-<i>b</i>-PBLG 	Micelle	DOX	PC3 <i>in vitro</i> ; PC3 tumour in male nude mice	ROS	462
PEG-<i>b</i>-PLTyr 	Nanoparticle	DOX	RAW 264.7 and HCT-116 <i>in vitro</i>	Enzyme	463
cRGD-PEG-<i>b</i>-PLTyr 	Nanoparticle	DOX	HCT-116 tumour in nude mice	Enzyme, cRDG for targeting to integrin	464
PEG-<i>b</i>-PNBLCys 	Polymersome	—	HeLa <i>in vitro</i>	UV, reduction	465

Table 3 (continued)

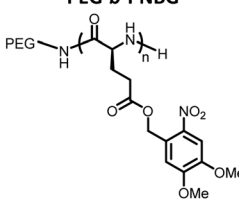
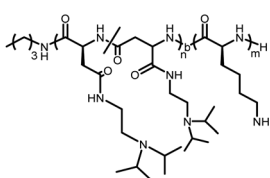
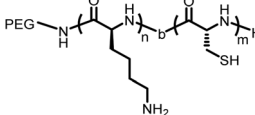
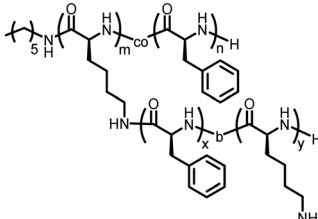
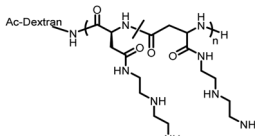
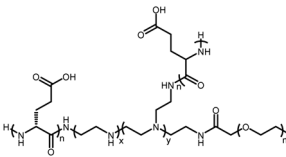
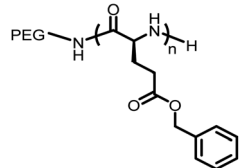
Polymer	Architecture	Drug/cargo	Cell/ <i>in vivo</i> testing	Targeting agent/stimuli	Ref.
PEG-<i>b</i>-PNBG  PNBG = poly(γ-(4,5-dimethoxy-2-nitrobenzyl)-L-glutamate)	Polymersome	—	—	UV	118
PLLys-<i>b</i>-PLAsp-<i>g</i>-Dip 	Polymersome	DOX, siRNA	HepG2 <i>in vitro</i>	pH	466
PEG-<i>b</i>-PLLys-<i>b</i>-PLCys 	Polymersome	DOX, microRNA	MCF-7, MCF-7/ADR <i>in vitro</i>	Reduction	467
P(LPhe-<i>co</i>-LLys-<i>g</i>-(PLPhe-<i>b</i>-PLLys)) 	Brushes, nanoparticles	Model drugs, siRNA	HEK 293 and HeLa <i>in vitro</i>	—	468
Ac-Dex-<i>b</i>-PLAsp-<i>g</i>-DET 	Polymersome	DOX and pDNA	BEL-7402 <i>in vitro</i>	pH	469
mPEG-<i>b</i>-OEI-<i>b</i>-(PLGlu)₂ 	Dendrimer/star polymer	DOX, cisplatin	B16F10 <i>in vitro</i> ; B16F10 tumour in male C57BL/6 mice	pH	470
PEG-<i>b</i>-PBLG 	Nanoparticle	Rapamycin	Aortic aneurysm model in Sprague-Dawley rats	—	471

Fig. 48 Synthesis of a cisplatin-crosslinked PEG-*b*-PLGlu polymersome with folic acid for cancer cell targeting. Reprinted with permission from *Biomacromolecules*, 2015, **16**(8), 2463–2474. Copyright 2015 American Chemical Society.

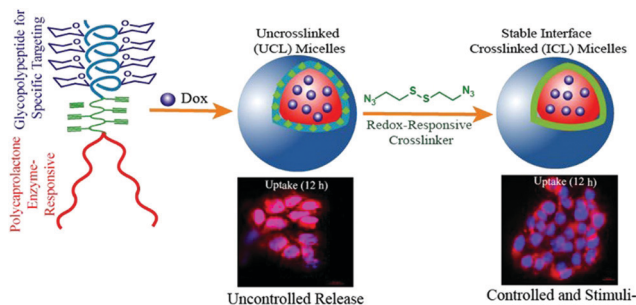


Fig. 49 Synthesis of enzyme and redox responsive interface crosslinked micelles for targeted, stimuli-responsive delivery of doxorubicin. Reprinted with permission from *Bioconjugate Chem.*, 2019, **30**(3), 633–646.

blocks self-assemble in aqueous systems. Interestingly, the Chen group has shown that the amino acid stereoisomer used in self-assemblies can influence self-assembly size, drug loading, and drug release.^{173,445}

Polypeptide micelles and vesicles provide excellent opportunity for pH responsive systems because of the electrically-charged side chains of some amino acids. Poly(L-glutamic acid) and poly(L-aspartic acid) are frequently used to form the interior block of self-assembled structures due to their change in solubility at biologically relevant pH. PLGlu is negatively charged and water-soluble at physiological pH of 7.4 but becomes less soluble when the acid group is protonated at lower pH. Chen *et al.* reported the synthesis of PEG-*b*-PLeu-*b*-PLGlu linear polymers that formed stable self-assemblies at pH 7.4 but were disrupted at endolysosomal pH of 5.⁴³⁴ Taking advantage of this change in conformation, the authors encapsulated DOX in the self-assembled nanocarriers and demonstrated *in vitro* that after 24 hours only 24% of drug was released at pH 7.4 while 76% of drug was released at pH 5.

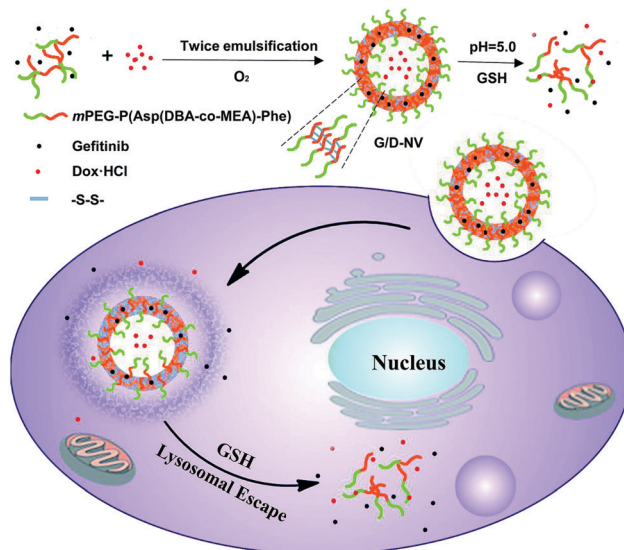


Fig. 50 The hydrophilic and hydrophobic regions of polymersomes enables co-delivery of hydrophilic and hydrophobic therapeutics, as demonstrated by Chen *et al.*⁴⁴² *RSC Adv.*, 2018, **8**, 2082–2091 – published by The Royal Society of Chemistry.

While both micelles and vesicles have shown great promise in drug delivery, vesicles offer additional opportunities as nanocarriers due to their hydrophilic and hydrophobic regions.⁴⁸⁵ As shown by Iatrou *et al.* using PEG-*b*-PBLG-*b*-PLLys linear polymers that formed polymersomes, water-soluble drugs such as DOX-HCl can be loaded into the aqueous cavity while hydrophobic drugs like paclitaxel can be loaded into the hydrophobic region, in this case the PBLG layer.⁴³⁶ Going one step further, Chen *et al.* demonstrated dual loading of hydrophilic and hydrophobic drugs into the same polymersomes for co-delivery of two chemotherapeutics *in vitro* and *in vivo* (Fig. 50).⁴⁴² While not specific to polypeptide-based nanocarriers, it is important to mention that pH-sensitive linkers have been used to conjugate therapeutics to polymeric nanocarriers, enabling pH-responsive release.^{446,449,486,487} The use of acid-labile hydrazone linkages in DDS is ubiquitous and has been eloquently reviewed by Sonawane *et al.*⁴⁸⁸

The more reductive intracellular environment of cells is another internal stimulus often exploited for cancer treatment using nanocarriers.^{231,234,343–347,351,442,452,454–457} Glutathione (GSH) is a tripeptide present at greater concentrations inside cells than outside, and is elevated in some forms of cancer cells.^{489,490} The greater reducing environment of cancer cells can be exploited through reduction-sensitive disulfide bonds, such as cystine, enabling degradation of the nanocarriers once taken up into the cell. Ding *et al.* reported the synthesis of PLG crosslinked star polymers with a sheddable PEG corona for delivery of camptothecin to cancer cells.²³¹ Linear PEG-*b*-PLG was synthesized with an acid-labile linker present between the PEG and PLG blocks, allowing PEG to be released in the tumour microenvironment. PEG-*b*-PLG and camptothecin formed self-assembled nanoparticles and H₂O₂ was added to oxidize the cysteine side chains, forming disulfide bonds

between polymer chains. *In vitro* experiments demonstrated the release of the PEG chains at pH 6.5 while the use of reducing agent dithiothreitol (DTT) caused dissociation of the star polymers. Interestingly, shedding of the PEG corona resulted in greater drug release than DTT treatment. As expected, the greatest drug release was observed at pH 6.5 plus DTT treatment.

The high concentration of reactive oxygen species in cancer cells offers another mechanism to selectively achieve drug release in cancer cells while healthy cells are less- or un-affected.⁴⁹¹ Sulfur-containing amino acids in particular are exploited for ROS-responsive release, as hydrophobic thioether groups can be oxidized to more hydrophilic sulfoxide and sulfone groups, leading to destabilization of self-assembled architectures.^{459–461} Using a similar approach, Zhang *et al.* modified the side chain of glutamic acid to synthesize 1,4-dithian-substituted L-glutamate (DTG) NCA, which was then polymerized from PEG amine (Fig. 51).⁴⁶¹ The PEG-*b*-PDTG polymers self-assembled to form micelles that could be loaded with Nile Red as a model drug. Oxidation of the pendant thioethers by addition of H₂O₂ resulted in disintegration of the micelles and release of the model drug. Yoo *et al.* developed a multiresponsive micellar system comprising a poly(methionine)-*b*-PLLys-*b*-PEG triblock containing MMP2/9 sensitive peptide linker between the PEG and PLLys blocks.⁴⁶⁰ Overexpression of MMP-2 and -9 within tumours led to cleavage of the PEG block, exposing a cell-penetrating PLLys block to facilitate cell internalization. Once internalized, ROS oxidation of the methionine thioethers caused disintegration of the micelle, releasing DOX into the cytosol.

Deepagan *et al.* exploited the high intracellular concentration of ROS species in cancer cells to oxidize diselenide crosslinked micelles (Fig. 52).⁴⁶² They synthesized a triblock containing PEG, selenium-functionalized poly(LGlu), and PBLG blocks which self-assembled to form micelles. DOX was loaded into the hydrophobic core and diselenide crosslinks were formed in the shell. These micelles were stable at pH 7.4 in

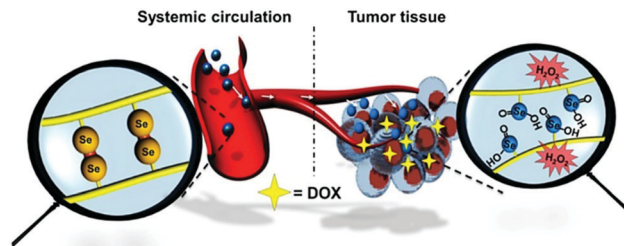


Fig. 52 Oxidation of selenide by intracellular ROS cleaves the micelle diselenide-crosslinks to allow intracellular DOX release within cancer cells.⁴⁶² Reprinted with permission from *Biomaterials*, 2016, **103**, 55–66. Copyright 2016 Elsevier.

PBS but showed rapid release of DOX in the presence of H₂O₂. *In vivo* assays in a PC3 tumour-bearing mouse model demonstrated significantly lower tumour volumes following treatment with DOX-loaded diselenide crosslinked micelles than with free DOX, highlighting the potential of ROS-responsive drug delivery systems for cancer therapy.

Multi-responsive polypeptide-based nanoparticles have shown excellent potential for drug delivery to tumour cells. Chen *et al.* reported a sequentially responsive nanoparticle for smart drug delivery, exploiting NCA ROP and the functionality of amino acids to synthesize a polypeptide shell-stacked nanoparticle (SNP) optimized for systemic circulation as well as the tumour and intracellular microenvironments (Fig. 53).⁴⁵⁴ The outer shell of the nanoparticles was designed to survive the circulatory system and reach the tumour through passive targeting. The outer shell was composed of PEG-*b*-PLLys with 80% of lysine side chains functionalized with dimethylmaleic anhydride *via* a pH sensitive bond. The nanoparticle core was composed of PLLys-P(LP-co-LCys) and loaded with doxorubicin. The outer shell was bound to the core through electrostatic interactions. At the tumour microenvironment pH of 6.8, complete detachment of the outer shell from the core was observed,

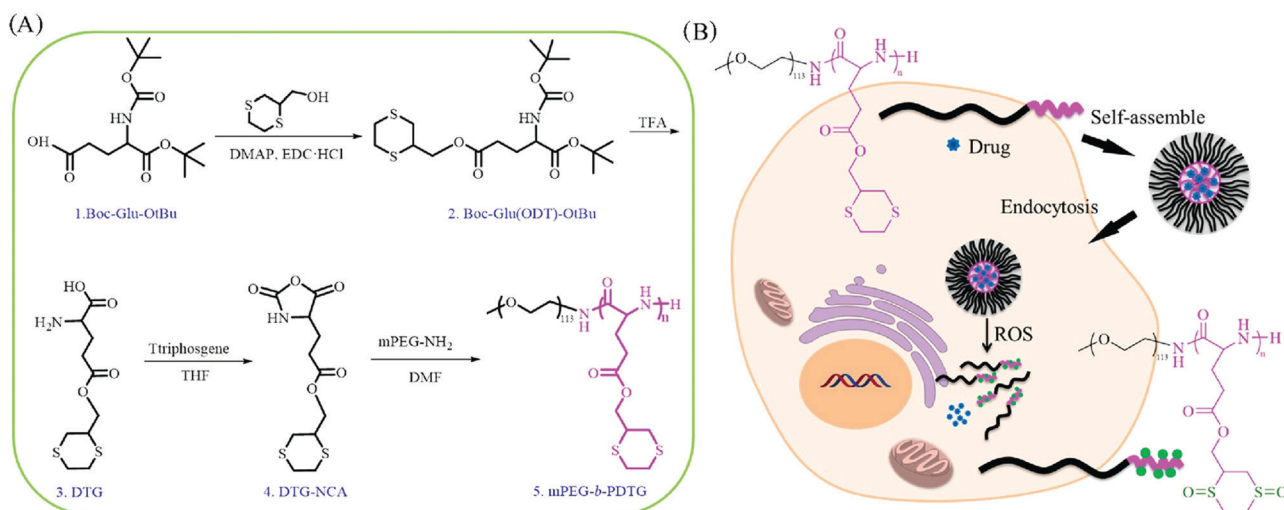


Fig. 51 Dithian moieties are susceptible to oxidation by reactive oxygen species (ROS), becoming more hydrophilic and destabilizing the PEG-*b*-poly(L-dithian substituted glutamic acid) (PEG-*b*-PDTG) micelles to enable drug release. Reprinted with permission from *Chin. Chem. Lett.*, 2020, **31**(5), 1229–1132.⁴⁶¹ Copyright 2020 Elsevier.

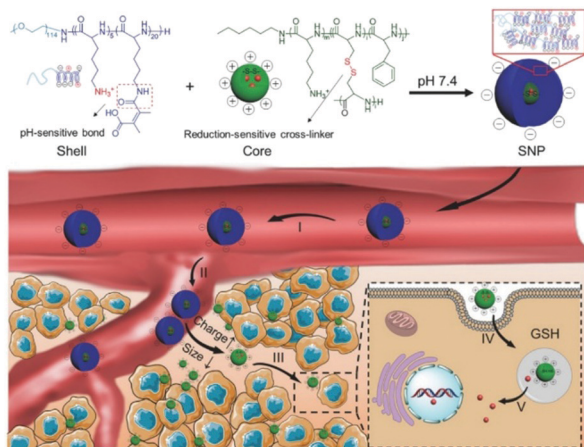


Fig. 53 The synthesis of sequentially pH- and redox-responsive nanoparticles enabled improved penetration into solid tumours.⁴⁵⁴ Reproduced with permission from *Adv. Mater.*, 2017, **29**(32), 1701170. Copyright 2017 John Wiley and Sons.

corresponding to a decrease in size from 145 nm in diameter to only 40 nm. The core disintegrated, enabling the release of doxorubicin, following incubation in 10 mM glutathione, mimicking the intracellular environment of cancer cells. *In vivo* studies in a murine model of lung cancer demonstrated the greater tumour penetration of the core compared to a non-transformable control nanoparticle, thought to be due to the much smaller size of the core following detachment of the outer shell. In comparison to free DOX, SNPs showed greater tumour accumulation, lower tumour volumes, and higher bodyweights indicating improved drug delivery with a reduction in adverse effects.

Due to their intrinsic nature polypeptide materials are sensitive to enzymes, which can be exploited to improve drug release.^{492–494} Gu *et al.* reported the ultra-high loading of DOX in PEG-*b*-poly(tyrosine) nanoparticles (PTNs) followed by rapid enzyme-mediated drug release.⁴⁶³ DOX-loading of 63 wt% was thought to be a result of π - π stacking of the tyrosine phenol side chain and DOX, which also led to high stability of the drug-loaded PTNs. Building on their work, Gu *et al.* later decorated the PTNs with cyclic RGD (cRGD-PTN) to improve cell uptake into colorectal cancer cells.⁴⁶⁴ DOX-loaded-cRGD-PTNs were found to have significantly greater accumulation in a HCT-116 tumour-bearing nude mice compared to non-decorated DOX-PTNs and 5-fold greater toleration than a clinically used liposome-DOX formulation. The DOX-loaded-cRGD-PTNs showed release of nearly 90% of encapsulated drug following 10 hours of incubation with cells while showing less than 19% release when incubated in PBS for 24 hours in the absence of cells, suggesting enzyme-mediated release of DOX.

Another mechanism for enzyme-stimulated drug release is the incorporation of known enzyme-sensitive sequences. These peptide sequences can be incredibly specific and are generally synthesized using solid phase peptide synthesis. Bacinello *et al.* reported the use of hybrid triblock polymers containing poly(trimethylene carbonate) and PLGLu blocks separated by a peptide with the sequence proline-valine-glycine-leucine-isoleucine-glycine (PVGLIG).⁴²⁹ The PVGLIG peptide is a known

substrate for matrix-metalloproteinases 2 and 9 (MMP-2 and MMP-9), enzymes overexpressed in cancer cells and commonly associated with tumour invasion and metastasis.^{495,496} While the polymersomes also contained a pH responsive PLGLu block to disrupt the self-assembled structure at low pH, incorporation of the PVGLIG peptide resulted in increased release of a model hydrophilic drug *in vitro*.⁴²⁹

The use of external stimuli such as light provides temporal control over drug release not possible with purely internal-responsive nanocarriers. In recent years, the use of UV-labile *o*-nitrobenzyl protecting groups on cysteine and glutamic acid NCAs has led to novel UV-responsive polypeptide-based materials.^{118,231,465,497} Lui *et al.* synthesized amphiphilic PEG-*b*-poly(*S*-(*o*-nitrobenzyl)-L-cysteine) (PEG-*b*-PNBLCys) that formed bilayered polymersomes.⁴⁶⁵ Cleavage of the *o*-nitrobenzyl group upon UV irradiation caused a conformational change from vesicles to micelles. Oxidation of the thiol side chains with H₂O₂ resulted in the formation of large aggregates due to disulfide bridges while the addition of 10 mM DTT reversed the aggregation. The authors also synthesized DOX-loaded polymersomes and demonstrated linear release of DOX over 12 hours *in vitro* when the polymersomes were irradiated with UV for 3 minutes every 2 hours, ultimately achieving 91% release. However, the limited penetration of light into human tissue/organs posts a key barrier for the clinical application of light-triggered drug delivery.⁴⁹⁸

4.2.2 Nanocarriers in non-cancer applications. Although polypeptide-based nanocarriers reported have primarily focused on cancer therapy, the treatment of other pathologies has been investigated.^{348,471,499} Feng *et al.* synthesized polypeptide-based nanogels loaded with methotrexate for the treatment of rheumatoid arthritis (Fig. 54).³⁴⁸ This disease causes cartilage and bone damage in small joints that can be treated with methotrexate (MTX), but adverse effects restrict its long-term use. Feng *et al.* synthesized PEG-*b*-P(LPhe-co-LCys) nanogels with a disulfide crosslinked core that were loaded with MTX. The disulfide core was incorporated to promote release of MTX in afflicted regions, which are known to have higher concentrations of glutathione. The nanogels demonstrated sustained release of

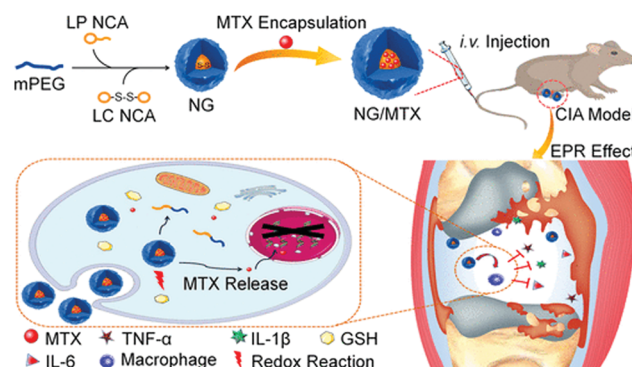


Fig. 54 Delivery of methotrexate (MTX) from PEG-*b*-poly(L-phenylalanine-co-L-cysteine) nanogels with a disulfide crosslinked core for the treatment of rheumatoid arthritis.³⁴⁸ Reprinted with permission from *ACS Biomater. Sci. Eng.*, 2018, **4**(12), 4154. Copyright 2018 American Chemical Society.

MTX over 70 hours and were effective at treating RA and reducing inflammation following IV injection in a mouse model.

Shirasu *et al.* reported the use of PEG-*b*-PBLG nanoparticles to deliver rapamycin in a rat model of abdominal aortic aneurysm (AAA).⁴⁷¹ Currently, aortic aneurysms are treated using invasive surgical procedures. The authors observed accumulation of the rapamycin-loaded nanoparticles in the AAA even at 24 hours post-IV injection. Analysis of treated rats showed the rapamycin nanoparticles were significantly more effective at inhibiting aneurysmal formation than free rapamycin, most likely due to accumulation of the nanoparticles in the AAA. Rats treated with the rapamycin nanoparticles were also found to have reduced accumulation of inflammatory cells, macrophages, and lower expression of inflammatory cytokines indicating successful delivery of rapamycin.

The wide range of morphologies and functional groups possible using polypeptides facilitates the synthesis of materials with multiple functions. Most commonly, drug delivery is performed concurrently with gene delivery^{466–470,500} to provide a combination therapy or with medical imaging agents^{455,501} in what is referred to as theranostics. Although these materials offer additional functionalities when compared to drug delivery alone, the same synthetic, targeting, and delivery strategies are applied. Readers interested in theranostics are directed to the above mentioned papers as well as a recent review by Augustine *et al.* on smart polypeptide-based nano-carriers as theranostics.⁵⁰²

4.2.3 Polypeptide hydrogels for drug delivery. Polypeptide hydrogels have also been investigated for drug and protein delivery and are optimal materials due to their biocompatibility and degradability.^{299,503–505} As opposed to nanocarriers, which are administered systemically, hydrogels are generally administered at the treatment site ensuring localized drug release. Ideally hydrogels should be injectable to enable minimally invasive insertion. Hydrogels can release their payload passively *via* diffusion or in response to stimuli, and a recent review by Liarou *et al.* gives an in-depth look at smart hydrogel synthesis and their use for a wide range of biomedical applications.²⁷⁶

The Chen group at the Chinese Academy of Sciences has published several papers in recent years investigating the release of proteins²⁸⁰ and anti-cancer drugs^{492,506,507} from polypeptide hydrogels. In line with a recent focus on combination therapies to achieve synergistic effects and reduced drug resistance,^{508,509} Chen *et al.* have reported the combined release of two therapeutic agents for cancer therapy.^{284,506} In one system for cervical carcinoma therapy, amphiphilic PEG-*b*-poly(L-alanine-co-L-phenylalanine) was dissolved in PBS with DOX and vascular disrupting agent Combretastatin A4 (CA4) at 4 °C (Fig. 55).⁵⁰⁶ The solution remained fluid below 25 °C and formed a gel following subcutaneous injection in a mouse model of cervical carcinoma. The system was designed to promote delivery of DOX to the tumour cells followed by CA4, which would disrupt the tumour vasculature and starve the tumour after DOX delivery. *In vitro* experiments demonstrated that nearly 50% of DOX was released within the first 2 days while most of the less hydrophilic drug CA4 was released between days 3 and 28.

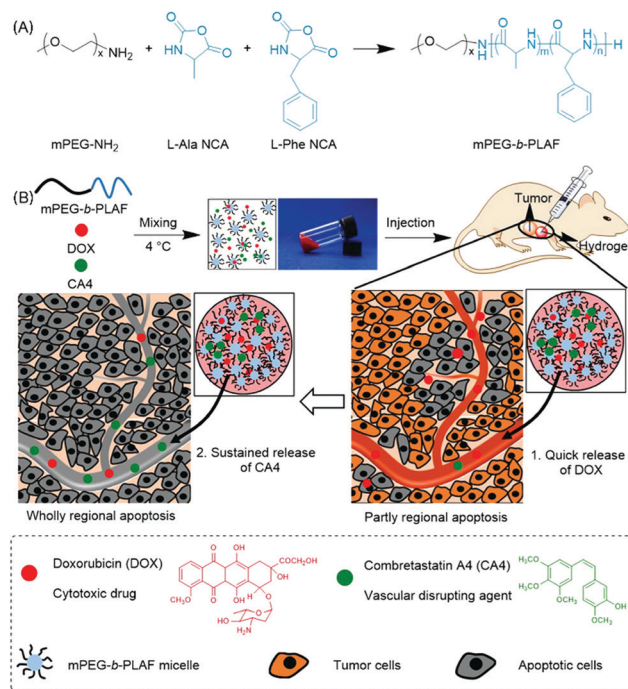


Fig. 55 Sequential delivery of DOX and CA4 from thermosensitive PEG-*b*-poly(L-alanine-co-L-phenylalanine) hydrogel for cancer therapy. Reprinted from *Acta Biomater.*, 2017, **58**, 44. Copyright 2017 Elsevier.

4.2.4 Future perspectives. Drug delivery from materials containing polypeptides synthesized *via* NCA ROP of α -amino acids continues to be a growing and promising field. For cancer therapy, several materials have reached clinical trials while the focus on targeted and stimuli-responsive delivery systems continues to yield results in which cancer cells are preferentially selected and killed. The advances made in the design and synthesis of these materials is inspiring, however more in-depth studies of the interactions of these materials with the biological environment are needed.

In the case of nanoparticles delivered systemically, *in vivo* mouse studies continually demonstrate accumulation in the liver, spleen, and kidneys. While the use of polypeptides aims to exploit the degradability of polypeptides and avoid long-term accumulation, this mechanism has not been studied enough. Although natural proteins are readily synthesized and degraded, most drug delivery systems depend on the modification of amino acid side chains which will likely play a role in enzymatic degradation. In addition, many delivery systems use long chains containing a single amino acid, which is a significant deviation from the proteins found in living organisms. The degradation of these homopolypeptide blocks has not been well studied in *in vivo* systems, and even many *in vitro* systems use inappropriate enzymes or enzyme concentrations. Therefore, it is important that future work include longer term *in vivo* studies to fully understand the long-term fate of administered polypeptide materials. While further work is needed, significant progress in the synthesis and design of polypeptides-based materials for DDS has been made, highlighting the potential clinical use of polypeptide-based materials into the future.

4.3 Polypeptides in nucleic acid delivery

Nucleic acid-based therapy entails the delivery of DNA or RNA into target cells with the aim of altering gene expression to treat a range of pathologies including cancer, infectious diseases, and immunodeficiency.⁵¹⁰ Exogenous nucleic acid materials such as plasmid DNA (pDNA), messenger RNA (mRNA), small interfering RNA (siRNA), and microRNA (miRNA) are examples of typical therapeutic nucleic acids. Nucleic acid-based therapy depends on the optimal transfer of DNA or RNA into targeted cells with high transfection efficiency and low toxicity;^{510,511} thus, development of effective and human-compatible delivery vectors is a key challenge in this field.⁵¹²

The need for a vector is due to the vulnerability of unprotected DNA or RNA to nucleases. In addition, genetic material is relatively large in size and carries negative charges. Though the charges render the material hydrophilic, they prevent the vectors from penetrating the plasma membrane, which is also negatively charged. Thus intracellular delivery of “naked” genetic materials is challenging, and two options are available to facilitate targeted delivery of genetic materials.⁵¹³ The use of viral vectors is highly efficient, but these present severe safety concerns, low loading capacity, and difficulties in scaling up.^{514,515} As an alternative, non-viral vectors can be employed. They include cationic polymers and lipids, which are considered desired alternatives to viral vectors for gene therapy due to their biocompatibility and minimal risk of mutagenesis.^{514,516} One of the most common ways of designing non-viral gene delivery vectors is the formation of an electrostatic complex between the positively charged functional groups of the cationic vector and the negatively-charged phosphate moieties of anionic genetic materials (polyplex) (Fig. 56).^{511,517} By formation of this complex, genetic materials condense and encapsulate into nanoparticles, which protect them from degradation by nuclease and facilitate their effective delivery to the intended site of action within the targeted cells. In the past decades, substantial progress has been achieved in the design and synthesis of cationic non-viral gene delivery vectors with precise control over architecture, molecular weight, and function. Some of the gene delivery systems have been tested in phase I clinical trials and some have reached phase II/III.^{510,518,519} Cellular uptake of the polyplex can be achieved through endocytic or non-endocytic pathways.^{514,520–522} Endocytosis mechanisms such as phagocytosis, micropinocytosis, clathrin-mediated endocytosis, and caveolae occur through at least three modes including fluid-phase, adsorptive, and receptor-mediated endocytosis. After endocytosis, the internalized polyplexes tend to be trapped in the intracellular vesicles termed endosomes which eventually fuse with lysosome, where

the pH reaches 4.5–5.0, and the polyplex is degraded. Therefore, a key design consideration is the necessary ability of the non-viral gene delivery vectors to escape from the endosome to reach either the cytoplasm or the nucleus. Non-endocytic strategies such as microinjection, permeabilization, electroporation, fusion, and penetration can be also utilized to deliver DNA or RNA into the intracellular environment because they avoid being degraded in lysosomes.^{514,520–522} Various cationic polymers such as polyethyleneimine (PEI), cationic chitosan, cationic dextran, PAMAM, and polypeptides with different architecture such as linear, brush, and star have been used as gene delivery vehicles. PEI, which is known as one of the most effective gene delivery polymers has shown high cell cytotoxicity due to its high cationic charge density.^{510,514,522} Therefore, the use of degradable cationic polymers with low cytotoxicity is preferred. Polypeptides synthesized by NCA ROP have been widely used as gene delivery cargos due to their biocompatibility, biodegradability, defined structure, and functionality which is reviewed here. Among the various polypeptide architectures obtained through NCA ROP, block copolymers and homopolymers in addition to a few star polypeptide examples have been mostly used for nucleic acid delivery. Table 4 shows the chemical structure, architecture, nucleic acid, polyplex particle size, and cell type used by some researchers for gene delivery. For further reading on gene delivery, readers are directed to excellent reviews in the field.^{510,511,514,522–525}

4.3.1 Complexes formed using poly(lysine). PLLys, a cationic polypeptide, has been widely used for polyplex formation in non-viral gene delivery carriers due to its degradability and biocompatibility.^{511,551–553} However, PLLys-based complexes have exhibited low blood circulation time, low transfection efficiency, high toxicity, and low ability for endosomal escape.^{511,551–553} Therefore, various strategies have been utilised to improve transfection efficiency, endosomal escape, and biocompatibility. These techniques include conjugating hydrophilic or amphiphilic segments to PLLys, incorporation of other cell selective or cell penetrating peptides, the design of α -helical, cationic polypeptides, or a combination of these techniques.

Johnson *et al.* synthesized a series of biocompatible block copolymers of poly((2-hydroxyethyl methacrylate)₄₀-*block*-(L-lysine)_{*n*}) (PHEMA-*b*-PLLys) as gene delivery vectors by varying the lysine repeat units (*n* = 40, 80, 120, and 150).⁵²⁶ Poly(L-lysine) is biodegradable and has binding ability to pDNA while poly(HEMA) is biocompatible. The block copolymers successfully formed complexes with pDNA and illustrated enhanced transfection efficacy with very low cytotoxicity.⁵²⁶ In another study, a block copolymer of poly(ethylene glycol) methacrylate (POEGMA) and PLLys (POEGMA-*b*-PLLys) was synthesized using a combination of ATRP and NCA-ROP.⁵²⁷ The synthesized hybrid copolymer formed a complex with pDNA and generated particles with sizes of approximately 100 nm. A high internalization and transfection of the developed polyplexes was observed into human liver cancer cells.⁵²⁷ The Qiao group synthesized 16- and 32-arm star polymers containing PAMAM dendritic cores and poly(lysine) arms *via* NCA-ROP.²⁰⁸ The star polymers were post-functionalized with PEG to improve biocompatibility

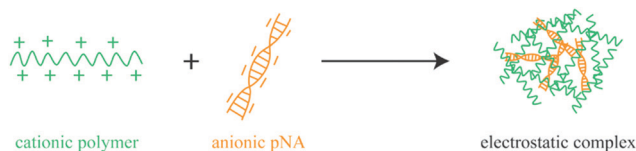


Fig. 56 Schematic presentation of the electrostatic complex formation between a cationic polymer and anionic genetic material (pDNA).

Table 4 Chemical structure, architecture, nucleic acid, particle size, and cell type used for nucleic acid delivery

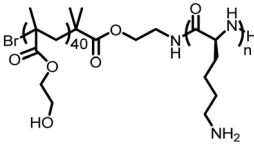
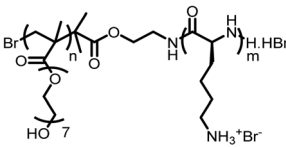
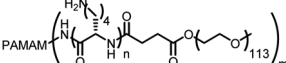
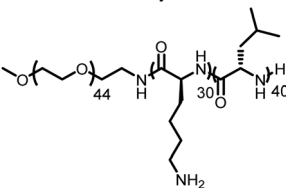
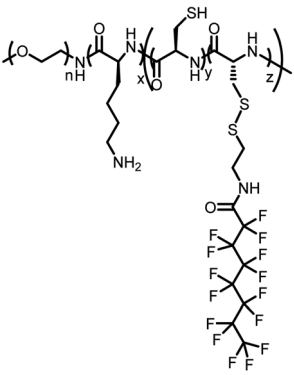
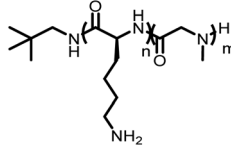
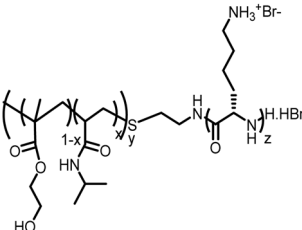
Polymer	Architecture	Nature of cargos	Polyplex particle size	Cell/animal type	Ref.
PHEMA-<i>b</i>-PLLys 	Block copolymer	pDNA	150–250 nm	Mouse embryonic fibroblast cell line (NIH 3T3)	526
POEGMA-<i>b</i>-PLLys 	Block copolymer	pDNA	100–180 nm	A549 alveolar epithelial cells and HepG2 cells (human liver cancer cells)	527
PAMAM-<i>g</i>-(PLLys-<i>b</i>-PEG) 	Star	siRNA	Not reported	HEK293T (derived from human embryonic kidney 293 cells)	208
PEG-<i>b</i>-PLLys-<i>b</i>-PLL 	Block copolymer	siRNA	142 nm	B16 melanoma cell line, and C57BL/6J mice	528
Fluorinated PEG-<i>b</i>-PLLys-<i>b</i>-PCys 	Block copolymer	pDNA	80 nm	293T cells (derived from human embryonic kidney 293 cells), MCF-7 cells (breast cancer cell), and HeLa cells (derived from cervical cancer cells)	529
PLLys-<i>b</i>-PSar 	Block copolymer	pDNA	40–43 nm	HEK 293T (derived from human embryonic kidney 293 cells)	530
P(HEMA-<i>co</i>-NIPAM)-<i>b</i>-PLLys 	Block copolymer	pDNA	95–285 nm	B-cell lymphoma and HEK-293 (human embryonic kidney 293 cells)	531

Table 4 (continued)

Polymer	Architecture	Nature of cargos	Polyplex particle size	Cell/animal type	Ref.
PEG-<i>b</i>-PAPA-<i>b</i>-PLLys + CPP33-PEG-<i>b</i>-PAPA 	Block copolymer	siRNA	90 nm	A549 cells (adenocarcinomic human alveolar basal epithelial cells) and orthotopic A549 human lung cancer tumour-bearing nude mice	532
PLLys-<i>b</i>-PLLeu-<i>b</i>-PLLys 	Block copolymer	pDNA	130–230 nm for protamine/pDNA polyplex	293T cell (derived from human embryonic kidney 293 cells)	533
PBLG-<i>g</i>-guanidine/amine 	Homopolymer	pDNA	100–200 nm	HeLa (derived from cervical cancer cells), and COS-7 (derived from the kidney of the African green monkey)	534
PS-<i>g</i>-P(BLG-<i>g</i>-guanidine) 	Homopolymer	DNA	100–250 nm	HeLa (derived from cervical cancer cells), RAW 264.7 (derived from mouse monocyte macrophage), and male C57/BL6 mice transfected with B16F10 (murine melanoma cell line)	535
R8-CD + Azo-PSar-<i>b</i>-PLLys 	Block copolymer	DNA	150 nm	HepG2 (human liver cancer cells)	536
PAMAM-<i>g</i>-(P(POBLG) modified with arginine) 	Homopolymer	siRNA	100–210 nm	MCF-7 (breast cancer cell line), Nude mice bearing orthotopic MCF-7 tumours	537

Table 4 (continued)

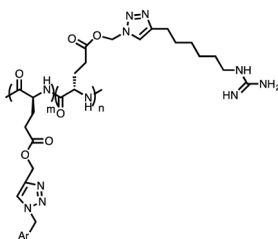
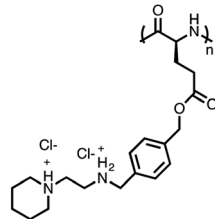
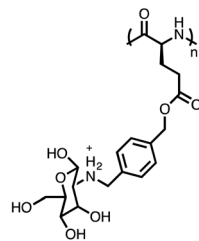
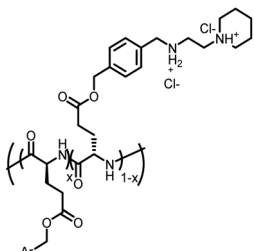
Polymer	Architecture	Nature of cargos	Polyplex particle size	Cell/animal type	Ref.
Guanidine and aromatic group-modified PPLG 	Random copolymer	DNA/siRNA	100–200 nm	HeLa (derived from cervical cancer cells), RAW 264.7 (derived from mouse monocyte macrophage), and male C57/BL6 mice	538
		siRNA	100–200 nm	H9C2 cells, and male Sprague-Dawley rats	539
PPABLG/PVBLG-8 	Homopolymer	siRNA	100 nm	RAW 264.7 cells, and male C57BL/6 mice	540
		pDNA	150 nm	HeLa, COS-7, and RAW 264.7	541
		siRNA	150 nm	U-87 MG cells (glioma cell line), HEK-293, and U-87 MG tumour-bearing mice	542
		sgRNA/Cas9 plasmid	87 nm	HEK293T, HeLa, K562 cells (chronic myelogenous leukaemia), mouse fibroblast cells (NIH 3T3), dendritic cells (DC2.4), normal human dermal fibroblasts (NHDF), human umbilical cord blood-derived endothelial progenitor cells (hEPCs), U2OS. EGFP cell line (human osteo carcinoma), and A549. GFP tumour-bearing mice, HeLa xenograft tumour-bearing mice	543
		pDNA	170 nm	hESCs H1 (H1 human embryonic stem cell line), MSCs (mesenchymal stem cells), and COS-7 cells	544
PVBLG-7 	Homopolymer	pDNA	150–200 nm	HeLa, COS-7, and RAW 264.7	541
PPABLG-Ar 	Block copolymer	pDNA	150 nm	HeLa, B16F10 (murine melanoma cell line), and female C57/BL6 mice	545

Table 4 (continued)

Polymer	Architecture	Nature of cargos	Polyplex particle size	Cell/animal type	Ref.
PPABLG-SH	Block copolymer	siRNA	200 nm	HeLa-Luc cells	546
PAsp-grafted chitosan-cyclodextrin conjugate	Homopolymer	pDNA	250 nm	HeLa, C6 (glial tumour cell), and HepG2 cell lines (human liver cancer cells)	547
PAsp-based triblock	Homopolymer	pDNA	260 nm	C6 and HepG2 cell lines, and male BALB/c nude mice	548
PPI-g-PLLys	Star	pDNA	140–200 nm	Mesenchymal stem cells (MSCs)	549
PPI = polypropyleneimine					

and used as a potential carrier for siRNA delivery, demonstrating that they could form complexes with siRNA and were indeed biocompatible. Luo *et al.* synthesized PEG-*b*-PLLys-*b*-PLLeu polypeptide micelles to co-deliver OVA antigen, PIC (a TLR3 ligand), and STAT3-siRNA.⁵²⁸ Polypeptide micelle-PIC/OVA/STAT3 siRNA nanovaccine effectively enhanced the anti-tumour efficacy of therapeutic vaccines and overcame dendritic cell dysfunction *in vivo* by deleting STAT3 gene *in situ*.⁵²⁸ In another study a poly(ethylene glycol)-*b*-poly(L-lysine)-*b*-poly(L-cysteine) (PEG-*b*-PLLys-*b*-PCys) triblock was synthesized by NCA-ROP polymerization followed by modification with fluoro-carbon chains and the polymer was used for pDNA delivery.⁵²⁹ The fluorinated polypeptides exhibited good gene transfection efficiency, low toxicity and serum resistance even in the presence of 50% fetal bovine serum.⁵²⁹ Poly(sarcosine), a polypeptoid, has also been combined with PLLys to decrease poly(lysine)'s inherent toxicity.^{530,536} Heller *et al.* synthesized PLLys-*b*-PSar by varying block lengths.⁵³⁰ The block copolymer formed polyplexes with pDNA and was used for transfection of HEK 293T cells with no detectable toxicity.⁵³⁰

An interesting design was performed by Veleva-Kostadinova *et al.*, who synthesized a series of thermally sensitive, polypeptide-based gene-delivery vectors by combination of free radical copolymerization and NCA ROP polymerization. The polymers were comprised of poly(2-hydroxyethyl methacrylate) (PHEMA), poly(*N*-isopropylacrylamide) (PNIPAM) and PLLys,

(PHEMA)₉-*g*-(PNIPAM)₇₇-*b*-(PLLys)_z (Fig. 57).⁵³¹ The degree of polymerization of poly(L-lysine) varied from 10 to 65 in order to find a proper balance between DNA delivery and cytotoxicity. Well-defined nanosized particles in the range of 95–285 nm were formed by abrupt heating of the mixture of the hybrid copolymers and DNA to 65 °C and then cooling to 25 °C. The copolymers had strong ability to form a complex with DNA, (also referred to as condensing DNA) in order to form well-defined polyplex particles. Additionally, both pure copolymers and polyplexes with the lowest degree of polymerization were devoid of intrinsic cytotoxicity. These findings revealed that thermo sensitive PLLys-based copolymers could be used as biocompatible gene delivery vectors.⁵³¹

As reviewed here, various strategies have been used to improve the transfection efficiency and cytotoxicity of PLLys. One of the common strategies is the addition of hydrophilic surface coating polymers such as PEG. Owing to the low-fouling properties of PEG, this incorporation plays a significant role in the protection of the nucleic acid against blood components. It also reduces cytotoxicity and aggregation of polyplex, whilst improving transfection efficiency and circulation time of the complex in blood.^{554–556} However, this incorporation also impacts nucleic acid delivery as a result of the positive charge shielding of poly(lysine). Some disadvantageous properties that are thus introduced include a reduced cellular uptake, and endosomal escape. To overcome the hurdles associated with

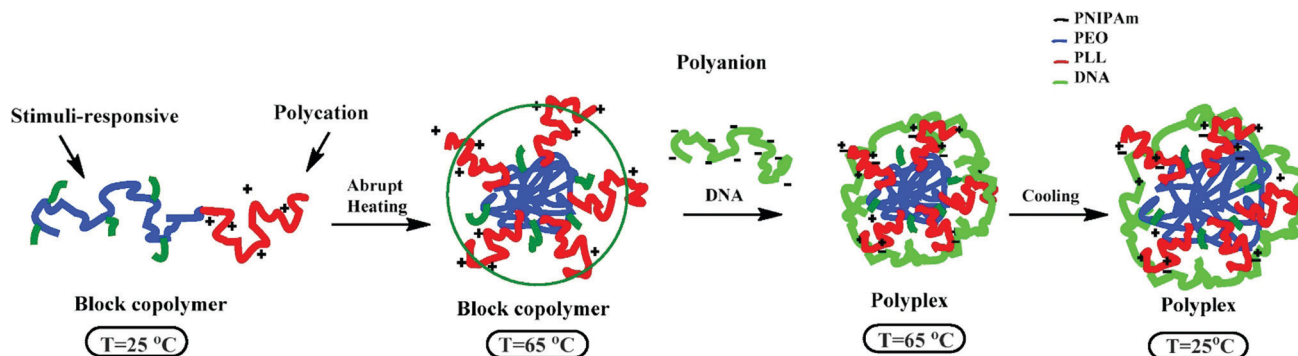


Fig. 57 Schematic illustration of particle formation upon abrupt heating and complex formation with pDNA. Reproduced with permission from ref. 531. Copyright 2018 American Chemical Society.

PEGylation the use of alternative strategies is necessary, such as incorporation of cell selective targeting peptides.

In a recently published work by Qiu *et al.*, chimeric lipopepsomes functionalized with selective cell penetrating peptide were developed to efficiently encapsulate and selectively deliver polo-like kinase 1 specific siRNA (siPLK1) to orthotopic A549 human lung tumour *in vivo*.⁵³² To prepare this system an asymmetric triblock copolymer of poly(ethylene glycol)-*b*-poly(α -aminopalmitic acid)-*b*-poly(L-lysine) (PEG-*b*-PAPA-*b*-PLLys) and a diblock copolymer (maleimide-PEG-*b*-PAPA) were synthesized *via* NCA ROP. Maleimide-PEG-*b*-PAPA was modified with lung cancer-specific cell penetrating peptide CPP33 (RLWMRWYSPRTRAYGC) and in order to increase the availability of CPP33 peptide on the outer surface of lipopepsomes, the PEG chain employed in CPP33-PEG-*b*-PAPA was longer than in PEG-*b*-PAPA-*b*-PLLys. The two polymers were then co-self-assembled in the presence of siRNA to generate siRNA-loaded CPP33-functionalized chimeric lipopepsomes (siRNA-CPP33-CLP) (Fig. 58). This design provided several advantageous properties for siRNA delivery including high encapsulation efficiency *via* charge complexation between siRNA and the PLLys segment, high protection of siRNA from *in vivo* degradation, selective internalization of siRNA by A549 human lung cancer cells, efficient

endosomal escape, rapid release of siRNA into the cytoplasm, and the remarkable subsequent sequence-specific gene silencing *in vitro*. Additionally, *in vivo* experiments demonstrated effective tumour growth suppression in A549 human lung tumour-bearing nude mice due to prolonged siPLK1-CPP33-CLP blood circulation and enhanced tumour accumulation. These results provided an attractive, simple, and safe delivery platform for siRNA delivery.⁵³²

4.3.2 Complexes formed using arginine or guanidine-modified polypeptides. Cell penetrating peptides (CPPs) are sequence-specific oligopeptides. They have helical structure or form helices in the proximity of cell membranes which gives them distinguished membrane penetrating properties. CPPs are able to facilitate cell internalization and endosomal escape of various cargos such as proteins, peptides, and nucleic acids, resulting in improved transfection efficiency. However, CPPs are often too short in length (fewer than 25 amino acid residues) and lack adequate cationic density. This limits their function as single delivery vectors to independently complex and deliver genes.^{534,557,558} Detailed study of CPPs has revealed arginine residues are often richly expressed in the primary structure of the CPPs where they are crucial for the cell penetration capacity. Arginine has guanidine groups on the side chain with pK_a of ~ 12.5 which

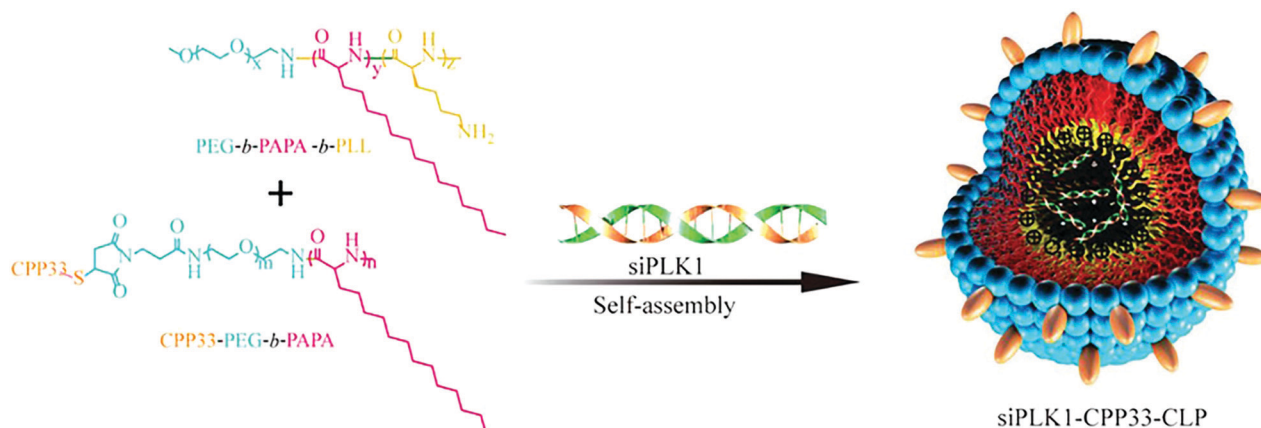
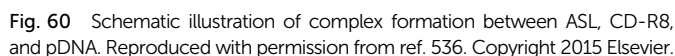
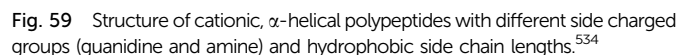


Fig. 58 Illustration of preparation of siPLK1-encapsulated CPP33-CLP from self-assembly of PEG-*b*-PAPA-*b*-PLLys and CPP33-PEG-*b*-PAPA. Reproduced with permission from ref. 532. Copyright 2019 John Wiley and Sons.

Zhang *et al.* designed and synthesized a range of cationic, α -helical polypeptides with different charged side groups (guanidine and amine) and hydrophobic side chain lengths and investigated the impact of polypeptide structure and functionality on the efficiency of pDNA delivery in two different mammalian cell lines (HeLa and COS-7) (Fig. 59).⁵³⁴ The results illustrated that the guanidine-containing polypeptides with helical conformation and hydrophobic content display high



membrane activities and transfection efficiencies. However, excessive hydrophobicity can cause damage to the cell membrane. Therefore, an optimal balance between membrane activities and cytotoxicity is required to maximize the gene delivery efficiency.⁵³⁴ Xu *et al.* developed photosensitizer (PS)-embedded guanidine-rich, four armed helical polypeptides *via* NCA ROP.⁵³⁵ The star polypeptide exhibited enhanced transfection efficiency and lower cytotoxicity compared to its linear analogue. In addition, almost complete endosomal release of the DNA cargo (~90%) was obtained due to the photochemical internalization mechanism. Their results demonstrated that using such a design, it is possible to overcome multiple membrane barriers against gene delivery.⁵³⁵

In another study, Du *et al.* synthesized azo end-capped PSAR-*b*-PLLys (ASL) polypeptide by NCA ring opening polymerization. ASL polypeptide formed a complex with DNA effectively and formed 150 nm spherical nanoparticles (Fig. 60).⁵³⁶ Importantly, a host-guest interaction between the azobenzene functionalities of ASL polypeptide and cyclodextrin-octa arginine (CD-R8) was used to introduce R8 on the nanoparticle surface and promote the internalization of gene into the cytoplasm and even nucleus due to the membrane penetrating impact of R8. The results illustrated that the ASL nanoparticles exhibited a comparable gene transfection level to PLLys with molecular weight of 50 kDa and good biocompatibility. This study also presented polysarcosine as an ideal alternative to PEG.⁵³⁶

Recently, Dang *et al.* developed a spherical, multivalent, helical, membrane-penetrating polypeptide for siRNA delivery which sensitizes photothermal ablation *via* inhibition of tumour glycolysis metabolism.⁵³⁷ A spherical helical guanidine-rich polypeptide was synthesized *via* ROP of 4-propargyloxybenzyl-L-glutamic acid (yielding PPOBLG). The third generation of PAMAM was used as macroinitiator and then functionalized by 6-azidoethylguanidine using azide-alkyne click reaction. The hydrophobic internal cavity of the spherical dendrimer-polypeptide (DPP) was then loaded with photothermal reagent ICG and condensed by siRNA against PKM2 (siPKM2) *via* electrostatic interactions (D-I/P NCs). Human serum albumin (HSA) was further used to coat the final nanoparticle and enhance serum stability and blood circulation (D-I/P@HSA NCs) (Fig. 61).

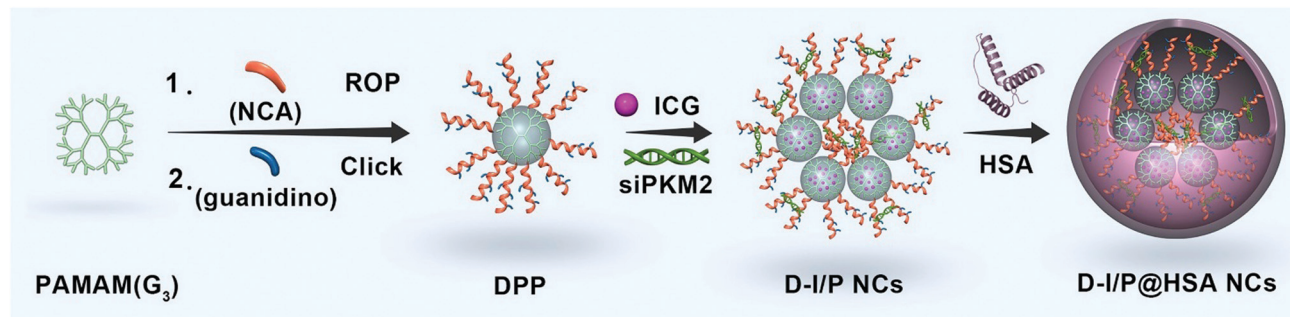


Fig. 61 Illustration of DPP synthesis, ICG loading, siPKM2 complexation, and interaction with HAS to produce final stable D-I/P@HSA NCs. Reproduced with permission from ref. 537. Copyright 2019 Elsevier.

Interestingly, the multivalent DPP exhibited higher membrane activities with lower cytotoxicity than its linear analogue. The tumour glycolysis metabolism was inhibited due to efficient PKM2 silencing in MCF-7 breast adenocarcinoma cells *in vitro* ($\sim 75\%$) and *in vivo* ($\sim 70\%$). Additionally, the energy supply for over-expressed heat shock proteins was depleted to strengthen the ICG-mediated photothermal ablation under NIR light irradiation. Synergistic anti-cancer effect with PTT was also imparted by cancer cell starvation due to siPKM2-mediated energy depletion. This study thus presented the design of a guanidine-rich DPP with multivalency-assisted strategy which has a strong membrane penetrating capability and effective RNAi delivery against tumour glycolysis metabolism to sensitize PTT and strengthen antitumour treatment.⁵³⁷

In another interesting study, a library of membrane penetrating cationic α -helical polypeptides was designed and synthesized to enhance DNA/siRNA delivery.⁵³⁸ A polypeptide with alkyne groups on the side chain terminals was synthesized *via* ROP of γ -propargyl-L-glutamate-based NCA and then guanidinium group and various aromatic groups including benzyl (Bn), naphthyl (Naph), biphenyl (Biph), anthryl (Anth), and pyrenyl (Py) were incorporated onto the side chains *via* the azide-alkyne cycloaddition reaction. An improvement in the siRNA knock down efficiencies *in vivo* and *in vitro* was observed by aromatic functionalization of the polypeptide chains. Polypeptides modified with Bn and Naph were identified as the top-performing materials by increasing the cellular uptake of DNA/siRNA cargos by ~ 4 fold and reducing the endosomal entrapment of gene cargo, resulting in up to 42-fold increase in the gene transfection efficiency. These polypeptides exhibited enhanced transfection efficiencies over PEI 25 kDa by several orders of magnitude. Additionally, they exhibited high TNF- α knockdown efficiencies for treatment of inflammatory disease.⁵³⁸ In another recently published paper, this group used the same polypeptide design to deliver siRNA against RAGE (siRAGE) for the treatment of myocardial ischemia reperfusion (IR) injury.⁵³⁹ The highest gene silencing (RAGE silencing) efficiency with the lowest cytotoxicity in H9C2 rat myoblast cells was observed with Bn-modified polypeptide. *In vivo* studies showed that Bn-modified polypeptide/siRAGE polyplexes exhibited remarkable RAGE knockdown by $\sim 85\%$ after intracardial injection; moreover, cardiac function was consequently recovered after IR

injury by suppressing myocardial inflammation, apoptosis, and fibrosis.⁵³⁹ Incorporation of aromatic domains with the characteristic π -electronic structure and flat-rigid shapes afford strong interactions with biological membranes which serve to enhance the transmembrane delivery efficiency of nucleic acids. Additionally, toxicity associated with membrane pore formation is reduced due to a direct transduction mechanism during cell penetration by aromatic groups. This study provided a promising and effective gene delivery approach by incorporating charged guanidine group and various aromatic groups to manipulate the membrane activities and penetrating mechanisms of polycations.

4.3.3 Complexes formed using designer amino acid residues.

Cheng's group, motivated by the membrane activity of cell penetrating peptides (CPPs), designed and synthesized a fascinating series of α -helical, cationic polypeptides with various side chain amino groups for non-viral gene delivery.^{14,561} The screening results showed that the poly(γ -(4-((2-piperidine-1-yl)ethyl)aminomethyl)benzyl-L-glutamate) (PPABLG, also called PVBLG-8) (Fig. 62A) is the most potent polypeptide in the library and its transfection efficiency is 12-fold higher than the

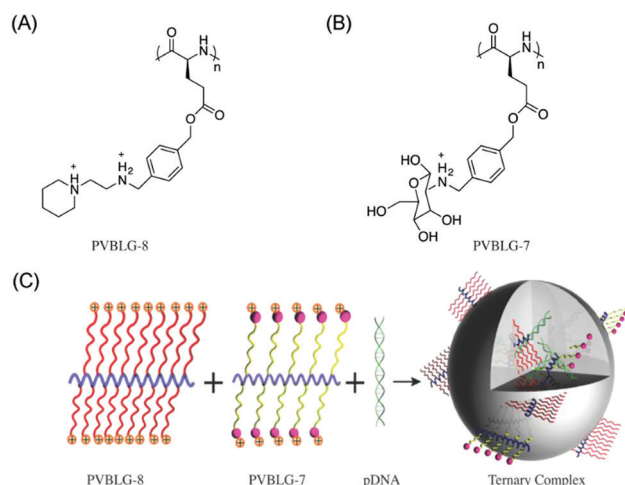


Fig. 62 (A) Chemical structure of PVBLG-8. (B) Chemical structure of PVBLG-7, and (C) schematic illustration of PVBLG-8/PVBLG-7/pDNA complex formation. Reproduced with permission from ref. 541. Copyright 2014 Elsevier.

commercially available transfection agent polyethyleneimine (PEI, 25 kDa). Interestingly, its racemic analogue PPABDLG (or PVBDLG-8) with random coil conformation exhibited negligible transfection efficiency.^{14,540,561} The Cheng group further incorporated various functionalities into PPABLG or PVBLG-8 system for efficient and safe gene delivery. For example, they developed an effective and safe non-viral gene delivery vector consisting of pDNA, a highly membrane-penetrating cationic helical polypeptide (PVBLG-8) (Fig. 62A), and a low-toxicity, membrane-inactive cationic helical polypeptide (poly(γ -glucosamine methyl) benzyl-L-glutamate, PVBLG-7) (Fig. 62B and C) capable of mannose receptor targeting.⁵⁴¹ The cellular uptake level, transfection efficiency, intracellular kinetics, and cytotoxicity of self-assembled PVBLG-8/PVBLG-7/DNA ternary complexes were investigated in a variety of mannose receptor-expressing cell types including HeLa cervical adenocarcinoma cells, COS-7 African green monkey kidney fibroblast-like cells, and RAW 264.7 mouse macrophages. The results were compared with the PVBLG-8/DNA binary complexes and a random copolypeptide PVBLG-8-r-7/DNA binary complex. The results illustrated that individual building blocks PVBLG-8 and PVBLG-7 work synergistically without compromising each other. Additionally, PVBLG-8-r-7 exhibited a decreased transfection efficiency compared to PVBLG-8. By changing the amount of each component, the ideal balance between membrane activity and the targeting efficiency was identified in each cell type in this study, and thus PVBLG-7 enhanced the gene transfection efficiency of PVBLG-8 without inducing additional cytotoxicity.⁵⁴¹

In another study by the same group, PPABLG was modified with various aromatic groups including phenyl, naphthyl, and anthryl (PPABLG-Ar) in order to increase the membrane activity and manipulate the membrane penetration mechanism for pDNA delivery.⁵⁴⁵ Incorporation of aromatic groups into the helical polypeptide design promoted the cell membrane penetration *via* direct membrane transduction, a non-endocytic and non-pore formation mechanism. Additionally, the designed polypeptide structure enhanced the gene delivery, avoided endosomal entrapment, and reduced the toxicity of the materials associated with excessive pore formation. Among the aromatic polypeptides used in this study the polypeptides with naphthyl side chains exhibited higher transfection efficiencies in melanoma cells *in vitro* and *in vivo*.⁵⁴⁵ The same group synthesized a thiolated cationic helical copolypeptide poly((γ -(4-((2-piperidin-1-yl)ethyl)-aminomethyl)benzyl)-L-glutamate)-co-poly(γ -(4-((2-mercaptoethyl)-aminomethyl)benzyl)-L-glutamate) (PPABLG-SH).⁵⁴⁶ Stable polypeptide/siRNA complexes were formed by loose electrostatic interaction between the cationic polypeptide and the negatively charged siRNA and were further stabilized by cross-linking the side chain thiol groups on the side chain terminals. Glutathione inside the cell triggers disulfide bond reduction and thus release of the siRNA from the complex. The polypeptide/siRNA exhibited effective cellular internalization, and endosomal escape.⁵⁴⁶

Recently, the Cheng group also designed a siRNA delivery system based on a cationic, helical cell-penetrating polypeptide PVBLG-8 and an anionic, random-coiled, modified

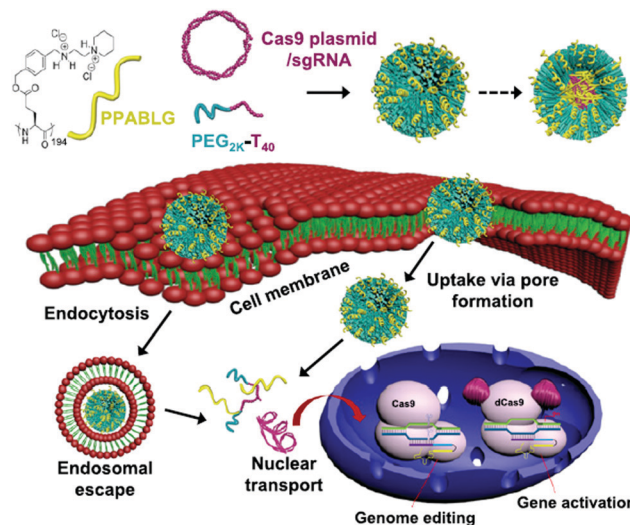


Fig. 63 Schematic presentation of PSPP nanoparticle formation and Cas9 plasmid/sgrRNA delivery for genome editing. Reproduced with permission from ref. 543. *Proc. Natl. Acad. Sci. U. S. A.*, 2018, **115**, 4903–4908.

poly(L-glutamic acid).⁵⁴² The PVBLG-8 was rigid with linear structure and showed weak siRNA condensation capability. PLGlu was used as a stabilizer to form PVBLG-8/siRNA/PLGlu nanoparticles (PSP NPs) with positive charge. Then, an additional amount of PLG was used to change the surface charge of the PSP NPs from positive to negative and form the metastable PVBLG-8/siRNA/PLG@PLGlu (PSPP) NPs. The PSPP NPs exhibited enhanced tumour accumulation due to the desired serum stability during circulation and enhanced permeability and retention.⁵⁴² In another work, PEGylated helical polypeptide nanoparticles were developed for nonviral gene editing *via* CRISPR/Cas9 (Fig. 63).⁵⁴³ α -Helical polypeptide (PPABLG) was used to form nano-complexes with Cas9 expression plasmids and single guide RNA (sgRNAs). PEG-Polythymine₄₀ (PEG-T₄₀) was incorporated into the formulation in order to enhance the extracellular stability and the potential application *in vivo*. The nanoparticles exhibited enhanced transfection efficiency, promoted endosomal escape, and transportation into the nucleus.⁵⁴³

4.3.4 Other designs of polypeptides for nucleic acid delivery.

Song *et al.* synthesized various types of folic acid-functionalized poly(aspartic acid)-grafted chitosan-cyclodextrin conjugates (CCPE-FA) by grafting poly(aspartic acid) (PAsp) to chitosan-cyclodextrin conjugates (CC).⁵⁴⁷ The primary amine groups of ethylenediamine-functionalized CC (CCE) initiated ring opening polymerization of BLA NCA and then were aminolyzed with ethylenediamine to produce CCPE polymers with high transfection and low toxicity. Host-guest interactions were used to prepare FA-functionalized CCPE. Three types of polyplexes of CCPE/pDNA, CCPE-FA/pDNA, and ternary CCPE-FA/CCPE/pDNA were prepared and their gene delivery efficiency using different cell lines was investigated. The ternary CCPE-FA/CCPE/pDNA polyplexes exhibited excellent gene transfection abilities in the folate receptor-positive tumour cells.⁵⁴⁷ Recently, the same group designed a novel ABA-triblock hydroxyl-rich cationic polyrotaxane PP-PGEA for nucleic acid delivery.⁵⁴⁸

The polymers consist of poly(ethylene glycol) backbone, one cyclodextrin (CD) core and three ethanolamine (EA)-functionalized poly(glycidyl methacrylate) arms (CD-PGEA), and degradable end capping poly(aspartic acid) polypeptide segments. The PP-PGEA polymers exhibited an excellent ability to condense plasmids such as the tumour-suppressor gene p53 and formed nanoparticles with low toxicity and degradability.⁵⁴⁸ Various other polymers have been also synthesized using NCA ROP and modified with different groups for gene delivery applications.^{562–565}

4.3.5 Nucleic acid delivery using polypeptides in the tissue engineering field. Polypeptides synthesized by NCA ROP can be also used as gene delivery vectors in tissue regeneration.^{549,550} Recently, Walsh *et al.* incorporated gene loaded star-shaped PLLs in collagen scaffolds to control gene delivery to host cells and investigated the regenerative capacity of the scaffolds for tissue engineering applications.⁵⁴⁹ Two star-PLLs vectors, 32-arm star-PLLs and 64-arm star-PLLs with 40 and 5 repeat units per arm, respectively, were synthesized and formed complexes with pDNA. The complexes were incorporated in the collagen scaffolds for *in vitro* and *in vivo* gene delivery. *In vitro* results demonstrated that 64-arm star-PLLs is capable of increasing gene expression in mesenchymal stem cells (MSCs) 44-fold over 32 arm star-PLLs, 2-fold over PEI, and 130-fold over linear poly(lysine). *In vivo*, star-PLLs-pDNA activated collagen scaffolds also exhibited extensive autologous host cell infiltration, nanomedicine retention, and transgene expression at the early time point of just seven days. These results illustrated the ability of star-PLLs incorporated in collagen scaffolds to transfect the autologous host cells *in vivo*.⁵⁴⁹

4.3.6 Future perspectives. To conclude, synthetic polypeptides prepared by NCA ROP have shown great potential as non-viral gene delivery vectors. These protein-mimetic polymers have excellent biodegradability, biocompatibility, versatile functionality, and the capability to adopt higher ordered structures. In this section, we reviewed how scientists were able to design polypeptides to achieve high transfection efficiency and high gene delivery with reduced endosomal entrapment and cytotoxicity.

Poly(L-lysine), a polypeptide with primary amine groups has been utilized widely for non-viral nucleic acid delivery because of its high affinity to form complexes with genetic materials. However, high toxicity, low transfection efficiency, and a low endosomal escape ability have limited its application. Researchers have controlled the charge density and the molecular weight, as well as tuned the hydrophilicity and hydrophobicity of PLLs by incorporating segments such as PEG, poly(HEMA), polysarcosine, aromatic structures, hydrophobic side chains, α -helicity, cell selective peptides, or a combination of these. These modifications created polypeptides with various architectures such as linear block copolypeptides or star polypeptides with improved transfection efficiency and reduced toxicity. The use of poly(L-arginine) or polypeptides with guanidine functional groups also reduced cytotoxicity and enhanced transfection efficiency due to improved endosomal escape ability. Cheng's group designed and synthesized α -helical and cationic polypeptides of PPABLG/PVBGL-8 using NCA ROP with high transfection efficiency as gene delivery vectors. They further

incorporated various functionalities such as glucosamine or aromatic groups into PPABLG/PVBGL-8 to improve transfection efficiency and endosomal escape with low cytotoxicity. NCA ROP enabled researchers to synthesize polypeptides with diverse amino acids and functionalities to improve targeting, cell penetration, and endosomal escape. However, the preparation of polypeptide-based materials still has some limitations due to the lack of sequence control and monodispersity. The type, ratio, amount, and location of the functional groups in the polymers also affects molecular weight, charge density, and hydrophilic/hydrophobic balance and subsequently impacts on the gene delivery ability of the polypeptide.

Additionally, although extensive work has been conducted to develop polypeptide-based systems for non-viral nucleic acid delivery, the challenges of the past decade still remain prevalent. Achieving both high transfection efficiency and low cytotoxicity simultaneously still limit the possible use of the current systems *in vivo*. High transfection and endosomal escape are the most important limiting factors for any delivery system. The future polypeptide-based gene delivery systems should be designed in a way to overcome these important obstacles by promoting cellular uptake of the polyplex through non-endocytic pathways while also keeping cytotoxicity low, for example through pore formation mechanism on the cell membrane. Another important issue for delivery systems is targeted delivery *in vivo*. Accumulation of delivery vehicles in the renal clearance organs reduces delivery efficiency to the target organs. Future design of polypeptides for nucleic acid delivery could focus more on the systems which enhance cellular targeting, for example by incorporation of cell specific peptides on the outer surface of the particle. Therefore, further research is necessary to obtain a deep and comprehensive understanding of the obstacles in nucleic acid delivery. This will allow the scientific community to manipulate and design intelligent functional polypeptides and hybrid carriers with the proper molecular architecture and secondary structure for clinical applications.

4.4 Tissue engineering

Biological functionality afforded by synthetic polypeptides extends to the field of tissue engineering.^{274,566,567} Tissue engineering relies on the use of engineered matrices as a scaffold to mimic the extracellular matrix (ECM) produced by host cells, providing physical support and biochemical cues for cells to attach to and proliferate, facilitating cellular regeneration, with the ultimate goal of synthesizing organ and tissue replacements. The majority of tissue engineering scaffolds are hydrogels due to their inherent ability to retain large amounts of water and mimic the mechanical properties of the natural ECM.¹⁸ However, microcarriers have also been used as scaffolds as they can easily be seeded with host cells before introduction into the body, where they assist with successful cell integration into the host.^{568,569} Research into new biomaterials focuses on the induction of different signalling cues to modulate cell fate and behavior. Polypeptide-based materials are a natural option as a scaffold mimetic since many of the natural ECM

components are proteins including collagen and proteoglycans. Within this class of biomaterials, NCA ROP offers the ability to create a wide variety of polypeptides with tunable properties and functionalities. Table 5 summarizes the recent studies which have shown desirable properties for tissue engineering. It should be noted that while the mechanical properties of the scaffold are important in determining cell fate (*i.e.* cell differentiation), these are not explicitly described in this review article as they are dependent on a variety of factors such as crosslinking density and chemical content^{566,567} which have been eloquently covered in the recent review on hydrogels by Zhou and Li.¹⁸

The wide range of amino acids developed for NCA ROP, combined with the large tool-kit of post-polymerization chemistries, enables the rapid modification of polypeptides as well as the tuning of their material properties to direct specific cellular interactions. Cell adhesion and proliferation can be promoted through the use of ionic residues which subsequently facilitate protein adsorption.^{330,578} These amino acid residues also provide functional handles for the introduction of signalling molecules or motifs such as the RGD sequence, which are well known to signal cell adhesion.^{333,337} Introduction of certain molecules in polypeptide-based scaffolds can aid to achieve the desired level of cellular regeneration and growth in cartilage and bone tissue.^{579–581} Despite the lack of sequence control exhibited by NCA ROP which affects the polypeptide's breakdown by native enzymes,⁵⁸² the integration of enzyme-identifiable sequences or easily cleavable bonds (*i.e.* disulfide bonds) is often pursued in polypeptide design to tune the degradation rate of scaffolds or cell behaviors on hydrogels.^{329,337}

Furthermore, hydrogels for tissue engineering can be designed such that their macrostructure is consolidated *in situ* or *in vitro*, to suit the application. The responsiveness of polypeptides toward environmental changes²⁷⁴ endows many polypeptide-based hydrogels with injectability.⁵⁸³ This responsiveness reduces the need for invasive surgical procedures in many cases; nonetheless, implantable scaffolds are still employed in instances where intricate scaffold geometries are necessary.¹⁸ The growing interest in personalized healthcare has led to early investigations of 3D printing for the manufacture of complex polypeptide-based scaffolds.^{317,571,572}

4.4.1 Tunable cellular interactions. The facilitation of cell adhesion and proliferation are both fundamental requirements for a tissue engineering scaffold.⁵⁶⁶ Introduction of synthetic polypeptides into said scaffolds is desirable as polypeptides obtained *via* NCA ROP can be designed using specific α -amino acid NCA monomers which will assist in cell adhesion and proliferation.^{18,274}

The introduction of polypeptide segments with ionic side chains—particularly PLLys (cationic) and PLGlu (anionic)—allows for cell adhesion without any side chain modification. Zhang *et al.* demonstrated this with the inclusion of PLLys in their poly(lactic acid-co-glycolic acid) microcarriers, resulting in improved cell adhesion.⁵⁷⁸ The use of ionic polypeptide side chains in scaffolds was also demonstrated in the P(LLys-co-LGlu)-based cryogels synthesized by the Qiao group which guided

adhesion and proliferation without any further modification.³³⁰ Seeded 3T3 fibroblast cells were found to form cell clusters after two days, as shown in Fig. 64, indicating protein adsorption to the scaffold due to the electrostatic interaction between proteins and peptides, and successful adhesion of the cells. Subsequent cell proliferation was evident after 14 days with the measured number of viable cells exhibiting a steady increase. The combined results of these studies demonstrate the potential of NCA ROP to create viable scaffolds without the necessity for further modification.

Further chemical modification can be nonetheless sought to direct cellular behaviours such as cell adhesion in scaffold materials. One such modification includes in the introduction of RGD – a peptide sequence which signals for cell adhesion.⁵⁸⁴ Ahrens *et al.* used a PLGlu-based scaffold where each glutamic acid side chain was modified to yield three different side chain groups: an inert PEG chain, a maleimide group and a norbornene group.³³³ The hydrogel was crosslinked *via* thiol-ene reactions between a 4-arm PEG-thiol and the maleimide and the norbornene side chains. Since the number of maleimide side chains remained in excess of the crosslinker, unreacted maleimide side chains provided a functional handle for the coupling to the terminal cysteine residue of an RGD peptide *via* thiol-maleimide click chemistry. These scaffolds exhibited a four-fold increase in the adhesion of human mesenchymal stem cells compared to a purely PEG-based control also possessing RGD. The increased adhesion was attributed to the modified PLGlu and subsequent ordering into α -helical conformations not present in the controls. To introduce further regulation of cell proliferation, Xu *et al.* attached the RGD sequence to their PLGlu-based hydrogel through a redox sensitive disulfide bond.³³⁷ Compared to a control lacking RGD, 3T3 fibroblasts were found to spread and proliferate at a greater rate as expected. *In vitro* testing showed that the disulfide bonds could be cleaved by addition of GSH, resulting in the detachment of RGD and associated cells from the scaffold. The redox responsivity thus granted the scaffold the ability to adapt to an extra cue that most other scaffolds would not be able to, demonstrating an ingenious strategy to control cell adhesion and proliferation.

Besides cellular interactions, biomaterial degradation is an especially important factor for tissue engineering as the ultimate goal of this application is to allow the body to produce its own ECM.⁵⁶⁷ Biomaterials should degrade at a rate which matches the rate of ECM production and cell growth of the target tissue, in order to allow implanted or native cells sufficient time to populate the space prior to degradation. Pore size and flexibility – both affected by crosslinking density – play a key role in the degradation rate of hydrogels.^{330,332} Pores of greater size and flexibility allow enzymes and other degrading molecules to interact with the polymeric backbone walls more readily, leading to a faster breakdown.³³⁰ Additionally, networks can be designed to contain functional groups or bonds with known degradability under specific conditions. Short amino acid sequences identified and cleaved by host enzymes (for example, matrix metallo-peptidases) represent one option which is yet to be explored thoroughly in polypeptide hydrogels.³²⁹ The use of redox-sensitive disulfide bonds also provides the ability to introduce labile crosslinks and tailor degradation.³²⁹ Depending on the

Table 5 Summary of different scaffolds with demonstrated tissue engineering potential

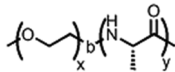
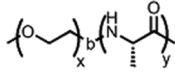
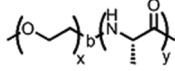
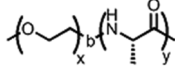
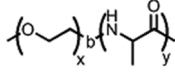
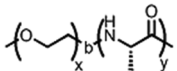
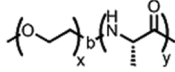
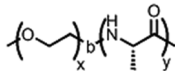
Scaffold	Gelation	Mechanical stiffness	Features	Ref.
PEG-<i>b</i>-PLA1a 	Thermal increase (to 37 °C)	Storage modulus up to 1 kPa	Cell proliferation with graphene oxide inclusion Injectability <i>via</i> thermal crosslinking Differentiation of tonsil-derived mesenchymal stem cells into adipocytes	306
PEG-<i>b</i>-PLA1a 	Thermal increase (to 37 °C)	Storage modulus up to 1 kPa	Facilitate cell proliferation Improved stiffness with Fe(III) ions Payload release of Fe(III) ions Differentiation of tonsil-derived mesenchymal stem cells into nerve cells	298
PEG-<i>b</i>-PLA1a 	Thermal increase (to 37 °C)	Storage modulus up to 1 kPa	Discrete microspheres with discrete polystyrene microspheres Differentiation of tonsil-derived mesenchymal stem cells into adipocytes, osteocytes and chondrocytes	570
PEG-<i>b</i>-PLA1a 	Thermal increase (to 37 °C)	Storage modulus up to 8 kPa	Injectability <i>via</i> thermal crosslinking Comparative differentiation study against commercial Differentiation of bone-marrow-derived mesenchymal stem cells into myocytes	295
PEG-<i>b</i>-PA1a 	Thermal increase (to 18–27 °C)	Storage modulus up to 10 kPa	Injectability <i>via</i> thermal crosslinking Biocompatibility Controllable inflammatory response based on monomer chirality	297
PEG-<i>b</i>-PLA1a within an electrospun silk fibroin mat 	Thermal increase (to 37 °C)	Storage modulus up to 100 kPa	Mimicry of a nerve conduit <i>via</i> spider silk tube Biocompatibility <i>In vivo</i> degradation	292
PEG-<i>b</i>-PLA1a 	Thermal increase (to 37 °C)	Storage modulus up to 3 kPa	Injectability <i>via</i> thermal crosslinking <i>In vivo</i> degradation and histological compatibility Payload release of recombinant human growth hormone <i>in vivo</i>	294
PEG-<i>b</i>-PLA1a 	Thermal increase (to 37 °C)	Storage modulus up to 700 Pa	Cell proliferation with graphene oxide inclusion Differentiation of tonsil-derived mesenchymal stem cells into chondrocytes	293

Table 5 (continued)

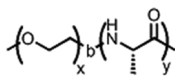
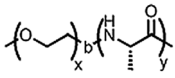
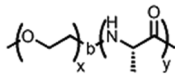
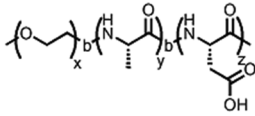
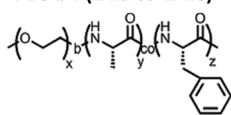
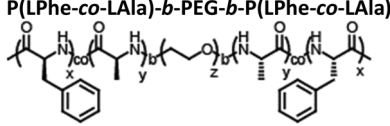
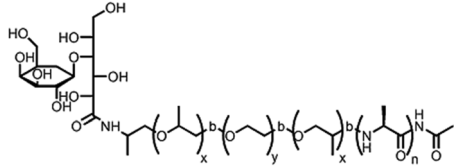
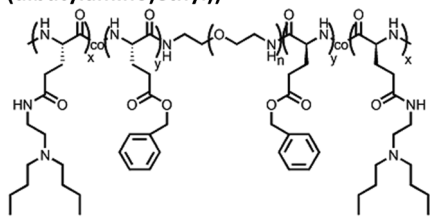
Scaffold	Gelation	Mechanical stiffness	Features	Ref.
PEG-<i>b</i>-PLA₁ 	Thermal increase (to 18–27 °C)	Storage modulus up to 1 kPa	Injectability <i>via</i> thermal crosslinking Hepatogenic differentiation due to loading of tauroursodeoxycholic acid (TUDCA), hepatocyte growth factor (HGF), and fibroblast growth factor 4 (FGF4)	305
PEG-<i>b</i>-PLA₁ with magnetic or hydroxyapatite nanoparticles dispersed within 	Thermal increase (to 37 °C)	N/A	Nanocomposite material Cell biocompatibility and <i>in vitro</i> cell proliferation Capable of magnetically inducing increased ECM secretion and proliferation	290
PEG-<i>b</i>-PLA₁ 	Thermal increase (to 30 °C)	Storage modulus up to 1 kPa	Injectability <i>via</i> thermal crosslinking Chondrocyte cell culture	301
PEG-<i>b</i>-PLA₁-<i>b</i>-PLAsp 	Thermal increase (to 30–40 °C)	Storage modulus up to 1 kPa	Cell proliferation with RGD-coated layered double hydroxide inclusion Payload release of kartogenin	302
PEG-<i>b</i>-P(LA₁-co-LPhe) 	Thermal increase (to 30–40 °C)	Storage modulus up to 2 kPa	Differentiation of tonsil-derived mesenchymal stem cells into adipocytes, osteocytes and chondrocytes	304
P(LPhe-co-LA₁)-<i>b</i>-PEG-<i>b</i>-P(LPhe-co-LA₁) 	Thermal increase (to 30–40 °C)	Storage modulus up to 5 kPa	Injectability <i>via</i> thermal crosslinking Tunable pore size and mechanical strength <i>via</i> adjustment of phenylalanine content <i>In vivo</i> regeneration of cartilage tissue using hydrogels with bone marrow mesenchymal stem cells encapsulated within at osteochondrol defect	296
PLX-PLA₁ with a lactobionic acid end-group 	Thermal increase (to 18 °C)	Storage modulus up to 10 kPa	Facilitate cell proliferation Improved hepatogenic differentiation with inclusion of actobionic acid	303
P(BLG-co-BLG-g-(2-(dibutylamino)ethyl))-<i>b</i>-PEG-<i>b</i>-P(BLG-co-BLG-g-(2-(dibutylamino)ethyl)) 	Thermal increase (to physiological temperature at pH 7.4)	N/A	Injectability <i>via in vivo</i> crosslinking <i>In vitro</i> biocompatibility <i>In vivo</i> testing and degradation <i>In vivo</i> payload delivery of human growth hormone	282

Table 5 (continued)

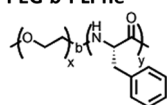
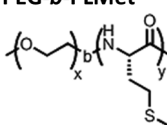
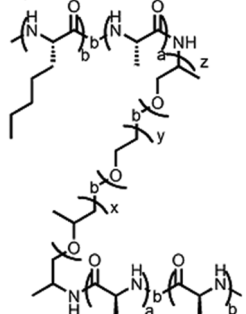
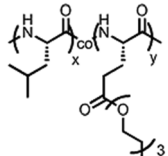
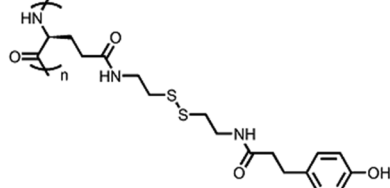
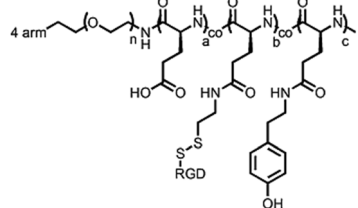
Scaffold	Gelation	Mechanical stiffness	Features	Ref.
PEG-<i>b</i>-PLPhe 	Thermal increase (to 37 °C)	Storage modulus up to 1 kPa	Suspension of different mesocrystals and nanocrystals Differentiation of tonsil-derived mesenchymal stem cells into osteocytes (improved with mesocrystals)	291
PEG-<i>b</i>-PLMet 	Thermal increase (to ~30 °C)	Storage modulus up to 4 kPa	Degradation <i>via</i> hydrogen peroxide Injectability <i>via</i> thermal crosslinking Cell viability	286
PLLys-<i>b</i>-PLAAla-<i>b</i>-PLX-<i>b</i>-PLAAla-<i>b</i>-PLLys 	Thermal increase (to 30–40 °C)	N/A	Injectability <i>via</i> thermal crosslinking Cell viability	300
P(LLeu-co-LGlu) with PLGlu side chains modified with oligoethylene glycol 	Thermal increase (to ~37 °C)	N/A	Neural stem cell viability <i>In vivo</i> transplantation Histological testing	285
PLGlu grafted with phloretic acid via disulfide bond 	Enzyme mediated crosslinking of phenol	Storage modulus up to 10 kPa	Cell viability <i>In vivo</i> testing and degradation Injectability <i>via in vivo</i> crosslinking	329
4 arm stars: PEG-<i>b</i>-PLGlu grafted with tyramine and disulfide bond linked RGD 	Enzyme mediated crosslinking of phenol	Storage modulus up to 2 kPa	Injectability <i>via in vivo</i> crosslinking RGD modified with cleavable disulfide bond allowing potential cell release	337

Table 5 (continued)

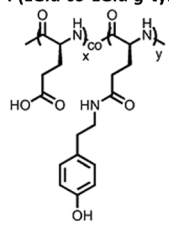
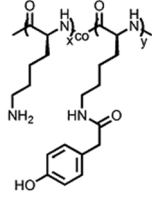
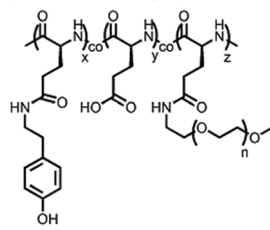
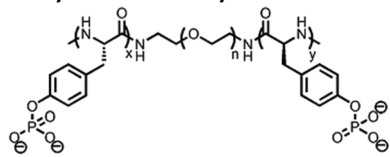
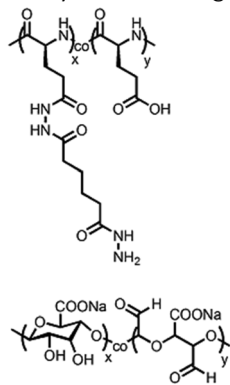
Scaffold	Gelation	Mechanical stiffness	Features	Ref.
P(LGlu-co-LGlu-g-tyramine) 	Enzyme mediated crosslinking of phenol	Storage modulus up to 20 kPa	<i>In vivo</i> testing and degradation Injectability <i>via in vivo</i> crosslinking Differentiation of bone-marrow-derived mesenchymal stem cells into chondrocytes	328
P(LLys-co-LLys-g-4-hydroxyphenylacetic acid) 	Enzyme mediated crosslinking of phenol	Storage modulus up to 20 kPa	Cell viability	334
P(LGlu-co-LGlu-g-tyramine-co-LGlu-g-PEG) 	Enzyme mediated crosslinking of phenol	Storage modulus up to 20 kPa	<i>In vivo</i> testing and degradation Injectability <i>via in vivo</i> crosslinking	338
PPLTyr-b-PEG-b-PPLTyr 	Enzyme mediated crosslinking of phenols and dephosphorylation	Storage modulus up to 1.5 kPa	Cell viability of PPLTyr prior to hydrogelation	69
P(LGlu-co-LGlu-g-hydrazide) and aldehyde-modified alginate 	Hydrazide-aldehyde coupling	Storage modulus up to 12 kPa	<i>In vivo</i> testing Injection <i>via</i> separate polymer injection Degradation in PBS	332

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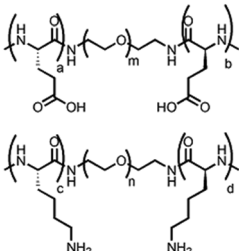
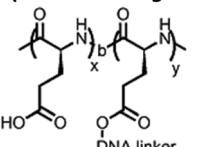
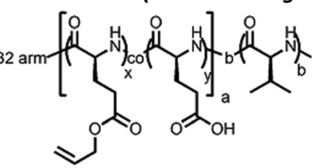
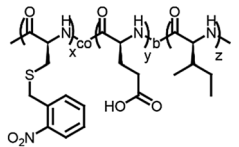
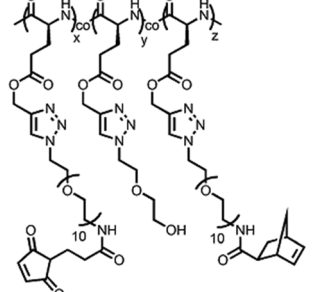
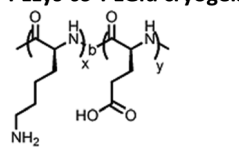
Scaffold	Gelation	Mechanical stiffness	Features	Ref.
PLGlu-<i>b</i>-PEG-<i>b</i>-PLGlu and PLLys-<i>b</i>-PEG-<i>b</i>-PLLys 	Ionic association	Storage modulus up to 10 kPa	pH dependent storage modulus <i>In vivo</i> testing	280
P(LGlu-<i>co</i>-LGlu-<i>g</i>-DNA) 	DNA linker	Storage modulus up to 6 kPa	Cell viability Two-ink 3D bioprinting	317
32 arm stars: P(LGlu-<i>co</i>-LGlu-<i>g</i>-allyl-<i>b</i>-LVal) 	Spontaneous formation during extrusion and UV crosslinking with <i>N,N'</i> -methylenebis-(acrylamide)	Storage modulus up to 3 kPa	Cell viability Degradation studies Drug loading with doxorubicin 3D printing	571
P(NBLCys-<i>co</i>-LGlu-<i>b</i>-Llle) 	Spontaneous formation during extrusion and UV crosslinking	Storage modulus up to 800 Pa	Cell viability 3D printing	572
P(LGlu-<i>g</i>-azide functionalized-<i>co</i>-LGlu-<i>g</i>-maleimide-<i>co</i>-LGlu-<i>g</i>-norbornene) 	4 arm PEG thiol	Elastic modulus of 3–17 kPa	RGD modified <i>via</i> thiol	333
PLLys-<i>co</i>-PLGlu cryogels 	Carbodiimide coupling of residue side chains	Compressive strength of ~35 kPa	Degradable by protease XIV Allows cell adhesion and proliferation <i>via</i> ionic residues	330

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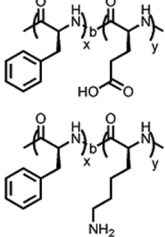
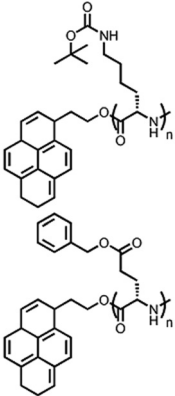
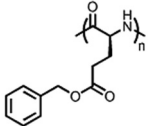
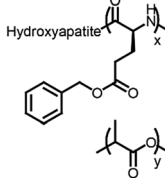
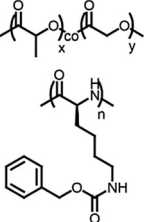
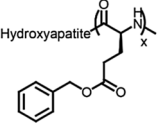
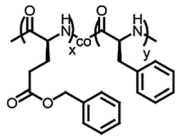
Scaffold	Gelation	Mechanical stiffness	Features	Ref.
Crosslinked P(LPhe-<i>b</i>-LGlu) and P(LPhe-<i>b</i>-LLys) micelles 	Genipin crosslinking	Storage modulus of 100 kPa	Drug loading with curcumin and amphotericin B Histological testing	573
PBLlys and PBLG grafted from reduced Glaisen graphene network 	Hot pressing	Compressive modulus up to 40 MPa	<i>In vitro</i> biocompatibility and cell proliferation	574
PBLG microcarriers 	Physical entanglement (discrete particles)	N/A	Microcarriers Tunable degradation in PBS buffer Seeding of chondrocytes <i>and in vivo</i> testing of chondrogenesis Histological analysis	568
Hydroxyapatite-<i>b</i>-PBLG suspended within PLLA scaffold 	Lyophilization of matrix	N/A	Nanocomposite material <i>In vitro</i> testing for cell proliferation <i>In vivo</i> testing and bone repair	575
High molecular weight PZLL, poly(lactic-co-glycolic acid) and bioglass 	Physical entanglement	N/A	Nanocomposite material <i>In vitro</i> cell testing and protein adsorption capabilities <i>In vivo</i> testing Histological testing Biomineralisation of scaffolds	576

Table 5 (continued)

Scaffold	Gelation	Mechanical stiffness	Features	Ref.
Strontium-substituted hydroxyapatite with PBLG grafts 	Physical entanglement (discrete particles)	Storage modulus up to 800 Pa	Microcarriers Tunable degradation in PBS buffer Ion carrier and release <i>In vivo</i> testing and bone repairation Histological analysis	569
P(BLG-co-LPhe) with bioglass dispersed within 	Physical entanglement	N/A	Nanocomposite material <i>In vitro</i> cell testing and protein adsorption capabilities Ion carrier and release <i>In vivo</i> testing Histological testing	577

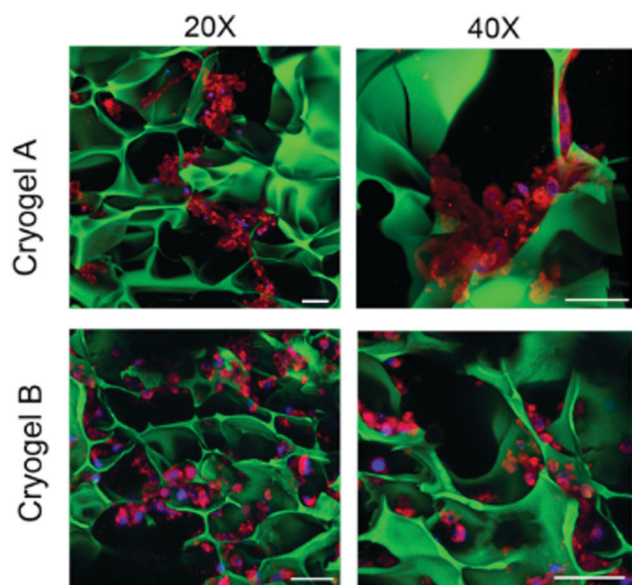


Fig. 64 CLSM imaging of cryogel scaffolds (cryogel A consisting of a 4 : 1 ratio of glutamic acid to lysine residues and cryogel B consisting of a 1 : 1 ratio of Glu to Lys residues). Scaffold walls shown in green from FITC tag, while cells and cell nuclei are stained red (Deep Red stain) and blue (DAPI), respectively. All scale bars represent 50 μ m. Reproduced with permission from *Biomacromolecules*, 2016, **17**(9), 2981. Copyright 2016 American Chemical Society.

concentration of reducing agent (*i.e.* GSH) these sites can cleave within a matter of minutes,³³⁷ hence hydrogels incorporating disulfide bonds may require the use of a robust primary cross-linking strategy to fully control the speed at which degradation occurs and to maintain or tune the mechanical strength of the scaffold.⁵⁸² As an example, Chen and colleagues examined the properties of poly(glutamic acid) hydrogels grafted with tyramine (PLGlu-*g*-TA) or cystamine-conjugated phloretic acid (PLGlu-*g*-CPA) (and their mixtures) as scaffolds for 3D cell

culture (Fig. 65A and B).³²⁹ Both kinds of hydrogels were enzymatically crosslinked by horseradish peroxidase in the presence of exogenous hydrogen peroxide, and displayed desirable cell encapsulation. Their degradations *in vitro* and *in vivo* were markedly different however. *In vitro* experiments demonstrated the PLGu-*g*-CPA hydrogel was susceptible to GSH and rapidly degraded, whereas PLGlu-*g*-TA remained stable under the same treatment conditions. Additionally, both types of hydrogel could be degraded by elastase *in vitro*, with PLGlu-CPA still exhibiting a more rapid degradation profile. *In vivo* degradation testing of the two polypeptide hydrogels demonstrated that the disulfide-containing hydrogel degraded more rapidly than PLGlu-*g*-TA (12 vs. 35 days) even at low physiological GSH concentrations (Fig. 65C and D).

4.4.2 Injectable hydrogels. In addition to the favourable properties of polypeptides relating to biocompatibility and biofunctionality, the polypeptides' ability to undergo environmentally-triggered changes *in vivo* has led to their implementation as injectable hydrogels for tissue engineering.⁵⁸³ Injectable hydrogels allow the facile handling of liquid solutions and the insertion of scaffolds at a target site with reduced invasive procedures, with *in vivo* consolidation of their macrostructure allowing them to mimic the required scaffold geometry at the site of injection.⁵⁸³ The majority of injectable synthetic polypeptide hydrogels can be subdivided into two categories – thermogels (hydrogels which undergo thermally-triggered crosslinking)^{294–297} and enzyme-mediated crosslinked hydrogels.^{328,329,334} While these two categories represent the majority of injectable synthetic polypeptides, examples of multi-solution systems exist where crosslinking occurs upon the mixing of two solutions.^{280,332}

4.4.2.1 Thermogels. Thermogels are designed to remain in solution state at cold temperatures before undergoing sol-gel transition at physiological temperature.⁵⁸³ In general, polypeptide thermogels are designed with blocks which undergo

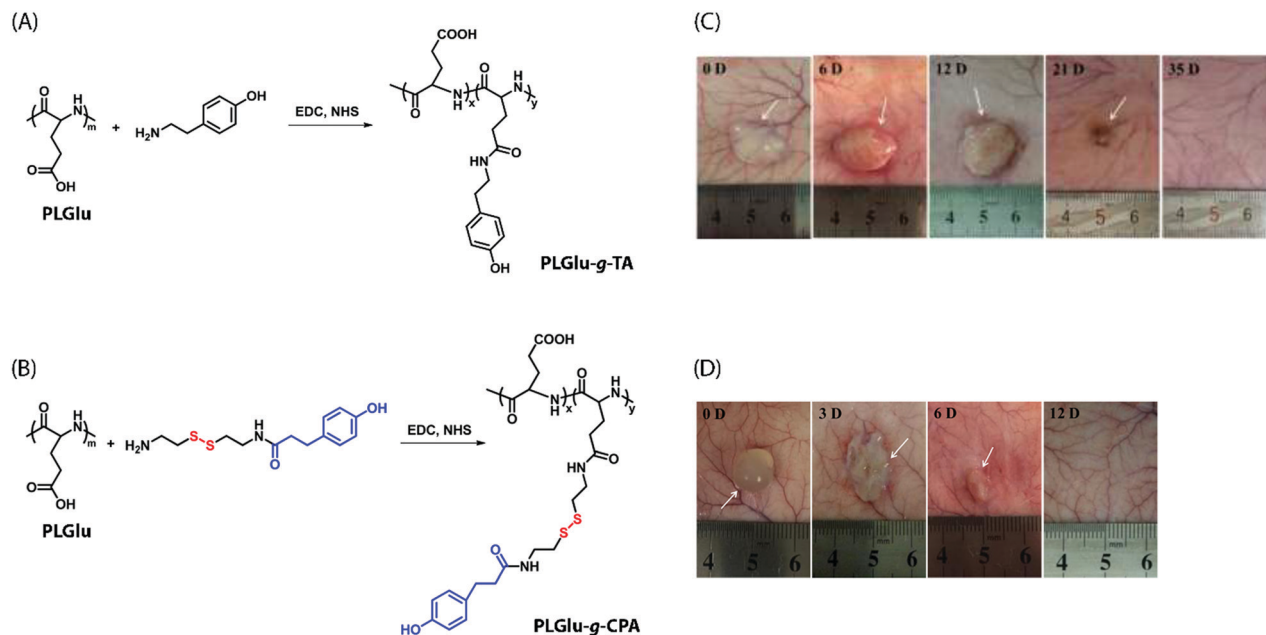


Fig. 65 The degradation profile of injectable hydrogels can be controlled through the inclusion of redox-sensitive disulphide bonds. Schematic representations of the coupling between PLGlu and tyramine (A) or cystamine-conjugated phloretic acid (B). Visual status of the gradual degradation of PLGlu-g-TA (C) or PLGlu-g-CPA (D) hydrogels after injection into the subcutaneous tissue of rats.³²⁹ Adapted with permission from *ACS Appl. Mater. Interfaces*, 2016, **8**(45), 30692. Copyright 2016 American Chemical Society.

transition from amorphous secondary structures to more rigid structures such as β -sheets at physiological temperature. For more information on the application and design of injectable thermogels in general, we refer readers to the review released by the Jeong group.⁵⁸³ Overall, the propensity towards β -sheet formation and subsequent cellular response are strong factors to consider in the design of thermally sensitive polypeptide hydrogels.

Amongst the reported polypeptide thermogels, poly(L-alanine) blocks are the most studied thermally sensitive blocks.^{294,295,297} Studies generally show that such hydrogels are able to undergo *in vivo* sol-gel transition under desirable physiological conditions (at 3.0–10.0 wt% in the temperature range of 30–40 °C). It is worth noting that the chirality of the alanine residues in these

hydrogel blocks and their subsequent propensity toward ordered secondary structure plays an important role in both the mechanical properties of the scaffold and host immune response. The Chen group demonstrated that the storage and loss moduli of PEG-*b*-PAla gels was largely unaffected by chirality as long as a single enantiomer was employed; in contrast, a racemic mixture yielded a less stiff gel.²⁹⁷ In addition, histological studies revealed hydrogels containing blocks of L-alanine led to low production of inflammatory markers in the subcutaneous tissue around the site of injection (Fig. 66).

In contrast, racemic analogues led to higher levels of inflammation, and exclusively D-alanine hydrogels elicited the highest immunogenic response. Overall, this study provides insight as to how to regulate levels of inflammatory response

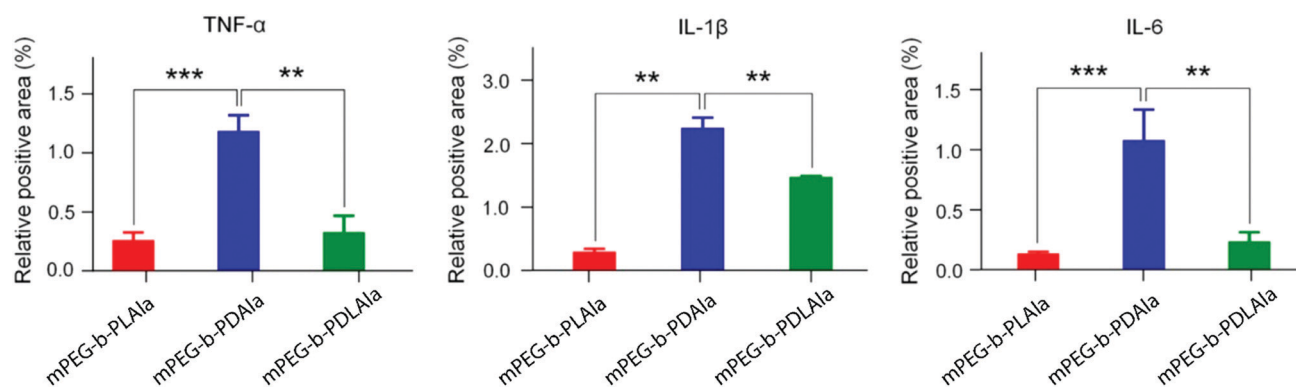


Fig. 66 Semiquantitative analyses of histological tests with PEG-PAla hydrogels with different stereosequence in the PAla block, determining production of tumour necrosis factor (TNF- α), interleukin-1 β (IL-1 β) and interleukin-6 (IL-6). Adapted with permission from *ACS Appl. Mater. Interfaces*, 2019, **11**(9), 8725. Copyright 2019 American Chemical Society.

in vivo and opens up potential consideration of hydrogels as adjuvants. Another study by Chen and co-workers reported a system with a far lower gelation temperature in the range of 16–33 °C.²⁸⁶ The lower gelation temperature of the PEG-*b*-poly(L-methionine) (PLMet) hydrogels can be attributed to methionine's greater propensity towards β -sheet formation. While advantageous in ensuring gelation occurs at body temperature, a trade-off of these hydrogel systems is the shorter handling time, risking *ex vivo* gelation. The PLMet hydrogels, interestingly, could act as a cytoprotective scaffold, shielding cells from the presence of hydrogen peroxide by sacrificially reacting with it.

4.4.2.2 Enzyme-mediated crosslinked gels. While synthetic polypeptidic thermogels are well-established, *in vivo* enzyme-mediated covalent crosslinking of polypeptide phenol side chains provides an interesting and less explored alternative to produce injectable gels.^{328,329,334} The primary method of enzymatic crosslinking is *via* peroxidase-mediated crosslinking of phenol side chains, using hydrogen peroxide as the substrate (Fig. 67).⁵⁸⁵ Briefly, this crosslinking is facilitated by proton abstraction of the hydroxyl group on the phenol side chain. This leads to radical formation and recombination, resulting in the covalent bonding of two phenol groups. Horseradish peroxidase is used to ensure enzymatic crosslinking *in vitro* before proceeding to any *in vivo* testing, where it is added to the polypeptide precursor solutions alongside H₂O₂. While knowledge of this chemistry indicates that introduction of PLTyr into polypeptide hydrogel scaffolds would be desirable, practically, PLTyr blocks are difficult to manipulate due to their high propensity toward β -sheet formation.⁵⁸⁶ Instead, numerous studies couple phenol-bearing molecules with primary amine functionality to a glutamic acid residue using carbodiimide chemistry post-polymerization.^{328,329,334,337,338} The Chen group has used tyramine conjugated to PLGlu, with gelation occurring rapidly after injection into rats.³²⁸

In addition to horseradish peroxidase, other enzymes native to the human body⁵⁸⁷ could also be exploited in the future for the successful design and introduction of complex scaffolds.⁶⁹ Although not investigated directly for tissue engineering applications, the Lu group was inspired by alkaline phosphatase (ALP) – an enzyme found primarily in the liver, placenta, kidney and bone – alongside horseradish peroxidase to induce gelation.⁶⁹ Biocompatible triblock copolymers containing a PEG_{5kDa} middle block and large poly(L-phosphotyrosine) (PPLTyr) outer blocks were obtained after a deprotection step. The PPLTyr blocks were susceptible to dephosphorylation by alkaline phosphatase, resulting in poly(L-tyrosine) blocks with a high propensity toward β -sheet formation and hence gelation. Hydrogel formation

occurred at 8 wt% in solution with 10 U μL^{-1} alkaline phosphatase in a Tris-HCl buffer (pH $\sim$ 8). The dual addition of ALP and HRP helped to further increase the storage moduli and the rate of gelation (1.5 h *versus* overnight) through the aforementioned crosslinking of phenolic side chains. Although this study focused only on hydrogel synthesis, it is easy to envision how the approach could be adopted in a range of other works for tissue engineering purposes.

4.4.2.3 Two-polymer systems. While the aforementioned methods of introducing injectability are by far the most common for synthetic polypeptide-based hydrogels, other studies have shown potential for injectability using systems where crosslinking is effectuated between two polymers initially prepared into two separate solutions.³³² Yan *et al.* investigated a hydrogel formulation consisting of two different polymer solutions which cross-linked upon mixing – one solution contained hydrazide modified PLGlu and another solution contained oxidized alginate.³³² The action by sodium periodate resulted in the breakdown of monomer units within the alginate backbone and revealed free aldehyde groups which were subsequently available for hydrazide-aldehyde coupling, leading to gelation.³³² Gelation occurred within 8 minutes for a 1:1 mixture at concentrations above 4 wt%. This rapid gelation time hence ensures gelation at the desired site as opposed to substance being lost to molecular diffusion upon introduction into the host.

4.4.3 Implantable hydrogels. While injectable hydrogels require minimal invasive procedures for introduction into a target site, their design must be tailored to conform to specific rheological requirements, and as a result may be unable to yield other desired features.⁵⁸³ For example, the specific macroporous structure yielded by cryogels requires *ex vivo* synthetic methods.^{330,588} Amongst the implantable hydrogels are 3D-printed structures which can print scaffolds with specific geometries for complex areas of implantation.^{317,336,571,572}

4.4.3.1 3D printing of scaffolds. 3D printing has garnered intense mainstream interest, with tissue engineering research beginning to now look at using this technology to create more personalized and intricate scaffolds and eventually organs.^{589,590} Specifically, in the field of extrusion-based bioprinting, shear-thinning hydrogels find particular interest as they are able to achieve flow at high stress and stabilize once static. With precisely known monomer origins and controllable degradation profiles, polypeptides present an opportunity to help revolutionize the 3D-bioprinting field.^{317,571,572} Murphy *et al.* from the Heise research group have investigated 3D extrusion printing of scaffolds in two different studies, both preforming the scaffold by extruding the NCA ROP-synthesized polypeptide with an appropriate photosensitive chemical handle or cross-linker, respectively, prior to post-printing curing under UV light.^{571,572} The first of these studies demonstrated the use of a 32-arm star with P(LGlu-*b*-LVal) arms.⁵⁷¹ To induce gelation, the design included two important features: outward hydrophobic poly(L-valine) blocks to assist with gelation through β -sheet aggregation, and allyl glutamate residues to secondarily crosslink upon

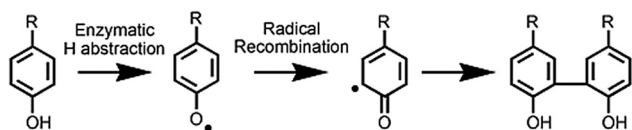


Fig. 67 Schematic of enzymatic phenol crosslinking.

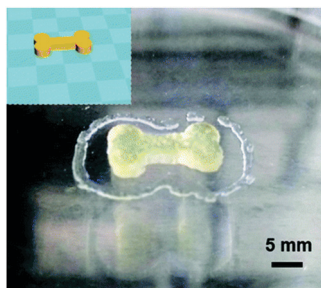


Fig. 68 3D-printed dog bone from photosensitive 4-arm polypeptide hydrogel scaffold based on CAD input (inset). Reproduced from ref. 572 with permission from The Royal Society of Chemistry. Copyright 2019.

UV irradiation, owing to the presence of Irgacure photoinitiator within the ink. The latter study opted for a different approach where linear diblock copolypeptide P(LGlu-co-NBLCys)-*b*-P(Ile), containing PLIle also to induce β -sheet formation, was mixed with 4-arm PEG-propiolate for further chemical crosslinking.⁵⁷² Post-printing UV removal of the *ortho*-nitrobenzyl protecting groups of cysteine allowed for crosslinking between PEG-propiolate and the freshly exposed thiols *via* thiol-yne chemistry in the absence of a photoinitiator. This study yielded scaffolds with complex geometries such as the example shown in Fig. 68. This ability to form geometrically complex structures was not observed in the earlier study despite the first scaffold being stiffer.

While the studies performed by Murphy *et al.* show promise with even cell viability being demonstrated after printing, they did not investigate bioprinting capabilities. This involves 3D printing with cell-laden ink solutions allowing for the precise seeding of cells within a scaffold. Li *et al.* demonstrated the fabrication of scaffolds using a two ink system, as already described in Section 3.4.1.³¹⁷ Briefly, one of the inks contained polypeptide-DNA while the other contained a complementary DNA sequence which allowed for the physical crosslinking of the extruded solutions. The resultant structures had noticeable defects, but nonetheless approximately retained the designed shape. To demonstrate the bioprinting capabilities, cell-laden 3D scaffolds were also fabricated with cells being suspended within the polypeptide-DNA bio-ink. A LIVE/DEAD assay revealed almost all cells survived the printing process (Fig. 69). While the given studies reviewed show promise for the future of NCA ROP-synthesized polypeptides in the space of 3D printing, there is still difficulty in controlling the resolution of these scaffolds and future studies should aim to address this challenge.

4.4.4 Scaffold design for directed cell differentiation.

While the above features are desirable for tissue engineering in general, scaffolds may also be designed with a specific target tissue in mind. Specific mechanical properties, the inclusion of specific compounds, medium of growth and other design factors all determine cell fate regardless of host cell origin. Work from the Jeong group using PEG-*b*-P(LAla-co-LPhe) showed tonsil-derived mesenchymal stem cells were able to undergo adipogenic, osteogenic and chondrogenic differentiation (fat cells, bone cells and cartilage cells respectively) by the addition of differentiation factors.³⁰⁴ Interestingly, a separate

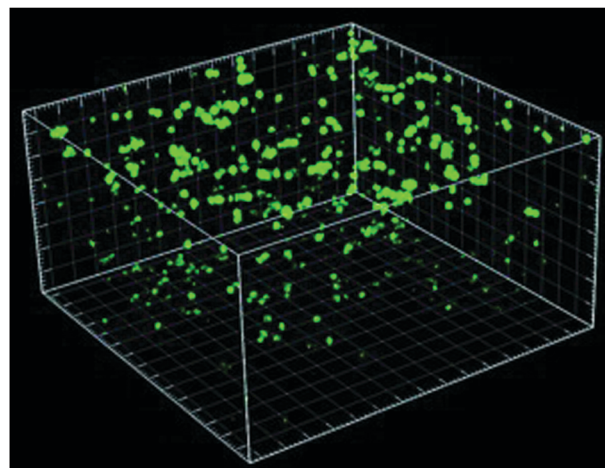


Fig. 69 3D stacks of cells printed within a PLGlu-DNA scaffold, stained via a LIVE/DEAD assay. Reproduced from ref. 317. Copyright 2015 John Wiley and Sons.

study from the same group used a similar polypeptide loaded with the growth factor necessary for tonsil-derived mesenchymal stem cells to undergo hepatogenic differentiation (liver cells), exemplifying the importance of both design and environmental features in dictating cell fate.³⁰⁶

Research in cartilage tissue engineering focuses on the differentiation and proliferation of mesenchymal cells into chondrocytes.⁵⁷⁹ The investigation performed by Peng *et al.* employed injectable thermosensitive PEG-PLAla scaffolds without any chemical modification, instead focusing on changing the length of the PLAla block.³⁰¹ An increase in β -sheet assembly controlled by increased PLAla block length resulted in an increase in the expression of genes relating to chondrogenic differentiation and proliferation, thus leading to increased organisation of cells into clusters. In contrast, the Ying group avoided the use of bulk hydrogels or scaffolds through the use of microcarriers.⁵⁶⁸ A W/O/W microemulsion was used to form particles containing PBLG and gelatin. Gelatin was removed after synthesis resulting in highly porous particles. Due to the highly porous nature, stem cells could be seeded after particle synthesis, protecting them from high fluid shear. Chondrocytes harvested from adult female C57 mice (*i.e.* the host for later *in vivo* testing) exhibited high proliferation within the carriers. As the structures are held together by physical entanglements as opposed to more stable crosslinking methods, they also displayed degradation in PBS, displaying continued degradation over 18 weeks *in vitro* (Fig. 70A) and 8 weeks *in vivo* (Fig. 70B). Furthermore, seeded chondrocytes were found to proliferate *in vivo*.

As one of the native components of bones, the integration of calcium phosphate-based compounds in nanocomposite scaffolds is particularly interesting for bone tissue engineering to promote biomineralization and osteoblast formation. The bone mineral makes up approximately 70% of the weight of a bone,⁵⁹¹ and has been integrated in scaffolds for tissue engineering previously as they signal biomineralization cues within seeded cells.^{290,569,575} Liao *et al.* and Yan *et al.* both took novel approaches by initiating NCA ROP with aminated

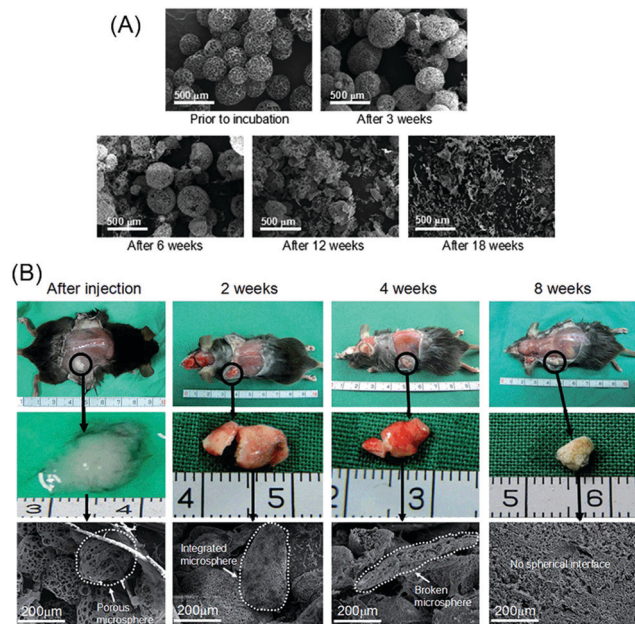


Fig. 70 PBLG microcarriers for cell delivery to bone defect sites. (A) SEM image of microcarriers after incubation in PBS over 18 weeks, (B) *in vivo* degradation and integration of microspheres. Reproduced from ref. 568 with permission from The Royal Society of Chemistry. Copyright 2015.

hydroxyapatite, resulting in PBLG chains grafting from the hydroxyapatite.^{569,575} Liao *et al.* dispersed these modified nanoparticles within a poly(L-lactic acid) scaffold, with the PBLG improving proliferation of mesenchymal stem cells during *in vitro* testing compared to the use of hydroxyapatite without PBLG.⁵⁷⁵ This led to increased bone repair with the volume fraction of bone defects being repaired jumping from approximately 70% to 85% over four weeks. Using the same W/O/W microemulsion approach as previously mentioned,⁵⁶⁸ Yin and co-workers instead used strontium-substituted hydroxyapatite with PBLG chains grafted from the nanoparticles to form microcarriers (Fig. 71A).⁵⁶⁹ Strontium was introduced into the system in order to assist with bone formation and reduce bone resorption. Adipose derived stem cells (ADSCs) were found to successfully penetrate the microcarriers, with the strontium-substituted

hydroxyapatite microcarriers (Sr10-HA-g-PBLG) significantly assisting cell proliferation over seven days compared to PBLG samples (Fig. 71B). The ability to culture cells with the microcarriers to seed them before injection proved advantageous as bone-like tissue could be observed in the seeded samples post-operation – a growth not observed in the unseeded control (Fig. 71C).

4.4.5 Future perspectives. Synthetic polypeptides are currently well-established within the tissue engineering field as they confer easily tunable functionality. Cell adhesion on the surface of materials through indiscriminate protein adsorption has been demonstrated using NCA ROP polypeptide-based hydrogels, but is not always an ideal technique as it can cause an immune response reaction and rejection of the implant or scaffold.^{592,593} To mitigate such issues, scaffolds can be loaded with molecules with the potential to cause targeted protein adsorption or reduce immune response with sustained release. Injectability is a powerful feature of many synthetic polypeptide-based scaffolds which will remain a staple selling point. Enzymatically crosslinkable synthetic polypeptides *via* phenolic side chains have emerged as an interesting method of inducing scaffold formation *in vitro* apart from the conventional use of environmentally triggered secondary structure formation. The side chain coupling of natural biomacromolecules with phenolic side chains provides an interesting avenue for injectable hydrogels. Furthermore, investigations into a broad range of endogenous enzymes for crosslinking such as alkaline phosphatase should also be explored to mitigate the introduction of foreign molecules.

In terms of innovation, the field of 3D printing and bioprinting has yet to thoroughly explore the use of synthetic polypeptides. Very few studies on the use of synthetic polypeptides in 3D-printed scaffolds currently exist, and within these even fewer can be classified as bioprinting. This research gap presents an opportunity and offers an exciting challenge for future research.

4.5 Other applications

In addition to the application of polypeptides as antimicrobial agents and as valuable materials/substrates or carriers in tissue

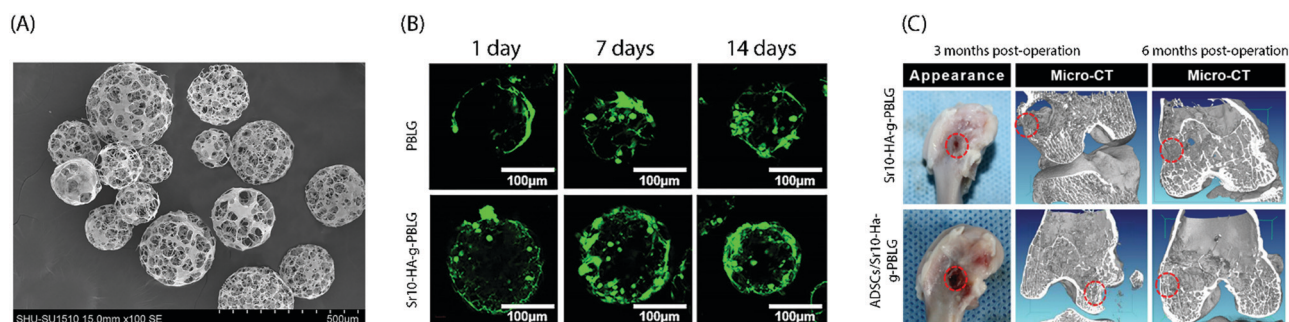


Fig. 71 PBLG-based microcarriers with strontium-substituted hydroxyapatite for cell delivery to bone defect sites. (A) SEM image of microcarriers, (B) confocal fluorescence images of adipose-derived stem cell proliferation on individual microcarriers with and without hydroxyapatite, (C) appearance and microcomputed tomography of repair of defected femora. Reproduced with permission from *ACS Appl. Mater. Interfaces*, 2018, **10**(19), 16270. Copyright 2018 American Chemical Society.

Table 6 Summary of synthetic polypeptides used for biosensors, antifouling surface and bioadhesives

Application	Polypeptide used	Approach	Ref.
Ethanol detection	Polypeptide from urethane derivative of <i>N</i> -Boc-L-lysine with bis-EDOT derivative	Electropolymerization	596
Cocaine detection	Thiophene functional PAla	Electropolymerization	600
Cocaine detection	PLPhe (EDOT-BTDA-PPhe)	Electropolymerization	598 and 599
Methamphetamine detection	PLA with bis-EDOT derivative	Electropolymerization	597
Glucose detection	Thiophene functional PLAla	Electropolymerization	602
Glucose detection	Thiophene-functionalized PLPhe	Electropolymerization	603
Glucose detection	PLLys bearing catechol	Electropolymerization	604
Catechol detection	Dendronized PLys bearing OEG and phenylboronic acid		606
Hydrogen peroxide	PBLG- <i>b</i> -PEG- <i>b</i> -PBLG	Nanoparticle	605
Protein anti-absorption	Zwitterionic PGLu bearing OEG and carboxybetaine	Coating	617
Protein anti-absorption	Zwitterionic sulfobetaine polypeptides	Coating	619
Protein anti-absorption	Copolypeptide bearing catechol	Coating	620
Protein anti-absorption	Methacrylate-ended PSar	Coating	394
Protein anti-absorption	PEGylated cationic PLys	Coating	393
Tissue healing	Functional side group polypeptides	Injectable	598 and 622
Tissue healing	Functional side group copolypeptides	Injectable	623 and 624
Tissue healing	Chitosan-graft polypeptides from DOPA, Cys and Arg	Injectable	625

engineering, drug delivery and nucleic acid delivery, synthetic polypeptide-based materials have been employed for other uses such as biosensors, antifouling coatings and bioadhesives. Some examples are given as below and summarized in Table 6.

4.5.1 Biosensors. Electrochemical biosensors have been widely developed due to their fast response and high sensitivity. Nanomaterials including metal nanoparticles and carbon nanomaterials have been intensively used and have shown excellent performance in electrochemical biosensing as they provide high surface area for fast sensing responses, good electrochemical properties and a low limit detection.⁵⁹⁴ However most of the reported nanomaterials are non-degradable and some of them are toxic to human cells. Synthetic polypeptides have thus been utilized to construct affinity-type biosensors, which can recognize a specific analyte through binding or an oxidation–reduction reaction recorded by a transducer. In the case of electrochemical biosensors, binding formation or a chemical reaction affects electron transfer. The difference in electron transfer to different concentrations of the analyte is measured as a current change and affords analytical detection.^{594,595} Generally, polypeptide macromonomers with electroactive end groups are synthesized, followed by electropolymerization onto the surface of electrodes. Thanks to the amino termini of the peptide chains, the biological recognition element (*i.e.* enzyme, antibody or aptamer) can be immobilized onto the polymer surface using crosslinking agents, such as glutaraldehyde or sulfo-SMCC, to recognize the corresponding bio-elements, and in turn achieve analytical detection. The common characterization technologies include cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS).

In 2014 Kesik *et al.* reported an amperometric ethanol biosensor employing polypeptides and ferrocene.⁵⁹⁶ The synthetic copolymer consisted of a ferrocene imidazole derivative of dithiophene and a bis-EDOT derivative which initiated the ROP of lysine NCA to bear polypeptide as a side chain. Both components were conductive in order to improve the interaction between the biomolecule and the conducting layer

and were electropolymerized on a graphite electrode. Alcohol oxidase was immobilized *via* the amino groups of the polypeptides using GA as the crosslinking agent for alcohol recognition (Fig. 72). The concentration of ethanol was determined by amperometric measurement (as it generates a current signal) and a good linear calibration curve was obtained. This amperometric ethanol biosensor was used to test the alcohol content in commercial wines and showed good agreement with the actual value. This system thus provided a simple and sufficient strategy for real time alcohol measurement.

Timur's group developed a polypeptide-based methamphetamine (METH) biosensor.⁵⁹⁷ The METH-selective antibody was immobilized in the polypeptide obtained from ROP of L-alanine NCA containing an end group of EDOT-BTDA for electroactivity, which was coated on glassy carbon electrodes. The coated surface was characterized by CV, EIS, SEM and fluorescence microscopy. The linearity for METH detection was in the range of 10–100 $\mu\text{g mL}^{-1}$ and the limit of detection was 13.07 $\mu\text{g mL}^{-1}$. Afterwards, the same research group developed a biosensor based on inclusion of an aptamer, a functional nucleic acid receptor with high ligand-binding affinity, using the synthetic poly-L-phenylalanine electroactive macromonomer (EDOT-BTDA-PPhe) as the matrix.⁵⁹⁸ Cocaine aptamers containing 32 base pairs with 5'-NH₂ and 3'-SH₂ were attached to the polypeptide chains by sulfo-SMCC crosslinker. The aptamer-based polypeptide with good orientation for binding was used for the detection of cocaine and its metabolite benzoylecgonine. The linear correlation sat at 1.0–10 nM and 0.5–10 μM for cocaine and BE detection respectively. In another publication, the same matrix was used where BE antibody was immobilized in the EDOT-BTDA-PPhe for BE and cocaine determination (Fig. 73).⁵⁹⁹ The linearity of BE and cocaine analysis was between 0.5–25 μM .

Another example of incorporation of aptamer into polypeptides for analytical cocaine detection was reported by Bozokalfa *et al.*⁶⁰⁰ The polypeptide they synthesized was a thiophene functional polypeptide macromonomer, which was subsequently further polymerized onto glassy carbon electrode

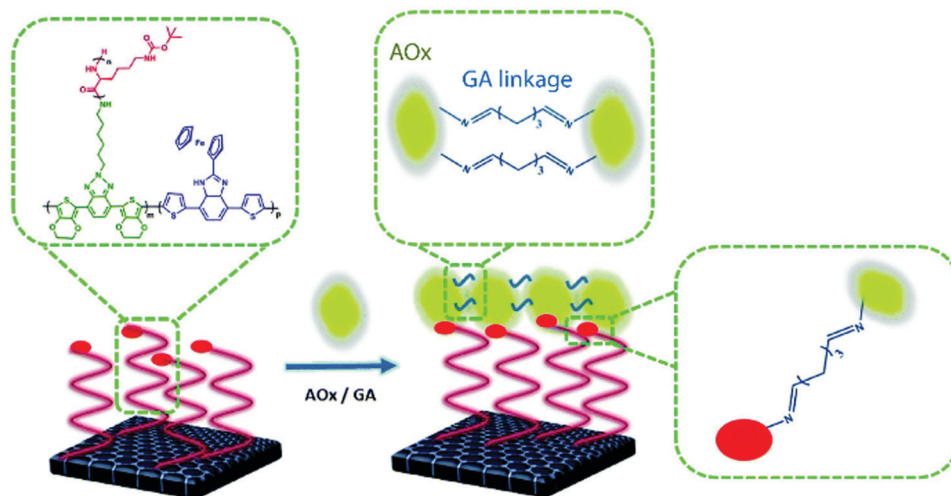


Fig. 72 Schematic illustration of amperometry ethanol biosensors. Reproduced from ref. 596 with permission from The Royal Society of Chemistry. Copyright 2014.

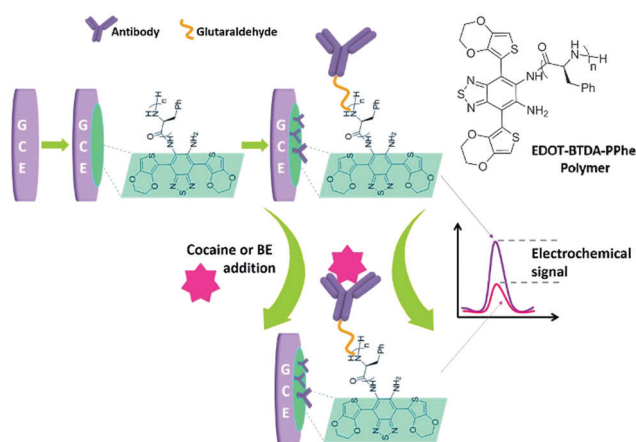


Fig. 73 Structure of polypeptide bearing functional macromonomer EDOT-BTDA-PPhe, surface modification and measurement principle of the cocaine or BE sensors. Reproduced from ref. 599 with permission from Elsevier. Copyright 2017.

surfaces, followed by aptamer conjugation *via* sulfo-SMCC crosslinker. Characterization by CV and EIS confirmed that the sensing interfaces were successfully obtained. The polypeptide chains provided surface attachment with good orientation and conformational change for the aptamer. The polypeptide-based cocaine sensor could detect trace amounts of cocaine and benzoyllecgonine at a linear range of 2.5–10 nM and 0.5–50 μ M respectively, providing a much greater sensitivity than the control sample with aptamer directly coated onto a gold electrode. This cocaine sensor provides potential for drugs of abuse testing. Enzyme-based electrochemical glucose sensors have been widely developed by using glucose oxidase which can selectively recognize and oxidize glucose to gluconolactone.⁶⁰¹ Timur and co-workers developed an approach for glucose detection by electrochemical polymerization of a thiophene-functionalized poly(alanine) macromonomer (T-PALA) on graphite electrodes and immobilization of glucose oxidase

(GOx).⁶⁰² The sensors displayed a good linearity for concentrations of glucose in the range of 0.01–1.0 mM. To explore the potential of using polythiophene for novel biosensors, the same group also constructed a similar glucose sensor by replacing the thiophene–polyalanine with thiophene–polyphenylalanine macromonomers (T-g-PPhe).⁶⁰³ The linearity for the glucose sensing was in the range of 0.025–1.0 mM with a fast response time of 12 s. A good stability with 65 repetitive tests only showed 27% reduction in the activity. Additionally, the T-g-PPhe coated on indium tin oxide modified glass electrode was further functionalized with RGD peptide for cell attachment. The obtained films showed better cell adhesion and spreading compared to unmodified films. More recently, Timur's group also synthesized catechol-attached polypeptide as a coating on electrode surfaces, followed by GOx and anti-immunoglobulin G (Anti-IgG) immobilization on the polypeptide using GA as a cross-linker.⁶⁰⁴ The obtained biosensor showed selectivity for GOx and IgG detection, and good linear curves between current signal and each analyte's concentrations were obtained by amperometric measurement, with linear ranges of 1.8–180 μ g mL⁻¹ and 0.05–2.50 ng mL⁻¹, respectively.

Several other materials bearing polypeptides which make use of their unique architectures have also shown great potential as sensors. Hybrid nanoparticles consisting of triblock polypeptides from γ -benzyl-L-glutamate and Prussian blue were synthesized *via* disassembly-assisted strategy to achieve nanosize (18 nm).⁶⁰⁵ The α -helical polypeptides were disassembled into small aggregates which were coordinated with Fe³⁺ to afford nanoparticles with core-shell structures. The nanoparticles exhibited an electroactive response to the reduction of hydrogen peroxide due to the redox current generated by Prussian blue/Prussian white conversion. The limit of detection for hydrogen peroxide was 18 nM, a 5-fold sensitivity increase over the response achieved in conventional Prussian blue nanoparticles, demonstrating an efficient electron mediation achieved by the hybrid nanoparticles. Thermo-responsive dendronized poly(lysine) was

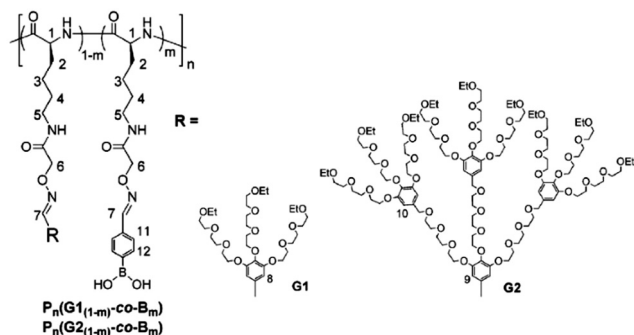


Fig. 74 Chemical structures of the dendronized polypeptides. Adapted with permission from ref. 606. Copyright 2016 American Chemical Society.

reported by the Zhang group.⁶⁰⁶ The poly(lysine) was modified with oxyamine functionality which can be grafted with aldehyde-cored oligoethylene glycol (OEG) dendrons (Fig. 74) and phenylboronic acid. α -Helix formation was enhanced by the dendronization of poly(lysines), leading to switchable thermal-driven phase transitions. The phenylboronic acid on the polypeptides could recognize catechol-containing compounds such as dopamine, and this function could be switched on or off due to the phase transition induced by changes in temperature. Cholesterol-decorated poly(L-cysteine) copolymers were designed to show micelle-to-vesicle transformation in response to oxygen species.⁶⁰⁷ The transition was due to the conformational change of polypeptides from β -sheet to α -helix, resulting from the oxidation of thioether groups in the side chains which changed the packing of cholesterol groups. The morphological transition provided an opportunity to detect oxygen species *in vitro* and *in vivo*, although the related study of biosensing has not been shown yet.

Overall, polypeptides with conjugated polymers have shown fast response, high selectivity, excellent durability and sensitivity, however there are still challenges and opportunities. Different architectures of polypeptides may influence the sensing performance but these effects remain to be further investigated. The detection limit of polypeptide-based biosensors is still relatively high compared to some other nanomaterials-based biosensors (pM or even fM). More repeatability tests need to be shown, and fouling could be a threat for signal loss (*i.e.* glucose in blood test). Detections *in vivo* and *in vitro* have not been attempted and there remains a huge potential to be explored. To achieve this, issues including the use of toxic components, adjustable size and reliability in biological media need to be tackled. For the enzyme-based biosensors specifically, stability has always been a big challenge as the activity of enzymes is strongly affected by environmental conditions such as pH and heat,⁶⁰⁸ issues which could not be solved by the incorporation of polypeptides as one of the components.

4.5.2 Antifouling. The antifouling coatings mentioned in this section resist the adhesion of biocontaminants through the use of polymeric coatings comprising of polypeptides. The antifouling characteristics imparted by careful choice of polypeptide composition are a desired feature in antibacterial and antimicrobial constructs to fend off biofilm formation as it is highly important to prevent the attachment of bacteria and

debris which can create an optimal substrate for further bacteria deposition. Antifouling properties are also sought after in drug delivery vehicles to prevent protein adsorption, which can lead to degradation of the drug vehicle or reduced blood circulation time.

PEG has been the most-widely used material to prepare antifouling surfaces for protein resistance since the first PEGylated protein therapeutic was approved by the FDA in 1990.^{609–611} PEG provides charming properties such as non-toxicity, low cost, uncomplicated synthesis and well-controlled molecular weight, yet despite these advantages, studies have shown that there are still some concerns when using PEG, especially *in vivo*.⁶¹¹ The biggest issue is its nondegradable character, which results in accumulative toxicity and possible immunological responses.⁹⁷ Recent findings of PEG antibodies in individuals have raised concerns about the potential immunogenicity of PEGylated therapeutics.^{612,613} Although these potential hazards have not been reported for the construction of antifouling surfaces against protein, the problematic oxidation of PEG under biological environments which results in a loss of protein resistance has been studied.^{614,615} Providing an alternative to PEG, zwitterionic polymers have been widely used as antifouling agents *via* coating of the target surface, due to their hydration capacity *via* ionic solvation and charge balance.⁶¹⁶ Specifically, in recent years synthetic zwitterionic polypeptides have shown great promise as antifouling materials. Polypeptides with zwitterionic components show excellent antifouling performance, and their inherent degradability and biocompatibility make them good candidates for antifouling applications in biological environments. The unique higher order structures of polypeptides, *i.e.* secondary structures, also provide opportunities for antifouling applications since an important factor for antifouling is the packing density of polymers, especially in brush structural polymers.

Hu's group synthesized a series of zwitterionic polypeptides containing carboxy betaine (CB) and varied the length of OEG linkers (by using Glu NCA modified with 1–3 repeat EG units during early stages of synthesis) in the side chains (Fig. 75A).⁶¹⁷ The polymers were then coated onto gold chips *via* thiol-gold bonding and their antifouling performance was examined. All the zwitterionic polypeptides showed outstanding antifouling activity, which was shown to be linker length dependent. The polymer with the longest length of EG linkers on the side chains performed best, with complete prevention of protein adsorption, 96–98% reduction of blood serum adsorption and ~99% reduction of cell/platelet/bacterial attachment. Additionally, further studies into the promising model system containing polypeptides with three pendant EG units were carried out to elucidate the effect of helical conformation and anchoring orientation on non-fouling properties.⁶¹⁸ Four polypeptides were prepared, a pair where the thiol positions were placed either at the C- or N-terminus, and another pair with helical or disordered conformation. The results showed that the gold surface modified with helical polypeptides was better at anti-fouling than that with unstructured polypeptides. The antiparallel anchoring effect generated by using mixtures of

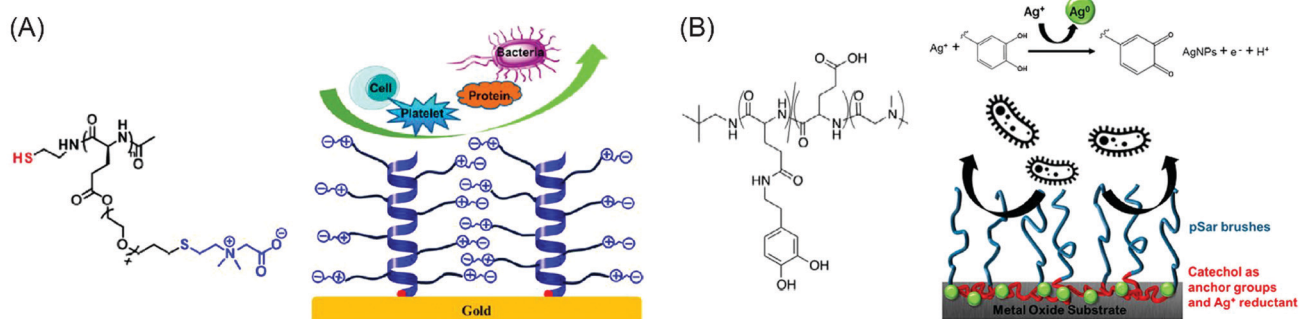


Fig. 75 Schematic structure of the antifouling surfaces bearing polypeptides. (A) Structure of the zwitterionic polypeptides P(CB-EG_xGlu) and illustration of the P(CB-EG_xGlu) coating on gold surfaces.⁶¹⁷ (B) Structure of pGlu(COONa)₁₁-*r*-pGlu(CODopa)₂₂-*b*-pSar_{62/124/264} (P(A/B/C)) copolymers, formation of Ag NPs by reduction by catechol and schematic depiction of TiO₂ surfaces modified with pSar brushes and Ag NPs.⁶²⁰ Adapted with permission from ref. 617 and 620, Copyright 2018 American Chemical Society.

thiol functionalized at the C- and N-terminus of the helical polypeptides further improved antifouling performance, presumably due to the antiparallel anchoring of the helices affording the optimal balance between hydration and surface packing. The best performing P(EG₃Glu) was applied to form zwitterionic poly(α -amino acid)s through conjugation with CB, resulting in ultra-low-fouling surfaces with no adhesion of mouse platelet and 98–99% reduction of human serum adsorption. Lu *et al.* reported a zwitterionic sulfobetaine polypeptide *via* a two-step synthesis for protein resistance.⁶¹⁹ The polypeptides were coated onto amino functional silica wafers for antifouling measurements and they showed good inhibition of protein absorption, confirmed by fluorescence microscopy. The absorption amount could be tuned by varying the concentration of the polypeptide solutions for coating. Overall, zwitterionic polypeptides offer conformational flexibility as their main advantage through the establishment and disruption of their secondary structures, which can affect the packing density. The usage of polypeptides obtained *via* NCA ROP as a component in antifouling coatings is still limited compared to short peptide-base materials, which have more freedom in sequence control to tune the conformation.⁶¹⁶

In addition to zwitterionic polypeptides, functional polypeptides have also been used to construct antifouling surfaces. Yoo *et al.* designed a dual-functional polypeptide brush for protein resistance and antimicrobial activity.⁶²⁰ Block copolymers containing catechol were obtained by combining protein-resistant pSar blocks with dopamine-modified poly(glutamic acid) blocks (Fig. 75B). The pSar provided protein resistant function while the poly(glutamic acid) offered the catechol functionalities for reduction of silver ions to Ag nanoparticles. The copolymers were coated on titanium dioxide surface for protein adsorption tests and the results showed that the brushes with shorter pSar block performed better in terms of anti-protein absorption. The formation of Ag nanoparticles also provided antibacterial function.

4.5.3 Bioadhesives. There are three key principles for the design of bioadhesives, including formation and retention of robust interfacial bonding and biocompatibility.⁶²¹ Wet adhesive strength is one of the most important features as

bioadhesives need to be applied in physiological environments where liquids such as blood exist. Due to their biocompatibility and degradability, polypeptide-based polymeric materials can be used in tissue adhesives *via* chemical conjugations, electrostatic bonding or crosslinking. Lu's group has incorporated different functional groups into the side chains of polypeptides to prepare bioadhesives.⁶²² They designed a series of hyperbranched polypeptides synthesized from levodopa, cysteine, arginine and lysine to give different side groups, such as catechol, guanidyl, thiol and double bonds (Fig. 76A). The initiator was thermo-sensitive Pluronic L-31, thus viscous fluid of the polymer solution solidified from 25 °C to 37 °C. Various chemical reactions or interactions also occurred, including Michael addition reactions, catechol oxidation or crosslinking reactions. The wet adhesive strength was dependent on the functional side groups, temperature, topological structure and curing time. The crosslinking formation *via* click reaction endowed the combination of poly(L-DOPA-*co*-Arg-*co*-Cys) and poly(DOPA-*co*-Arg-*co*-Lys acrylamide) with the strongest wet adhesive strength (138 kPa) compared to other samples. The hyperbranched polypeptides were degraded by enzyme (Protamex) at 37 °C, and the results showed that 70% degradation was achieved after 16 hr. In a follow-up study, the branch number in the hyperbranched polypeptides was increased using the same method of NCA ROP initiated by alcohols (Fig. 76B).⁵⁹⁸ *In vitro* testing showed that the adhesive strength on porcine skin and the tensile adhesion strength on bone were up to 114.5 kPa and 786 kPa respectively. *In vivo* tests on rabbit kidney models showed a successful closure of arteries up to 16 days.

In another work, the same group reported a series of surgical adhesives based on thermo-responsive polypeptides (block copolymers) derived from 3,4-dihydroxy-L-phenylalanine, diethylene glycol monomethyl ether-L-glutamate (EG2-Glu), L-arginine (Arg), L-cysteine (Cys), and ϵ -N-acryloyl-L-lysine (Ac-Lys) (Fig. 77).⁶²³ Poly(EG2-Glu) is thermoresponsive and shows sol-gel transition at body temperature. The multiple functional groups in the side chains endowed the adhesives with good strength and healing effects due to various crosslinking reactions, including thiol-ene click reaction, crosslinking between catechol and sulfhydryl and crosslinking by forming disulfides. The maximum lap-shear

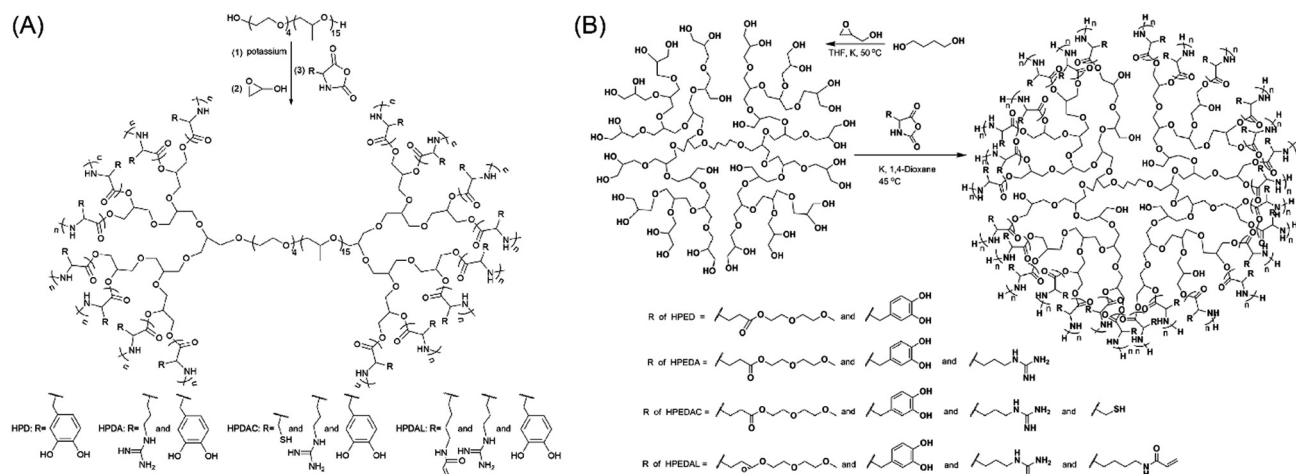


Fig. 76 Synthesis of hyperbranched polypeptides for surgical adhesives. (A) Hyperbranched polypeptides with different functional groups on the side chains and (B) increased branched numbers. Reproduced from ref. 598 and 622 with permission from The Royal Society of Chemistry. Copyright 2016, 2018.

strength of the adhesive on porcine skin was 101.2 kPa and the tensile strength on bone was 603 kPa. Antibleeding and tissue adhesive tests on a rat model showed good hemostatic properties and healing effects with osteotomy gap remodelling completed in 2–9 weeks. They also designed a similar triblock copolymer initiated by thermoresponsive Pluronic 1-31 which incorporated Arg, Cys, and Ac-Lys to improve the adhesion strength and curing rate.⁶²⁴ Lap-shear adhesion strengths and tensile adhesion strengths in this case were up to 106 kPa and 675 kPa respectively. In a more recent work, chitosan was utilized as an initiator for the synthesis of co-polypeptides from DOPA-NCA, Cys-NCA and Arg-NCA *via* ROP.⁶²⁵ The selection of chitosan was due to its non-toxicity, biocompatibility, biodegradability and antibacterial properties. The widely used DOPA in bioadhesives provides binding capability to tissues

and enhances adhesive strength, while the Cys and Arg also improved the adhesion toughness and fastened the curing rate. The healing test performed well on metal adherends, porcine skin and bone tissue, with the tensile adhesion strength on bone up to 642.70 kPa.

These studies have shown reliable wet adhesive strength achieved by using polypeptides as backbones, but the issues surrounding polypeptides synthesis and scalability for clinical applications still exist and need to be addressed. Although the studies showed that the degradation products (incomplete degradation) are nontoxic, the toxicity of products from full degradation in the long-term need to be investigated. How to adjust the degradation rate is also a requirement that needs to be further explored in order to meet clinical needs. The polypeptide-based thermo-responsive bioadhesives have expanded the family

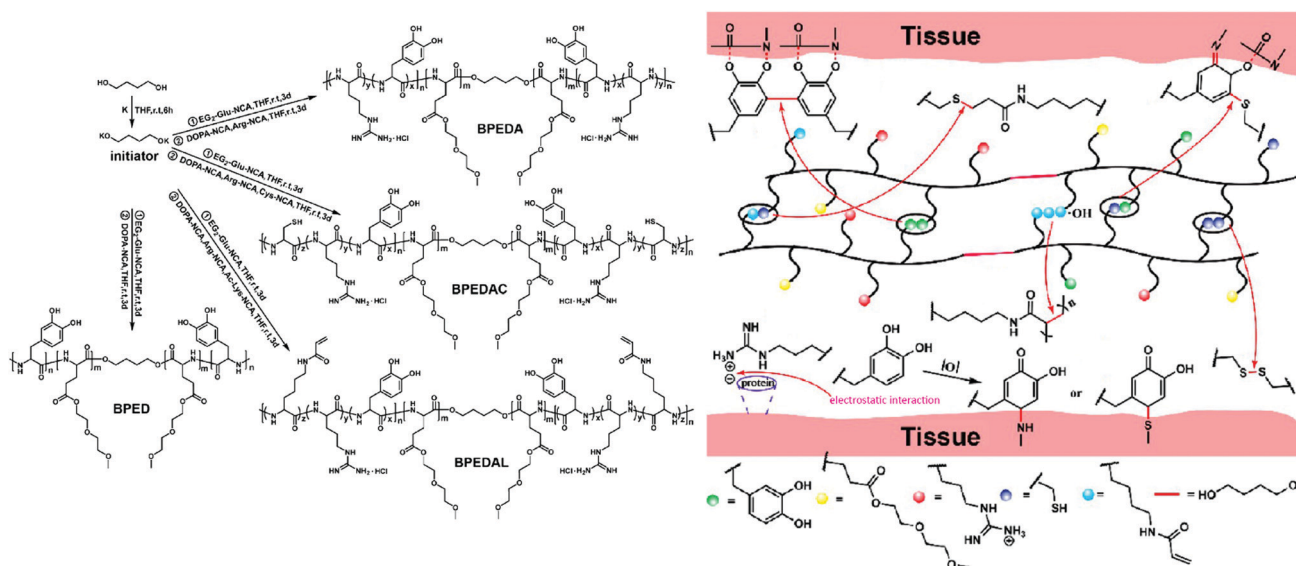


Fig. 77 Synthesis of polypeptides with multiple functional groups in the side chains, and the possible crosslinking reactions with the functional groups. Adapted with permission from ref. 623. Copyright 2017 American Chemical Society.

of multi-functional bioadhesives, yet more functionalities on bioadhesives remain to be developed, such as for instance through a combination of antimicrobial properties, self-healing ability and drug release capability.⁶²¹

5. Conclusion and future perspectives

This article provides a comprehensive review of the latest synthetic methods available to produce polypeptides of various architectures *via* NCA ROP and has surveyed the recent literature on biomedical applications. Poly(α -amino acids), with their varied composition, attainable secondary structures, attractive self-assembly behaviour and desirable biocompatibility and biodegradability have not only captured researchers' interest in their own right but also as part of polymer conjugates. Because of these characteristics polypeptides have extensively been used in the biomedical fields, especially in drug and nucleic acid delivery, tissue engineering and more recently as an emerging class of promising antimicrobials.

Overall, commendable synthetic advances in the ring opening polymerization of *N*-carboxyanhydrides have been reported in the past five years. These developments are increasingly allowing scientists to employ a larger arsenal of initiating molecules, opening up avenues for complicated architectures, self-assemblies and exciting development into varied applications. Moreover, the academic community has shown the production of polypeptides is now possible with remarkable control under less stringent conditions, as demonstrated by the very recent reports of NCA ROP carried out in open atmosphere, emulsion, and aqueous PISA. The use of flow chemistry to produce NCA monomers is also worth highlighting and exploring further as a means of polymerization. These developments are truly exciting and together they will help researchers turn a traditionally complicated synthesis into a straightforward process, as well as reduce time and cost. Advances in NCA ROP are key to bringing polypeptides closer to realizing their true commercial, industrial and biomedical value. To date, NCA polypeptide studies have predominantly explored linear polypeptides or made use of linear macroinitiators to form peptide polymers since more refined architectures are difficult to achieve controllably. Further refinement to produce polypeptides with very narrow polydispersities and reproducibility is necessary given that this is a great challenge for medical regulatory processes. This review highlights the new chemistries that will allow for the synthesis of more complex polypeptide architectures to better mimic the tertiary and quaternary structures of proteins. It is expected that attaining such mimicry will in turn unlock some of the incredible features displayed by natural proteins.

Two other great challenges that remain in the field of polypeptides obtained *via* NCA ROP are control over the peptide sequence, and finetuning of the affinity of said synthetic polypeptides towards mammalian and bacterial cells using complementary chemistries. Currently, polypeptides have limited selectivity between eukaryotic cells and prokaryotes, leading to low therapeutic indices which impact the real applicability of

the NCA ROP chemistry. Similarly, selectivity between cancer cell targets and healthy cells needs to be addressed, as an imbalance between cationic and hydrophobic charges within the polypeptide can broaden activity leading to non-specific cell membrane lysis and other disruptive processes. Nucleic acid delivery is also impacted by some of these challenges. Gene-based therapy has gained significant attention as a potential method for treating genetic disorders, infectious disease, and cancer. Therefore, efforts in the design of non-viral gene delivery vectors with high transfection efficiency and low cytotoxicity need to be continued to understand the structure-function relationship and the biology behind the delivery systems for clinical applications. Polypeptides obtained by NCA ROP are a class of antimicrobial agents increasingly gaining attention, as they have so far shown great promise as alternative treatments for serious and multi-drug resistant bacteria strains *in vitro* and *in vivo*. However, it is also imperative to continue exploring their optimal composition and structural variation, possible modes of delivery and pharmacokinetics, to overcome issues such as protein fouling and circulatory clearance. Overall, it is of utmost importance to continue developing a body of knowledge about the optimal polypeptide composition and suitable additives that will palliate adverse cell interactions for the wide range of biomedical applications. Though the difficulty in addressing these challenges is evident, combinatorial and orthogonal chemistries can be performed on polypeptides, which opens addition of moieties that would be difficult to incorporate through polymerization. Introduction of these techniques will enable addition of different ligands to target cells, aid with biocompatibility, and solubility as an example. Thus new chemistries involved in NCA ROP polypeptide formation in combination with orthogonal chemistries have the potential to revolutionize these fields.

Lastly, an application yet to be explored for polypeptides obtained by NCA ROP is their potential use as plastic replacements. The facile ring opening nature of this chemistry, production of tailored molecular weight materials of chosen composition, in addition to pursuit of specific secondary structures all lend themselves as desirable features to exploit in the production of flexible or stiff "bioplastics". Unlike current plastic waste with long hydrocarbon backbones, the poly(amido) backbone would make these materials bioavailable and compostable. To achieve this ambitious goal, it is necessary to find inexpensive sources of amino acids, in addition to developing revolutionary synthetic methods which will allow facile scale-up and a reduction in the use of harmful starting materials for NCA synthesis.

List of abbreviations

AA	α -Amino acid
AAMMA	Accelerated amine mechanism through monomer activation
AFM	Atomic force microscopy
ALP	Alkaline phosphatase
AMM	Activated monomer mechanism

AMP	Antimicrobial peptide	MM	Macromonomer
ATRP	Atom transfer radical polymerization	MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
BLG	γ -Benzyl-L-glutamate	MSA	Methanesulfonic acid
CA4	Combretastatin A4	MW	Molecular weight
CCS	Core-crosslinked star	NAM	Normal amine mechanism
CD	Circular dichroism	NBCys	<i>S</i> -(<i>o</i> -Nitrobenzyl)-L-cysteine
CD	Cyclodextrin	NCA	<i>N</i> -Carboxyanhydride
CFU	Colony-forming unit	NHC	<i>N</i> -Heterocyclic carbene
CGC	Critical gelation concentration	NIR	Near infrared
CHLG	γ -(6-Chlorohexyl)-L-glutamate	NDM-1	New Delhi metallo-beta-lactamase 1
CMC	Critical micelle concentration	NMM	<i>N</i> -Methyl morpholine
COD	1,5-Cyclooctadiene	NMP	Nitroxide-mediated radical polymerization
CPP	Cell penetrating peptide	NTA	<i>N</i> -Thiocarboxyanhydride
CTA	Chain transfer agent	OEG	Oligo(ethylene glycol)
CuAAC	Copper-catalyzed azide-alkyne cycloaddition	OEI	Oligo(ethyleneimine)
CV	Cyclic voltammetry	<i>o</i> NB	<i>O</i> -Nitrobenzyloxycarbonyl
DCM	Dichloromethane	PA	Poly(alanine)
DDS	Drug delivery system	PAMAM	Poly(amidoamine)
DIPEA	<i>N,N</i> -Diisopropylethylamine	PAPA	Poly(α -aminopalmitic acid)
DLS	Dynamic light scattering	PBLA	Poly(γ -benzyl-L-aspartate)
DMC	Dimethylcarbonate	PBLG	Poly(γ -benzyl-L-glutamate)
DMEA	<i>N,N</i> -Dimethylethanolamine	PBLL	Poly(<i>tert</i> -butoxy-L-lysine)
DMEDA	<i>N,N'</i> -Dimethyl-1,2-ethanediamine	PCL	Poly(ϵ -caprolactone)
DMF	<i>N,N'</i> -Dimethylformamide	PDGA	Poly(D-glutamic acid)
DNA	Deoxyribonucleic acid	PDI	Polydispersity index (M_w/M_n)
DOPA	3,4-Dihydroxyphenylalanine	PDLLeu	Poly(D,L-leucine)
DOX	Doxorubicin	PDLeu	Poly(D-leucine)
DP	Degree of polymerization	PDLVal	Poly(D,L-valine)
DPPC	Dipalmytoil phosphatidylcholine	PE	Phosphatidylethanolamine
ECM	Extracellular matrix	PEG	Poly(ethylene glycol)
EDOT	3,4-Ethylenedioxythiophene	PEGA	Poly(ethylene glycol) acrylate
EDTA	Ethylenediaminetetraacetic acid	PEI	Polyethyleneimine
EG	Ethylene glycol	PG	Phosphatidylglycerol
EIS	Electrochemical impedance spectroscopy	PGA	Poly(glutamic acid)
ELG	γ -Ethyl-L-glutamate	PHEMA	Poly(2-hydroxyethyl methacrylate)
ELP	Elastin-like polypeptide	PHLG	Poly(γ -6-chlorohexyl-L-glutamate)
EPR	Enhanced permeability and retention	PHDLG	Poly(γ -6-chlorohexyl-D,L-glutamate)
ESI	Electrospray ionization	PISA	Polymerization-Induced Self-Assembly
FDA	Food and drug administration	PIXE	Proton-induced X-ray emission
FRP	Free radical polymerization	PLAla	Poly(L-alanine)
GA	Glutaraldehyde	PLAsp	Poly(L-aspartic acid)
GOx	Glucose oxidase	P β LAA	Poly(β -aspartic acid)
GPC	Gel permeation chromatography	PLCys	Poly(L-cysteine)
HFAB	1,3-Bis(2-hydroxyhexafluoroisopropyl)benzene	PLCys-Cys	Poly(L-cystine)
HMDA	Hexamethyldiamine	PLGlu	Poly(L-glutamic acid)
HMDS	Hexamethyldisilazane	PLHis	Poly(L-histidine)
HSA	Human serum albumin	PLIle	Poly(L-isoleucine)
IHC	Imidazolium hydrogen carbonate	PLLys	Poly(L-lysine)
LPS	Lipopolysaccharide	PLLA	Poly(L-lactide)
MA	Methacrylate	PLLeu	Poly(L-leucine)
MBA	<i>N,N'</i> -Methylenebisacrylamide	PLMet	Poly(L-methionine)
MBC	Minimum bactericidal concentration	PLPhe	Poly(L-phenylalanine)
MDR	Multi-drug resistant	PSar	Poly(sarcosine)
METH	Methamphetamine	PLTyr	Poly(L-tyrosine)
MIC	Minimum inhibitory concentration	PLVal	Poly(L-valine)
		PNBLCys	Poly((<i>o</i> -nitrobenzyl)-L-cysteine)

PNIPAM	Poly(<i>N</i> -isopropylacrylamide)
POSS	Polyhedral oligomeric silsesquioxane
POBLG	Poly(γ -(4-propargyloxybenzyl)-L-glutamate)
POEGMA	Poly(oligo(ethylene glycol) methacrylate)
PPABLG	Poly(γ -(4-((2-piperidine-1-yl)ethyl)aminomethyl)benzyl-L-glutamate)
PPLG	Poly(γ -propargyl-L-glutamate)
PPep	Polypeptide
PPLTYr	Poly(L-phosphotyrosine)
PS	Polystyrene
PVBLG	Poly(γ -(4-vinyl-benzyl)-L-glutamate)
PZLL	Poly(ϵ -carbobenzyloxy-L-lysine)
RA	Radially amphiphilic
RAFT	Reversible addition–fragmentation chain transfer polymerization
RDRP	Reversible-deactivation radical polymerization
RNA	Ribonucleic acid
ROMP	Ring opening metathesis polymerization
ROP	Ring opening polymerization
ROP	Ring opening polymerization
ROS	Reactive oxygen species
SEC	Size exclusion chromatography
SEM	Scanning electron microscopy
SMCC	Succinimidyl 4-(<i>N</i> -maleidomethyl)-cyclohexane-1-carboxylate
SNAPP	Structurally nanoengineered antimicrobial peptide polymer
SNP	Shell-stacked nanoparticle
SPPS	Solid-phase peptide synthesis
TEA	Triethylamine
TEM	Transmission electron microscopy
THF	Tetrahydrofuran
TMG	1,1,3,3-Tetramethylguanidine
TMS	Trimethylsilyl
TNPC	TiO ₂ nanospike surface coating
TREN	Triethylaminetriamine
TTC	Trithiocarbonate
TU-S	<i>N,N'</i> -Bis[3,5-bis(trifluoromethyl)phenyl]-thiourea
VRE	Vancomycin-resistant <i>Enterococcus</i>
XRD	X-ray diffraction
ZLL	ϵ -Carbobenzyloxy-L-lysine

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This work was generously supported by the Australian Government Research Training Program Scholarship, the Melbourne International Engagement Award and the Melbourne International Research Scholarships. GGQ acknowledges the supports of the Australian Research Council (ARC)'s Discovery Project

Grants (DP160101312, DP170104321), and the Australian National Health and Medical Research Council (NHMRC)'s Project Grant (APP1142472). NMOS is the recipient of NHMRC funding (APP1142472, APP1158841, APP1185426), ARC funding (DP160101312, LE200100163), Cancer Council Victoria funding (APP1163284), and Australian Dental Research Funding in anti-microbial materials and research is supported by the Centre for Oral Health Research at The Melbourne Dental School. ARM wishes to thank Matthew D. Biviano for assistance.

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Highly Living Stars via Core-First Photo-RAFT Polymerization: Exploitation for Ultra-High Molecular Weight Star Synthesis

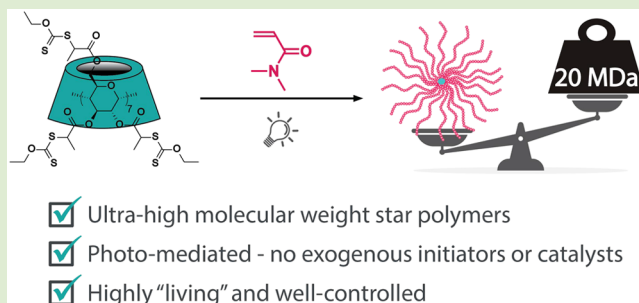
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Supporting Information

ABSTRACT: Star polymers are highly functional materials that display unique properties in comparison to linear polymers, making them valuable in a wide range of applications. Currently, ultra-high molecular weight (UHMW) star polymers synthesized using controlled radical polymerization are prone to termination reactions that have undesirable effects, such as star–star coupling. Herein, we report the synthesis of the largest star polymers to date using controlled radical techniques via xanthate-mediated photo-reversible addition–fragmentation chain transfer (RAFT) polymerization using a core-first approach. Polymerization from xanthate-functionalized cores was highly living, enabling the synthesis of well-defined star polymers with molecular weights in excess of 20 MDa.



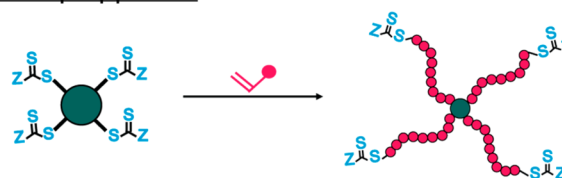
Star polymers are highly functional materials due to their compact, spatially defined, three-dimensional structure that leads to unique physical properties and increased opportunity for functionalization. They are currently used industrially as viscosity modifiers and lubricants, while potential applications in areas as varied as drug nanocarriers and interfacial stabilizers are also being investigated.^{1–5} Star polymers consist of linear arms that radiate from a central core and can be synthesized using a variety of controlled radical polymerization methods. Synthesis via reversible addition–fragmentation chain transfer (RAFT) polymerization has received significant attention and can be performed using either an arm-first or core-first approach.⁶ Arm-first star polymers are synthesized either by conjugation of the linear arms to a multifunctional species or through sequential polymerization with a cross-linker that then forms a gel-like core of the star polymer. The core-first approach requires the use of a multifunctional core from which the arms can be polymerized. Although both arm-first and core-first approaches can lead to well-defined star polymers, only the core-first approach allows for an accurate and predetermined number of arms and facile purification.⁷

Star polymers synthesized via the core-first approach have been synthesized using many different polymerization techniques including atom transfer radical polymerization (ATRP), nitroxide-mediated polymerization (NMP), RAFT polymerization, and ring-opening polymerization (ROP).^{8–13} The core-first approach via RAFT polymerization is complicated by the asymmetric nature of RAFT agents, which have both a fragmenting (R) and a nonfragmenting

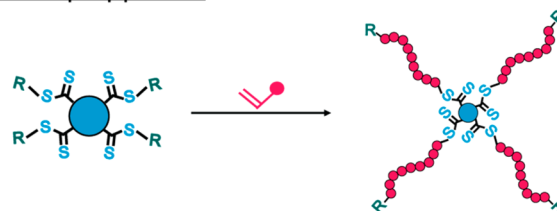
(Z) group. The particular synthetic approach is determined by which of these is conjugated to the core (Scheme 1). The Z-group approach (when the Z-group is covalently conjugated to

Scheme 1. Core-First Star Polymers Can Be Synthesized via RAFT Polymerization Using the R-Group or Z-Group Approach Depending on Orientation of the Asymmetrical RAFT Agent

R-Group Approach



Z-Group Approach



Received: August 16, 2019

Accepted: September 16, 2019

Published: September 20, 2019

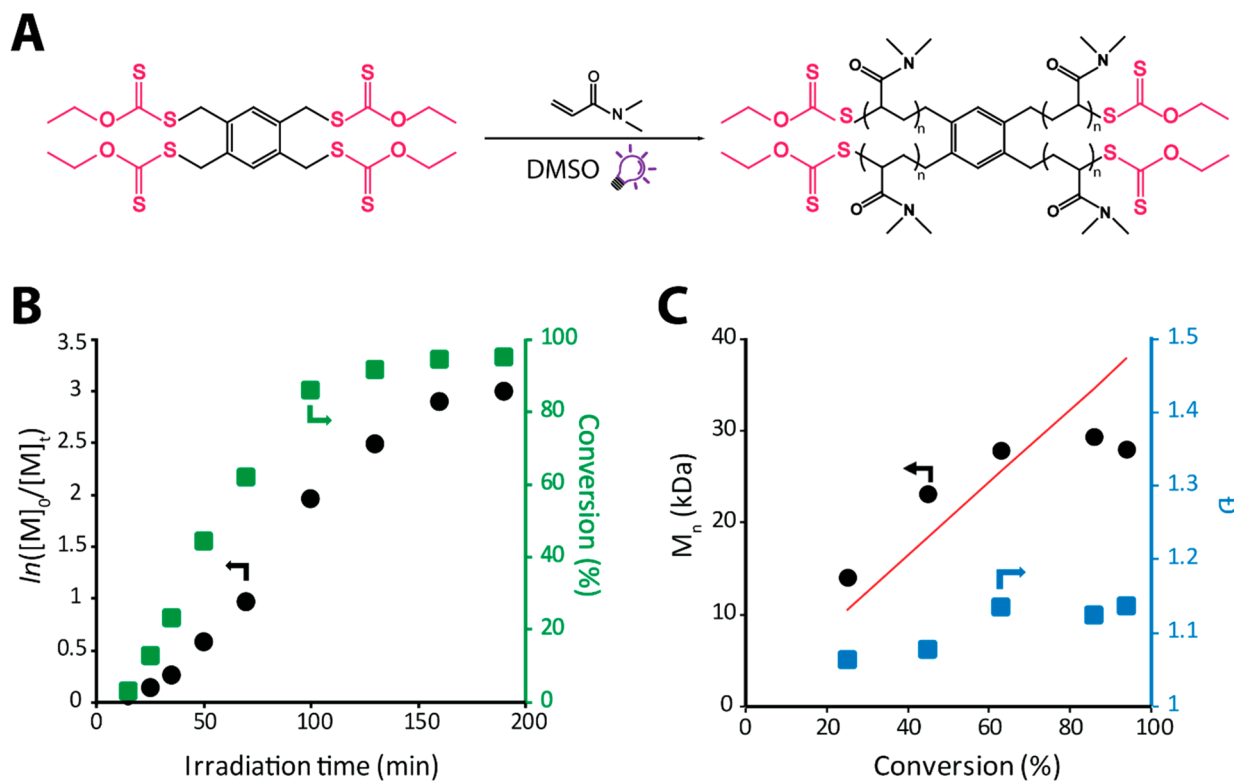


Figure 1. Core-first star polymers synthesized via xanthate-mediated photo-RAFT polymerization. (A) Schematic of DMA polymerization DP 100 per arm from 4-XAN under purple light, (B) kinetic studies of polymerization, and (C) dispersity, theoretical molecular weight (red line), and M_n versus conversion.

the core) prevents star–star coupling, a common side reaction when using the R-group approach.^{6,11,14} However, synthesis of high molecular weight star polymers is difficult due to steric hindrance and congestion around the core, as the chain length of the growing arms and/or the number of arms is increased.^{1,6}

Star–star coupling is often observed in reversible-deactivation radical polymerization (RDRP) when a core-first synthetic approach is used, including for RAFT polymerization via the R-group approach. This is due to the location of the propagating radical species at the end of each arm.¹⁵ The R-group approach does have advantages, including the presence of the thiocarbonylthio group at the star periphery, offering additional opportunities for postpolymerization functionalization. Moreover, if star–star coupling could be avoided, then this approach would offer the potential to achieve high molecular weight star polymers with precise arm numbers, which would be challenging for the Z-group or coupling-to techniques.

In recent years, photo-mediated RAFT polymerization (photo-RAFT) has been shown to result in highly “living” polymers.^{16–18} As is convention, we use the term “living” here to specifically refer to a high retention of ω -chain end functionality—in our case the thiocarbonylthio moiety—that allows the polymer chain to be reinitiated and to continue chain growth. However, the use of photo-mediated RAFT polymerization has not yet been investigated for the synthesis of star polymers using the core-first approach. We hypothesized that photo-RAFT polymerization would help to minimize termination reactions, enabling us to reach high molecular weights. Additionally, using a photoiniferter-based initiation approach allows us to eliminate exogenous radical initiators

which may initiate chains that are not attached to the star core.¹⁹

To test this theory, we synthesized a tetrafunctional xanthate (4-XAN) core and investigated the polymerization of dimethyl acrylamide (DMA) under purple light (Figure 1A). A target degree of polymerization (DP) of 100 monomer units per arm was used, with polymerization kinetics shown in Figures 1B and 1C. The rate of polymerization was rapid, with 95% conversion obtained in less than 3 h of irradiation. Near-linear pseudo-first-order kinetics were observed until 95% conversion, indicating a constant radical concentration and suggesting high chain-end fidelity. The dispersity remained low throughout the polymerization, and the theoretical molecular weight (determined from NMR conversion) (Figure 1C red line) was similar to the molecular weight determined using GPC paired with light scattering.

A tetrafunctional trithiocarbonate (4-TTC) core was also synthesized and used to synthesize PDMA star polymers (Figure S8). Well-defined PDMA stars were obtained, although the rate of polymerization was much slower, with 94% conversion reached after 96 h. In addition, star polymerization of methyl acrylate (MA) was also attempted from both the 4-XAN and 4-TTC cores, albeit with less control (Figures S9 and S10, respectively). Polymerization of MA from 4-XAN was extremely rapid with 96% conversion achieved in 15 min. The experimental molecular weight from GPC analysis is significantly greater than the theoretical molecular weight, particularly at low conversions, suggesting free radical polymerization was the dominating mechanism, instead of xanthate-mediated chain transfer. The poor control observed in the polymerization of MA from 4-XAN is not surprising due to poor compatibility of acrylates and xanthate

RAFT agents. In general, more activated monomers such as MA are generally paired with more reactive RAFT agents such as trithiocarbonates and dithiobenzoates to achieve control.^{20,21} The higher propagation rate of acrylamides is better suited to the longer active period observed for xanthate RAFT agents, whereas MA is slower propagating, which leads to greater opportunity for termination. The 4-TTC core led to PMA stars with improved dispersities and molecular weights that matched theoretical molecular weights; however, a high molecular weight shoulder was observed, indicating star–star coupling had occurred (Figure S10).

Using the described photo-RAFT polymerization approach with the tetrafunctional xanthate 4-XAN, we were able to synthesize a range of well-defined star polymers with differing chain lengths per arm ($DP_{n,arm} = 100, 200, 500, \text{ and } 1000$) (Figure 2A, Tables S2 and S3). We further investigated the

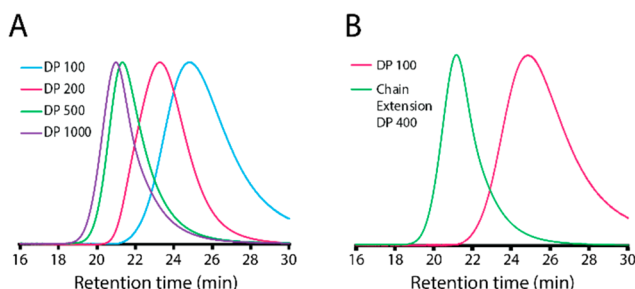


Figure 2. PDMA star polymers synthesized from the 4-XAN core via photo-RAFT polymerization under visible light are highly living. (A) GPC refractive index (RI) trace of star polymers synthesized with 4-XAN under purple light with target DPs of 200, 500, and 1000 per arm. (B) GPC trace of diblock polymer synthesized with 4-XAN under purple light with DMA DP 100 per arm for the first block and DMA DP 400 for the second block.

livingness of the star polymers synthesized from 4-XAN through *in situ* chain extension experiments, with a targeted degree of polymerization (DP) of 100 for the first block and chain extension of DP 400 (Figure 2B, Table S4). The GPC trace remained monomodal after chain extension, and the near-complete shift to lower retention times demonstrates the highly living property of the xanthate initiator.

Due to previously mentioned issues with core-first star polymer synthesis, star polymers with high MW arms and precise arm numbers have long been considered out of reach. However, inspired by the livingness observed in our experiments and recent work from the Sumerlin group that used a similar xanthate photo-RAFT polymerization method to synthesize ultra-high molecular weight (UHMW) linear polymers, we decided to attempt the synthesis of UHMW star polymers.²²

The 4-XAN core was used to polymerize DMA with a target DP of 10 100 per arm, the equivalent of 1 MDa per arm, using both purple and UV light irradiation. The use of UV was employed to increase the rate of polymerization, enabling reactions to reach 96% conversion in 6 h, while irradiation with purple light required 102 h to reach the same conversion. The corresponding theoretical molecular weights were 0.96 MDa per arm with a total molecular weight of 3.8 MDa (Figure 3B and Table 1). GPC traces of 4-XAN DP 10 100 stars initiated with purple light are shown in Figure 3A at 25, 43, and 96% conversion, while the trace of the UV-initiated star is shown in black at 96% conversion. The UV-initiated star trace appears

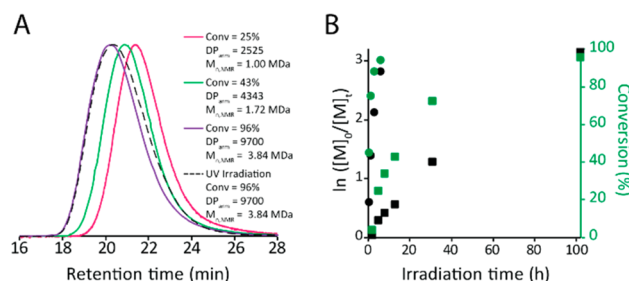


Figure 3. Synthesis of 4-arm UHMW star polymers. (A) GPC traces of 4-XAN DP 10 100 irradiated with purple light at 25, 43, and 96% conversion and UV at 96% conversion and (B) kinetic study of star polymerization using purple (squares) and UV (circles) light.

slightly broader than the purple-light-initiated star. This increased dispersity may result from direct activation of monomer by UV irradiation resulting in a less controlled polymerization than observed with purple light irradiation, where direct activation of the monomer does not occur.

Due to the ultra-high molecular weights of the polymers synthesized—beyond the limits of our GPC-coupled light-scattering detector—additional methods of characterization were investigated. Static light scattering (SLS) requires the use of a high precision laser whose light is scattered upon interaction with a sample and has been previously employed to determine the absolute molecular weight of star polymers.²³ A range of accurately determined concentrations are required to successfully carry out SLS measurements, with the intensity of the scattered light measured at multiple angles. A Zimm plot is generated that can provide the weight average MW and the radius of gyration (although not dispersity) (Figures 4C and S11). Therefore, using the assumption that our polymers were monomodally distributed, SLS indicated that the molecular weights of the 4-XAN DP 10 100 stars were a close match to their theoretical values, with observed MWs of 2.5 and 3.8 MDa for the UV and purple light, respectively (Figure 3 and Table 1).

To further study the limits of this photo-mediated star polymerization, β -cyclodextrin was modified so that each of its 21 hydroxyl groups was functionalized with a xanthate, resulting in a 21-xanthate core (21-XAN) (Figure S5). Star polymerization of DMA from the 21-XAN core with DPs 50, 200, and 500 per arm resulted in well-defined stars with monomodal, symmetrical GPC traces (Figure 4A). A star polymer with a per arm target DP of 1900 (target molecular weight of 4 MDa) was synthesized, and conversion of 95% was observed following 3.5 h of UV irradiation (Figure 4B). Once again, the theoretical M_n of 3.8 MDa and the experimental MW of 5.3 MDa from SLS were in relatively close agreement.

The largest molecular weight star polymer was synthesized using the 21-XAN core and a DP of 11 000 per arm. NMR indicated 96% conversion after 18 h of UV irradiation, corresponding to a total molecular weight of 21.9 MDa. NMR and SLS results were in agreement with the $M_{w(SLS)}$ equal to 20.4 MDa.

Additionally, the arms were cleaved from the core via acid-catalyzed hydrolysis and resulted in polymers with a M_n of 723 kDa (expected arm M_n was ca. 1043 kDa) and dispersity of 1.5 (Figure 4D), indicating a relatively high initiation efficiency from the core. To our knowledge, 20.4 MDa is the largest molecular weight reported for a star polymer synthesized via any RDRP.

Table 1. UHMW PDMA Star Polymers Synthesized with the Largest Known Star Polymer Synthesized to Date via RDRP Highlighted

Number of arms	DP per arm	Target MW (MDa)	$[M]_0$ (M)	Time (h)	Conversion (%) ^a	$M_{n(\text{theo})}$ (MDa) ^b	$M_{w(\text{SLS})}$ (MDa) ^c	R_g (nm) ^c
4	10 100	4	1	6	96	3.8	2.5	71.1
4 ^d	10 100	4	1	102	96	3.8	3.8	74.7
4	20 200	8	3	6	94	7.5	6.3	124.1
21	1 900	4	1	3.5	95	3.8	5.3	105.7
21	11 000	23	0.5	18	96	21.9	20.4	205.1
21	22 000	46	0.5	18	94	43.3	3.2	79.6

^aConversion was determined using ¹H NMR spectroscopy. ^bTheoretical molecular weights ($M_{n(\text{theo})}$) were determined via monomer conversion values. ^c $M_{w(\text{SLS})}$ and R_g were determined by static light scattering. ^dThis polymerization was performed using purple light irradiation, while all others were irradiated with ultraviolet light. Polymers where experimental and theoretical results were in agreement are highlighted in purple. The largest reported RDRP star polymer, with $M_{w(\text{SLS})}$ of 20.4 MDa, is highlighted in dark purple and bold text.

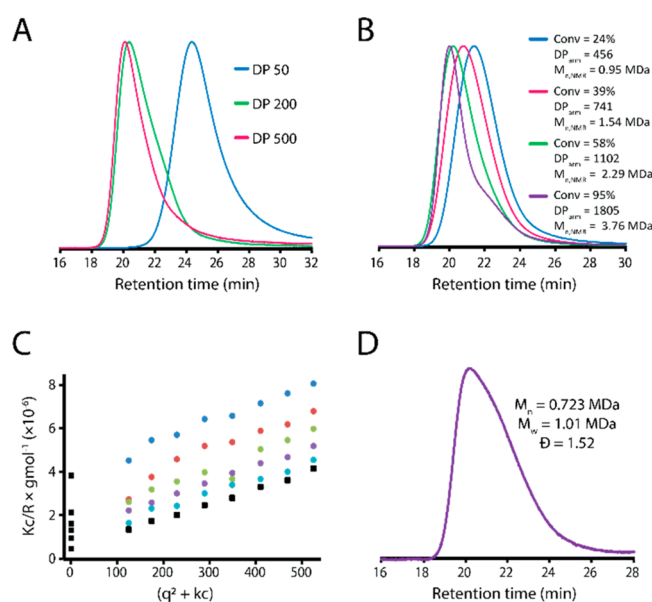


Figure 4. PDMA star polymers with 21 arms. GPC traces for star polymers with (A) DP 50, 200, and 500 per arm and (B) DP 1900 per arm at 24, 39, 58, and 95% conversion. (C) Zimm plot of 21-XAN DP 11 000 generated from SLS ($M_{w(\text{SLS})}$ 20.4 MDa, $M_{n(\text{theo})}$ 21.9 MDa). (D) GPC trace of PDMA arms cleaved from 21-XAN DP 11 000 after 16 h of stirring in 5 M HCl.

A range of polymers were synthesized to determine the limits of polymerization, in terms of both degree of polymerization and molecular weight (Table 1). The DP per arm was increased for both the 4-XAN and 21-XAN cores until theoretical and experiment molecular weights disagreed by more than 50%. The maximum successful degree of polymerization was obtained using the 4-XAN core with a target DP of 20 200, resulting in a star polymer with a $M_{n(\text{theo})}$ of 7.5 MDa and a $M_{w(\text{SLS})}$ of 6.5 MDa. Polymerizations from 4-XAN with a DP of 50 000 per arm achieved high conversion and theoretical molecular weights of nearly 18 MDa. However, SLS demonstrated the resulting polymers had molecular weights of 2.9 and 2.5 MDa when using purple and UV light, respectively.

We hypothesize this could be due to the shear stress of stirring, causing star arms to be broken from the core. Control experiments without a RAFT agent showed no conversion following purple light irradiation, supporting that initiation was only occurring from the star core. The Matyjaszewski group has previously reported the cleavage of arms from a brush-arm star polymer core, which they hypothesized was a result of congestion and the highly strained architecture of the star polymer core.²⁴

To determine the limit in terms of total molecular weight, we attempted to synthesize a star polymer from the 21-XAN core with a target DP of 22 000 per arm. Following 18 h of UV irradiation, monomer conversion was 94% for a theoretical MW of 43.3 MDa, yet SLS analysis revealed an apparent experimental molecular weight of 3.2 MDa. We propose that this discrepancy is due to the extreme dilution of the photoactive (xanthate) species, leading to background polymerization. Control experiments demonstrated free radical polymerization of DMA in DMSO with UV irradiation, explaining how high conversion could be achieved, while a much lower than expected MW was observed. These observations indicate that it is more likely an effect of the arm chain length rather than the overall molecular weight that results in these synthetic limitations, as can be seen in Figure S12.

In conclusion, we report the synthesis of well-defined, monomodal UHMW star polymers via photo-mediated RAFT polymerization to conversions of over 95%. The highly “living” nature of these star polymers enabled the synthesis of UHMW stars of extremely long arm lengths, including the largest star polymer synthesized to date using RDRP techniques. The facile synthesis of well-defined UHMW star polymers may provide access to unique star-based polymeric materials that were previously unattainable.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmacrolett.9b00643.

Additional experimental and characterization data for core and star polymer products including absorbance data, polymerization kinetics, Zimm plots, ^1H NMR spectra, and gel permeation chromatograms (PDF)

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors acknowledge the Australian Research Council (ARC) for funding in the form of a Discovery Project (DP140100002). S.A.-L. is the recipient of a Melbourne International Engagement Award, and F.K. is the recipient of an International Postgraduate Research Scholarship. G.B. acknowledges the Australian Microscopy & Microanalysis Research Facility at RMIT University. The authors would also like to thank Prof. Krzysztof Matyjaszewski for his insightful suggestions and discussions.

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From UV to NIR: A Full-Spectrum Metal-Free Photocatalyst for Efficient Polymer Synthesis in Aqueous Conditions

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Abstract: Photo-mediation offers unparalleled spatiotemporal control over controlled radical polymerizations (CRP). Photo-induced electron/energy transfer reversible addition–fragmentation chain transfer (PET-RAFT) polymerization is particularly versatile owing to its oxygen tolerance and wide range of compatible photocatalysts. In recent years, broadband- and near-infrared (NIR)-mediated polymerizations have been of particular interest owing to their potential for solar-driven chemistry and biomedical applications. In this work, we present the first example of a novel photocatalyst for both full broadband- and NIR-mediated CRP in aqueous conditions. Well-defined polymers were synthesized in water under blue, green, red, and NIR light irradiation. Exploiting the oxygen tolerant and aqueous nature of our system, we also report PET-RAFT polymerization at the microliter scale in a mammalian cell culture medium.

Photo-mediation enables spatiotemporal control of controlled/living radical polymerizations (CRP) while maintaining excellent dispersity and chain-end fidelity.^[1] For this reason, visible light-mediated reversible addition–fragmentation chain transfer (RAFT) polymerization has been studied extensively since its discovery in 2014.^[2] Such RAFT polymerization encompasses two distinct mechanisms known as photoiniferter and photo-induced electron/energy transfer (PET)-RAFT approaches. Photoiniferter RAFT polymerization requires direct activation of the RAFT agent via light irradiation and is therefore limited to wavelengths absorbed by the RAFT agent, generally UV, blue, or green light.^[3] The PET-RAFT mechanism depends on the excitation of a photo-

redox catalyst or a photosensitizer by light irradiation. The excited catalyst/photosensitizer interacts with the RAFT agent via energy or electron transfer to generate the initiating radical for polymerization.^[2,4] Numerous compounds have been employed as photoredox catalysts or photosensitizers for PET-RAFT polymerization including organic dyes,^[5] metalloporphyrins such as zinc tetraphenylporphyrin (ZnTPP),^[6] and naturally-occurring photosynthetic pigments,^[7] enabling PET-RAFT polymerization across the visible light spectrum. Interestingly, a photoenzymatic RAFT mechanism using flavin-dependent enzymes was recently reported, with visible light causing photoexcitation of FADH[−] to facilitate the PET process and mediate polymerization.^[8] Furthermore, PET-RAFT polymerization is typically less sensitive to oxygen and can therefore be performed under less stringent conditions.^[8,9]

As PET-RAFT polymerization has gained popularity due to its versatility and less stringent reaction conditions, there has been a push to develop photocatalysts with broadband and near-infrared (NIR) absorption, owing to potential green chemistry and biomedical applications. The solar spectrum covers wavelengths from 250–2500 nm, with IR energy alone making up nearly 50 % of solar energy, while photons from visible light and NIR constitute 95 % of the solar flux at sea level.^[10] Therefore, broadband and NIR absorbance are necessary to achieve efficient solar energy utilization. Furthermore, NIR can penetrate opaque materials including human tissues, and therefore has potential use in biomedical applications and even in vivo polymerization.^[11] Boyer and co-workers reported the first NIR-mediated CRP, using bacteriochlorophyll a as a photocatalyst.^[12] More recently, they reported the use of aluminum naphthalocyanine as a photosensitizer for peroxides to initiate RAFT polymerization under NIR irradiation.^[13] The authors demonstrated temporal control and achieved high conversions even with paper, chicken skin, and pig skin barriers. These reports are significant developments in NIR-mediated photopolymerization and potential in vivo polymerization but to date have been limited to organic solvent.

Focusing on broadband photopolymerization, Matyjaszewski and co-workers exploited the localized surface plasmon resonance (LSPR) effect of nanostructured silver orthophosphate (Ag₃PO₄) photocatalysts.^[14] PET-RAFT polymerization of methyl acrylate demonstrated high conversions following blue, green, and red light irradiation as well as when exposed to natural sunlight. Furthermore, polymerization of benzyl acrylate was possible using NIR irradiation. However, polymerization was again only limited to organic solvent.

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Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under:
<https://doi.org/10.1002/anie.202007196>.

To date, all NIR-mediated CRP polymerization systems reported have been employed in organic solvents.^[12–15] However, the development of aqueous systems is essential for any potential *in vivo* applications and could also offer less expensive and more environmentally friendly options for industrial use. To aid with the development of aqueous broadband- and NIR-responsive systems, we looked to recent advances in the field of photocatalysis for solar-driven hydrogen evolution. Our group has previously reported efficient PET-RAFT polymerization with organic semiconductor graphitic carbon nitride (g-C₃N₄),^[16] an organic photocatalyst previously used in hydrogen evolution.^[17] With the use of g-C₃N₄, RAFT polymerization can be carried out with no degassing or monomer purification.^[16] Due to the limited absorbance spectrum and relatively hydrophobic surface functional groups of g-C₃N₄, its activity for polymerizations was only studied under UV irradiation in DMSO. More recently, novel photocatalysts such as covalent triazine-based framework-1 (CTF-1)^[18] and formic acid-treated carbon nitride (FAT)^[19] have shown narrower band gaps and red-shifted absorbance spectra in comparison to g-C₃N₄. Due to the improved function of these new catalysts, our previous successful PET-RAFT polymerizations using g-C₃N₄, and the need of aqueous PET-RAFT systems under NIR irradiation, we decided to investigate the use of these photocatalysts for PET-RAFT polymerization under various wavelengths in an aqueous system.

Furthermore, we synthesized a novel self-assembled carboxylated porphyrin (SA-TCPP) photocatalyst. Although it has been previously shown that nanometric SA-TCPP particles had a high solar spectrum efficiency and enhanced hydrogen and oxygen evolution from water under visible light irradiation,^[20] our prepared micro-size SA-TCPP photocatalyst displayed a broader absorbance spectrum from 300 to 950 nm (Figure 1A; Supporting Information, Figure S1). Owing to the micro-fiber-like morphology, the lifetime of photoexcited electron–hole pairs is further extended, which in turn can lead to an improved polymerization efficiency compared to other recently reported photocatalysts for hydrogen evolution. As our PET-RAFT polymerization with this catalyst is in aqueous conditions under broad wavelengths, we further tested its polymerization ability in biological cell culture media, investigating its potential for *in situ* biomedical applications as well as highly efficient green controlled polymerizations.

We prepared the SA-TCPP photocatalyst through a modified approach. Commercially available 5,10,15,20-tetra (4-carboxy-phenol) porphyrin (TCPP) was deprotonated in an aqueous 1M KOH solution and heated to 80 °C, yielding a purple solution. 0.1M HCl was added dropwise until the pH became neutral, following which the precipitate was purified via dialysis and freeze-dried. The resultant self-assembled TCPP (SA-TCPP) showed a dark green color and formed long fibers/rods as seen using SEM (Scheme 1; Supporting Information, Figure S2). The obtained rods displayed a non-crystalline X-ray diffraction (XRD) pattern and this result was further confirmed by TEM diffraction measurement (Figure 1B). Two obvious peaks at 7° and 22° represent the 1.26 nm width of an aggregated TCPP molecule and the

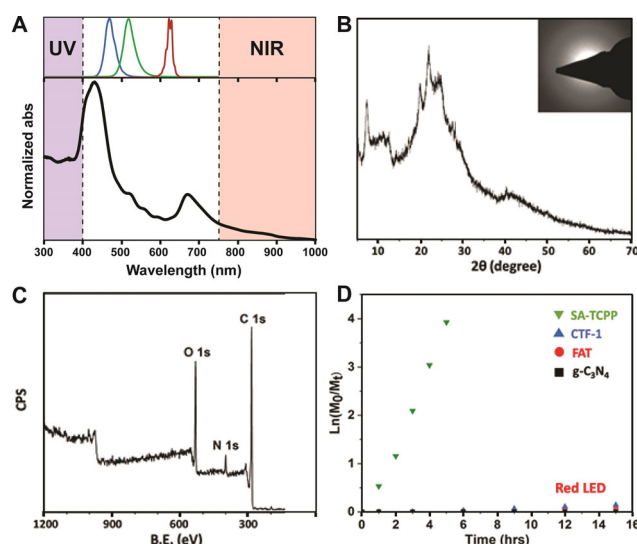
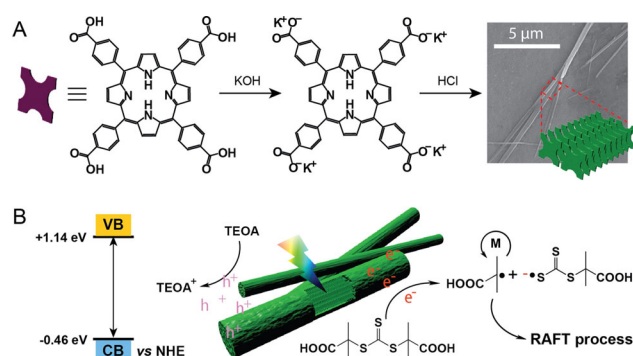


Figure 1. A) UV/Vis normalized absorption spectrum of SA-TCPP (bottom) and LED light source emission spectra (top). B) XRD and electron diffraction patterns of SA-TCPP. C) Full XPS spectrum of SA-TCPP. D) Polymerization kinetics of DMA using SA-TCPP and previously reported photocatalysts under red light irradiation.



Scheme 1. A) The synthetic strategy for rod-like SA-TCPP and B) schematic mechanism of the photocatalytic PET-RAFT polymerizations by SA-TCPP.

0.4 nm face-to-face distance between TCPP molecules. X-ray photoelectron spectroscopy (XPS) peaks assigned to C 1s, N 1s, and O 1s were located at 285, 400, and 533 eV, respectively, and were consistent with the molecular structure of SA-TCPP (Figure 1C; Supporting Information, Figure S4). Furthermore, the electrochemical experiment further revealed that the prepared SA-TCPP rods exhibit a band gap of −0.46 to +1.14 eV (Supporting Information, Figure S5).

The broad absorption spectrum of SA-TCPP allowed us to investigate its efficiency in comparison to previously reported H₂ evolution photocatalysts g-C₃N₄, CTF-1, and FAT, in water ([photocatalyst] = 2.5 mg mL^{−1}) under blue, green, and red light irradiation (Supporting Information, Figure S7). In all cases, polymerizations performed with SA-TCPP resulted in the highest conversion. All polymerizations showed linear semi-logarithmic plots and dispersities of 1.1 or below. While all polymerizations achieved high conversions under blue

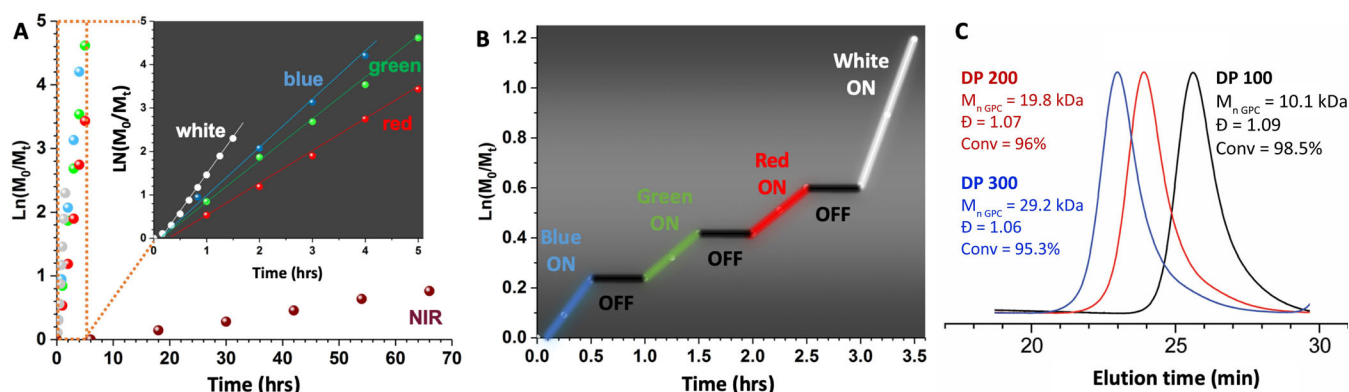


Figure 2. Kinetics of SA-TCPP-catalyzed RAFT polymerizations of DMA under A) various LED irradiation (white to NIR, 4 mWcm⁻²) and B) in an ON/OFF experiment. C) GPC evolution of a *pseudo*-pentablock PDMA by chain extension.

light irradiation, SA-TCPP increasingly outperformed the other catalysts as the irradiation wavelength increased and was the only catalyst that led to polymer formation under red light irradiation (Figure 1D).

The polymerization kinetics of DMA using SA-TCPP under blue, green, red, white, and NIR irradiation are shown in Figure 2A. The use of CTF-1, FAT, and g-C₃N₄ was not investigated under NIR irradiation owing to their limited absorbance spectra and poor performance under red light. In a typical reaction, DMA (100 equiv), trithiocarbonate (TTC) RAFT agent (1 equiv), triethanolamine (TEOA, 13.4 equiv), and SA-TCPP (5 mg) were dissolved in DI water (50 % v/v). Reaction mixtures were bubbled with argon for 20 minutes prior to light irradiation to afford faster polymerization and reduced induction period, although degassing and addition of TEOA were not essential, as previously reported (Supporting Information, Figure S8).^[16]

The rate of polymerization was highest using white light, followed by blue, green, red, and NIR (Figure 2A). Monomer conversions of 90 % or greater were achieved in 90 min using white light, 3 h with blue or green light, and 4 h with red light. Although the NIR-mediated polymerization was significantly slower, 53 % conversion was reached in 66 h. These results are consistent with the absorbance spectrum of SA-TCPP shown in Figure 1A, where absorbance was greatest at blue-shifted wavelengths, as well as the higher energy of lower wavelength photons. In all cases, linear pseudo-logarithmic plots were observed indicating a constant radical concentration.

The temporal control of the system was then demonstrated using an on/off experiment, where the light source was turned on and off at 30 minute intervals (Figure 2B). Several cycles were performed with a different wavelength of light used during each on period, highlighting the broadband absorption of the photocatalyst. No polymerization was observed during the off period, demonstrating instantaneous temporal control. After 2 hours of irradiation, 70 % monomer conversion was achieved. Pleasingly, the rates of polymerization during the on periods were in agreement with those observed in Figure 2A, with white light showing the fastest rate of polymerization, followed by blue, green, and red.

Crucially, retention of the TTC RAFT agent and its living nature were demonstrated via two chain extension reactions

with greater than 95 % monomer conversion attained for each block (Figure 2C; Supporting Information, Figure S9). GPC traces showed symmetrical, monomodal peaks after each chain extension. Due to the symmetrical nature of the RAFT agent employed (S,S'-Bis(α , α' -dimethyl- α'' -acetic acid)trithiocarbonate), the TTC moiety remained at the center of the polymer and the two chain extensions experiments resulted in a well-defined *pseudo*-pentablock polymer.

Successful NIR-mediated polymerization with SA-TCPP suggested PET-RAFT polymerization could be performed through barriers. NIR is known to penetrate barriers, including mammalian tissues, and the Boyer and Matyjaszewski groups have previously reported successful NIR-mediated PET-RAFT polymerization through materials such as paper and animal skin.^[13,14] Interestingly, Boyer and co-workers observed minimal change in the apparent rate propagation coefficient when polymerizing methyl acrylate through 0.1 mm paper, 1 mm chicken skin, and 2.5 mm pig skin barriers, mediated by NIR (850 nm).^[13]

Therefore, we set out to determine if we could perform our polymerization through a barrier using NIR (850 nm) and SA-TCPP in an aqueous environment. We carried out our investigation using a piece of opaque paper wrapped around the reaction vessel as a barrier. An induction period of 6 hours was observed before achieving 30 % conversion after 66 h (Figure 3B). The GPC trace of the resulting polymer was monomodal and symmetrical, with a low dispersity of 1.07, as shown in Figure 3C. To our knowledge, this is the first time NIR irradiation has been used for CRP polymerization in water. These results signify an essential step towards biocompatible NIR-mediated polymerization, where an aqueous system would be essential.

Any *in vivo* polymerizations would also need to be biocompatible and performed with a low volume. To this end, we attempted a preliminary study of the PET-RAFT polymerization using PEG methacrylate (PEGMA, 480 Da) in the presence of mammalian fibroblast cells in a 96-well plate (Figure 4A). Six wells each contained 0.13 mg SA-TCPP, 22 mg PEGMA, 0.7 mg TTC, and 0.5 mg TEOA in complete DMEM cell culture medium to a total volume of 100 μ L. The 96-well plate was irradiated with red light for 45 minutes at 37 °C. The conversion of PEGMA was 11 % and

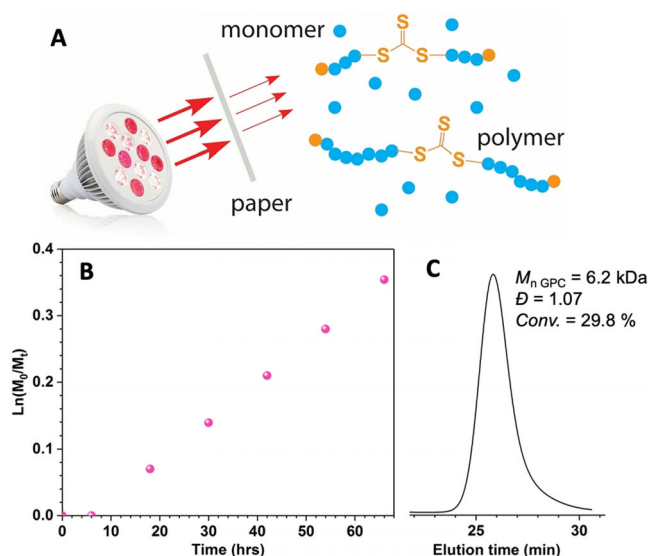


Figure 3. A) Illustration of an opaque paper barrier employed for polymerizations under NIR LED light ($\lambda_{\text{max}} = 850 \text{ nm}$). B) Kinetic study of SA-TCPP catalyzed RAFT polymerization of DMA under NIR LED irradiation (4 mWcm^{-2}). C) GPC curve of the resultant PDMA.

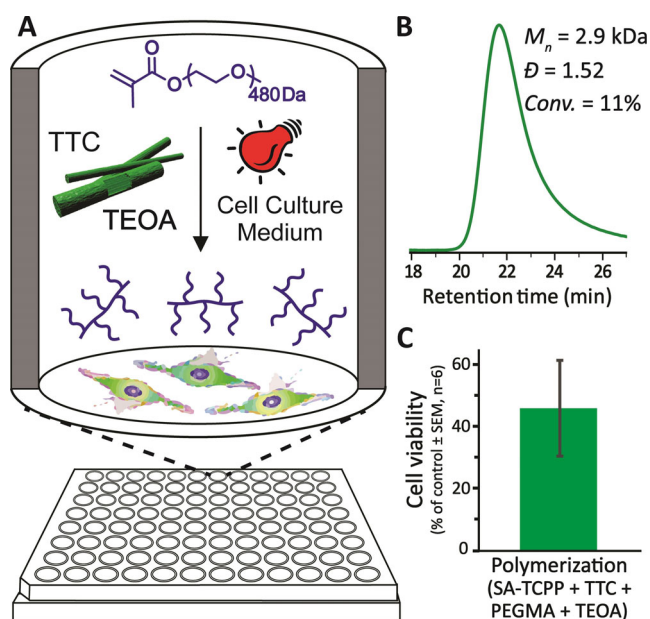


Figure 4. A) Microliter-scale PET-RAFT polymerization of PEGMA₄₈₀ in a 96-well plate containing fibroblast cells, 45 min of red light irradiation. B) GPC trace of poly(PEGMA), and C) cell viability following PET-RAFT polymerization.

GPC characterization showed a monomodal peak with a small tail (Figure 4B). Significantly, this work demonstrates our system can conduct polymerization without degassing at microlitre scale, offering the future potential for machine programmable multi-block polymerization via computer-controlled microliter injection systems. We also measured cell viability immediately following light irradiation and obtained 46% cell viability (Figure 4C). While optimization of reac-

tion conditions and reagents is needed to reduce the toxicity of the system, these preliminary results suggest a biocompatible, NIR-mediated PET-RAFT polymerization may soon be possible.

The recent reports by the Boyer,^[12,13] Matyjaszewski,^[14] and Yagci^[15a] groups demonstrate exciting developments in broadband- and NIR-mediated CRP. Building on these critical advances, we report for the first time an aqueous NIR-mediated CRP. The use of a metal-free, porphyrin-based organic photocatalyst with an absorbance spectrum covering 300–950 nm enabled broadband- and NIR-mediated PET-RAFT polymerization in water. SA-TCPP was compared to three other photocatalysts previously reported for hydrogen evolution and was found to have the highest rate of polymerization when irradiated with white, blue, green, and red light. Preliminary investigations resulted in the successful microliter-scale polymerization of PEGMA in the presence of mammalian cells in complete cell culture medium, with future works to focus on improving cell viability. These findings have broad implications for potential in vivo polymerizations for biomedical applications as well as green polymerizations.

Acknowledgements

G.G.Q. acknowledges the Australian Research Council's Discover Project (DP170104321). S.A.-L. is the recipient of a Melbourne International Engagement Award. M.L. acknowledges support from the China Scholarship Council-University of Melbourne Research Scholarship (201606260063). Q.F. acknowledges the Australian Research Council under the Future Fellowship (FT180100312). J.X. and J.T. are thankful for the financial support from the Royal Society Newton Advanced Fellowship grant (NAF/R1\191163) and Leverhulme Trust (RPG-2017-122).

Conflict of interest

The authors declare no conflict of interest.

Keywords: NIR · photocatalysis · photopolymerization · RAFT polymerization

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Manuscript received: May 18, 2020

Revised manuscript received: June 20, 2020

Accepted manuscript online: August 5, 2020

Version of record online: ■■■■■, ■■■■■

Communications

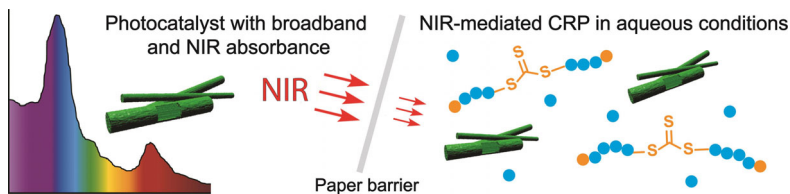
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Photopolymerization



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From UV to NIR: A Full-Spectrum Metal-Free Photocatalyst for Efficient Polymer Synthesis in Aqueous Conditions



By employing a metal-free photocatalyst and PET-RAFT approach, we present the first broadband- and NIR-mediated controlled radical polymerization in aqueous

media, including cell culture medium, marking a crucial advance towards in vivo polymerization for biomedical applications.