Nanoengineered Templated Polymer Particles: Navigating the Biological Realm

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CONSPECTUS:

Nanoengineered materials offer tremendous promise for developing the next generation of therapeutics. We are transitioning from simple research questions, such as “can this particle eradicate cancer cells?” to more sophisticated ones like “can we design a particle to preferentially deliver cargo to a specific cancer cell type?” These developments are poised to usher in a new era of nanoengineered drug delivery systems.

We primarily work with templating methods for engineering polymer particles, and investigate their biological interactions. Templates are scaffolds that facilitate the formation of particles with well-controlled size, shape, structure, stiffness, stability, and surface chemistry. In the past decade, breakthroughs in engineering new templates, combined with advances in coating techniques, including layer-by-layer (LbL) assembly, surface polymerization, and metal-phenolic network (MPN) coordination chemistry, have enabled particles with specific physicochemical properties to be engineered. While materials science offers an ever-growing number of novel synthesis techniques, a central challenge of therapeutic delivery has become understanding how nanoengineered materials interact with biological systems. Increased collaboration between chemists, biologists, and clinicians has resulted in a vast research output on bio-nano interactions. Our understanding of cell-particle interactions has grown considerably, but conventional *in vitro* experimentation provides limited information, and understanding how to bridge the *in vitro/in vivo* gap is a continuing challenge. As has been demonstrated in other fields, there is now a growing interest in applying computational approaches to advance this area. A considerable knowledge base is now emerging, and with it comes new and exciting opportunities that are already being capitalized on through the translation of materials into the clinic.

In this Account, we outline our perspectives gained from a decade of work at the interface between polymer particle engineering and bio-nano interactions. We divide our research into three areas: (i) biotrafficking, including cellular association, intracellular transport, and biodistribution; (ii) biodegradation, and how to create controlled, responsive release of therapeutics; and (iii) applications, including drug delivery, controlling immunostimulatory response, biosensing, and microreactors. There are common challenges in these areas for groups developing nanoengineered therapeutics.

A key “lesson-learned” has been the considerable challenge of staying informed about the developments relevant to this field. There are a number of reasons for this, most notably the interdisciplinary nature of the work, the large numbers of researchers and research outputs, and the limited standardization in technique nomenclature. Additionally, a large body of work is being generated with limited central archiving, other than vast non-specific databases, such as Web of Science and PubMed. To help address these points, we have created a web-based tool to organize our past, present, and future work [Bio-nano research knowledgebase. http://bionano.eng.unimelb.edu.au/knowledge_base/ (accessed May 2, 2016)]. This tool is intended to serve as a first step toward organizing results in this large, complex area. We hope that this will inspire researchers, both in generating new ideas, and also in collecting, collating and sharing their experiences to guide future research.

1. INTRODUCTION

The central goal of particle-based therapeutics is to increase therapeutic efficacy while decreasing side effects. Recently, there has been considerable research into the interactions between biological systems and nanostructured materials (bio-nano interactions), which will guide the engineering of the next generation of therapeutic vehicles.^{1,2} Nanotechnology is an important bridge between engineering and biotechnology, and has advanced the design and characterization of particles for biomedical applications.^{3,4} As a result, a range of delivery systems, including numerous types of polymer particles, have been generated over the past three decades. Importantly, the physicochemical properties of these polymer particles, such as the size, shape, rigidity, structure, and surface chemistry, are crucial in guiding our understanding of bio-nano interactions and related biomedical applications.^{5,6}

The nanotechnology-driven templating method for the fabrication of polymer particles can be achieved via coating, growing, or infiltrating polymers on the surfaces or into the pores of a template, followed by polymer stabilization or cross-linking, and finally template removal. Templating is a versatile technique that provides a means of controlling the physicochemical properties of the resultant polymer particles. For example, size and shape are usually dependent on the morphology of the template;^{7,8} rigidity can be controlled by cross-linking density or polymer layer thickness;^{9,10} hollow or network structures can be tuned by using solid or porous templates;^{11,12} and the surface chemistry can be varied depending on polymer composition and post-functionalization.¹³ Different techniques, such as layer-by-layer (LbL) assembly,¹⁴ surface polymerization,¹⁵ and mesoporous silica (MS) templating¹⁶ have been developed to engineer these physicochemical properties.

This account will focus on our recent endeavors in studying bio-nano interactions governed by the physicochemical properties of templated polymer particles. In the past 10 years we have transitioned from focusing on particle design to focusing on both *in vitro* and *in vivo* studies (Figure 1). The first section includes the effects of physicochemical properties of particles on cellular association, including cellular binding and uptake, intracellular trafficking, biodistribution, and pharmacokinetics. Degradation is crucial for particles in controlled therapeutic delivery, therefore, the second section highlights particle degradability due to biological stimuli (e.g., pH, redox potential, and enzymes) during microenvironment changes in the cellular uptake process. In the final section, we discuss applications of templated polymer particles in drug and vaccine delivery, as well as in the design of sensors and bioreactors.

Figure 1. Timeline providing examples of bio-nano interactions studied using particles assembled through templating methods in the Caruso group. Parts of this figure are adapted with

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2. *IN VITRO* AND *IN VIVO* INTERACTIONS

Bio-nano interactions of polymer particles at the cellular level include cellular binding, cellular uptake, and intracellular trafficking, which are all influenced by the physicochemical properties of particles (Figure 2). Particle size and shape have been widely reported to influence uptake pathways and intracellular trafficking *in vitro*.^{2,26} Additionally, size and shape influence particle flow, margination, and adhesive properties in blood vessels.²⁷ Particle stiffness is another complex factor that influences both cellular association and uptake.²⁸ The precise effect of varying stiffness depends on cell type, surface chemistry, size, and geometry.²⁹ For instance, we observed higher cell binding and uptake with soft, hollow particles, which we attribute to their deformation upon contact with the cell membrane, leading to higher contact area.^{10,30} In contrast to this, others have reported higher uptake when using stiffer particles.³¹ It is also important to note that terms such as elasticity, hardness, rigidity, and flexibility are widely and often interchangeably used in the literature, though they are not equivalent.²⁹

Figure 2. A summary of polymer particles with controlled size, shape, rigidity, surface chemistry, stability, and structure that have been explored for bio-nano interaction *in vitro* and *in vivo*.

Cellular association is not only governed by the physical properties of particles, but can also be tuned by varying the chemistry of the particle surface. For example, polymer particles composed of low-fouling materials (e.g., PEG) or modified with low-fouling polymers show decreased cellular association.³²⁻³⁴ To increase specific cellular binding or uptake, targeting molecules can be functionalized onto polymer particle surfaces either by electrostatic adsorption or covalent conjugation.^{17,18,22,34,35} Particles are exposed to a complex environment in cell cultures and *in vivo*, and without low-fouling surfaces, they will be rapidly coated with various biomolecules. This coating, termed the protein corona, is strongly influenced by particle size and surface chemistry.³⁶ Furthermore, the protein corona can dramatically alter the bio-nano interactions depending on targeting ligand and cell type.^{23,36-38} Interestingly, targeting to cells increases association, but this may not actually increase uptake, which is highly dependent on the receptors targeted.^{22,39} It is important to draw a distinction between “particles binding to the surface of cells” and “particles internalized by cells” (cellular uptake); cellular association includes both of these.

The cellular uptake mechanism (e.g., phagocytosis, macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin/caveolae-independent pathways) is influenced by particle properties, and this can vastly impact on biological outcomes.²⁶ Cellular uptake is a time-dependent process, and longer incubation times result in higher numbers of

internalized particles.²⁰ Internalized particles are usually transported from endosomes to lysosomes in a matter of minutes,⁴⁰ and less stiff particles have demonstrated faster transport to lysosomes.²⁸ Interestingly, polymer particles located in the lysosome are not distributed equally between the two daughter cells upon cell division, with ubiquitylation playing a central role in the overall cell response to particles.⁴⁰ Although hollow polymer particles with different aspect ratios or rigidities can have different uptake kinetics, these differences do not appear to influence intracellular fate.^{10,41} Combined with flow cytometry, polymer particles equipped with a switch that responds to microenvironmental changes in pH allows for sensitive, high-throughput investigation of particle-cell interactions *in vitro*.⁴²

The reason for studying physicochemical effects on particle association and trafficking is both to increase our understanding and also to engineer particles for specific *in vivo* applications. In terms of biodistribution, smaller poly(ethylene glycol) (PEG) particles composed of large molecular weight PEG demonstrate extended *in vivo* circulation time when compared to larger particles and particles with lower molecular weight PEG.⁷ PEG particles without templates show significantly higher blood retention compared to MS@PEG particles with mesoporous silica templates. This may be because of differences in particle stiffness, as softer particles could minimize the filtration effect experienced during circulation *in vivo* by compressing similar to red blood cells.^{24,31,43} Another example of physicochemical properties affecting function is the observation that PEGylating polymer particles dramatically improves their lymphatic drainage *in vivo*.²⁵ Still, there is a large gap between *in vitro* predictions and *in vivo* behavior. A compromise may be *ex vivo*: assays based on whole human blood are more predictive of PEG particle behavior *in vivo* than traditional cell-line based *in vitro* assays.⁷

3. BIODEGRADATION

One crucial material requirement of therapeutic polymer particles is the capacity to degrade or disassemble to release the encapsulated cargo via biological stimuli in targeted microenvironments (Figure 3). During cellular internalization, particles will often be exposed to a decrease in pH, an increase in the amount of reducing agents, and an increase in enzyme concentration as they proceed from the extracellular fluid into the endosomes/lysosomes.²⁶ The acidification of the endosome/lysosome can occur minutes after internalization, leading to a pH of ~4-6. Similarly, various enzymes are pumped into the endosome, lysosome or cytoplasm to further degrade and destroy both foreign and natural biological materials. Finally, cells also contain reducing agents, such as glutathione (GSH), that break disulphide bonds. These three biological degradation mechanisms (pH, enzymes, reducing agents) allow for a broad spectrum of engineered responses in polymer particles, though other less common triggers, such as sugar gradients⁴⁴ or shear,⁴⁵ also exist.

A Examples of extracellular microenvironments

B Examples of intracellular microenvironments

C Examples of responsive polymer particle design

Figure 3. Examples of (A) extracellular and (B) intracellular microenvironments. (C) Rational design of responsive polymer particles and mechanisms of triggered particle degradation and/or cargo release via biological stimuli (e.g., pH, enzyme, and redox) after internalization. The examples and values included are intended to provide a general overview and are not exhaustive.

As polymer particles are internalized, the pH drops from 7.4 in the extracellular environment to a range of ~6.5-4.5 in endosomes or lysosomes. Therefore, materials that are either protonated or deprotonated in this range are promising candidates for engineering degradable particles. Synthetic polymers with a pK_a below 7.4, such as poly(2-diisopropylaminoethyl methacrylate) (PDPA), are useful for forming pH responsive, degradable polymer particles.⁴⁶⁻⁴⁸ Similarly, biotin-modified PEG can be used as a capping polymer to form neutravidin-iminobiotin bonds that rapidly decompose around pH 4-6, as a result of the lower affinity of the protonated iminobiotin to neutravidin.⁴⁹ Additionally, pH labile cross-linkers, such as 8-arm-PEG-methylmaleic anhydride, can be used to form protein replica particles capable of disassembling below pH 6.5 and after cellular internalization.⁵⁰

Unlike synthetic polymers, the multiple charge states of both polyphenols (e.g., tannic acid, TA), and metal ions allow for metal-phenolic network (MPN) particles to exhibit varied pH-disassembly profiles.^{51,52} Generally, MPN particles composed of higher valence metal ions are more stable under acidic pH than those composed of lower valence metal ions ($Zr^{4+} > Al^{3+} > Cu^{2+}$). Interestingly, Fe^{3+} results in one of the most stable MPN particles, likely due to specific affinity differences between polyphenols and certain metal ions. Al^{3+} has been shown to have favorable disassembly kinetics for drug delivery, and can degrade and release anticancer drugs in the endosome/lysosome.⁵³ Polymer-phenol conjugates can also form MPNs, which allow for the assembly of pH-degradable low-fouling PEG hydrogel particles.³² Boronic acid, rather than a metal, can be combined with TA to form boronate-phenolic network (BPN) particles that are responsive to both pH and sugar.⁴⁴

Polymer particles composed of biomolecules or containing biomolecule cross-linkers can be degraded by enzymes encountered in the bloodstream and during cellular internalization. Hollow particles composed solely of proteins are promising candidates for protease degradable carriers,¹² and the chemical properties of the proteins (reduced, native, or oxidized) can play a role in their degradation profiles.⁵⁴ Similarly, particles containing or composed of poly(L-lysine), poly(L-glutamic acid), poly(L-arginine), poly(L-histidine), or gelatin can also be degraded by proteases in solution and in the proteolytic environment of endosomes/lysosomes.⁵⁵⁻⁶⁰ By cross-linking synthetic polymer particles with an enzyme-cleavable peptide sequences, degradation can be engineered in response to specific proteases (e.g., cathepsin B or thrombin), allowing for *in vitro* degradation within cancer cells,⁶¹ or in the thrombus microenvironment.⁶²

The intracellular environment contains reducing agents (e.g., GSH), which can help to degrade polymer particles stabilized with disulfide bonds. Disulfide bonds are usually assembled by cross-linking polymers with a disulfide cross-linker, or by cross-linking thiolated polymers using oxidizing reagents or thiol-disulfide exchange.^{9,21,48,63} Cargo can also be conjugated to particles via disulfide bonds, and can then be released in the presence of reducing agents after internalization.⁶⁴ However, the majority of polymer particles are internalized into acidic compartments within the cell, where redox-responsive materials are less effective.⁴⁷ Therefore, designing responsive particles effective at lower pH is of interest. To achieve this, dual-responsive hollow LbL polymer particles – using the synergistic effects of pH and redox-potential – can be engineered which allow for rapid and efficient cargo release, even at extremely low concentrations of GSH.⁴⁶ In addition, the degradation rates in both simulated cellular conditions and in cells can be finely tuned by varying the amount of disulfide cross-linker for redox sensitive particles.⁴⁸

4. BIOMEDICAL APPLICATIONS

Our increasing ability to engineer polymer particles with specific properties, combined with our increasing understanding of their bio-nano interactions, has provided the impetus for studying their application within biomedicine. Example applications that have been investigated for polymer particles include: (i) drug and gene delivery, (ii) vaccination and immunostimulation, (iii) sensing of bacteria and viruses, and (iv) confined bioreactions (Figure 4).

The advantage of using templated polymer particles for drug and gene delivery is the high level of control that can be exerted over particle properties. A drug carrier can deliver and release a drug in response to changes in redox potential, pH, or enzyme concentration: multiple templated particle systems have successfully demonstrated intracellular delivery of therapeutics (Figure 4A). Examples include hollow or porous polymer particles with either hydrophobic drugs (e.g., paclitaxel and thiocoraline) or with hydrophilic drugs (e.g., doxorubicin) conjugated to the polymers used to assemble the particles.^{13,63,65,66} One of these particles has demonstrated the ability to circumvent drug resistance mechanisms in multi-drug resistant cancer cells.⁶⁷ Protein therapeutics can also be embedded in the capsule during LbL assembly, and these particles have been used to induce bone formation in mice with the help of embryonic stem cells.⁶⁸ In addition, hollow polymer particles loaded with enzymes (e.g., urokinase plasminogen activator) have been used to target activated platelets and release the cargo for fibrin degradation in the presence of thrombin.⁶² Intracellular delivery of drugs can also be performed using polydopamine particles modified with a drug-polymer conjugate, or by using MPN particles assembled on drug-loaded calcium carbonate templates.^{53,69} Both of these systems demonstrate pH-responsive drug release

with cytotoxicity at similar, and even lower, concentrations than free drug. Gene delivery with resulting gene silencing can be achieved using a MS templating technique wherein albumin or polypeptide particles are used to deliver small interfering RNA (siRNA).^{11,57}

A Drug and gene delivery

B Vaccination and immunostimulation

C Biosensing

Detection and differentiation sensor

Internalization sensor

D Confined bioreactions

Localized reactions

Encapsulated reactions

Figure 4. Potential applications of polymer particles include: (A) drug and gene delivery, (B) vaccination and immunostimulation, (C) biosensing, and (D) confined bioreactions.

Templated polymer particles are additionally of interest for immunostimulation and vaccination, as the immune system inherently recognizes, investigates, and processes exogenous particles in the size range of viruses and bacteria (Figure 4B).⁷⁰ For example, hollow LbL polymer particles loaded with antigens (e.g., KP9 or ovalbumin) can be processed by antigen presenting cells (APCs) to activate antigen-specific T cells.^{64,71} MS-mediated ovalbumin particles and self-adjuvanting polypeptide particles conjugated with CpG have been used for T cell response in immunized mice and human dendritic cell activation, respectively.^{9,54} LbL capsules with different shapes can result in different degrees of cytokine secretion.⁷² In addition, surface engineering of polymer particles with PEGylation not only improves their lymphatic drainage but also increases the priming of antigen-specific T cells *in vivo*.²⁵ Finally, nanoengineered synthetic vaccine particles can mimic microbial structures and functions to induce protective immunity against cancer and viral infection.⁷³

Sensors used to detect and report changes in their environment can also be assembled through templating (Figure 4C). Hollow LbL polymer particles can be backfilled with liquid crystal emulsions followed by disassembly of the templating particles. When these liquid crystals come into contact with Gram negative bacteria or lipid-enveloped viruses they transition from a bipolar to a radial configuration, a change that is easy to observe with polarized light.⁷⁴ This transition is not seen for Gram positive bacteria or non-enveloped viruses, and can therefore be used to distinguish between different types of bacteria and viruses. For probing carrier internalization by

cells, current technologies are limited by either low throughput or low sensitivity due to the nature of the fluorescent probes. Hollow LbL particles with a fluorescence probe that switches “on” and “off”, depending on the pH, can be used to probe endocytosis (Figure 4C).⁴² These hollow particles are assembled with a fluorescent dye and a quencher incorporated into the multilayers. At extracellular pH the dye and quencher are close to each other and the fluorescence is quenched. At lower (intracellular) pH the particles swell and the quenching is lost, which leads to recovery of fluorescence.

Templated polymer particles can be used as bioreactors, for example using biomimetic multi-compartmentalized colloidal assemblies (Figure 4D).⁷⁵ This concept is inspired by the structure of biological cells, where reactions are highly controlled both spatially and temporally with the help of compartmentalization. Triggered enzymatic degradation of DNA or continuous synthesis of RNA can both be performed inside hollow polymer particles.^{76,77} Using a multi-level hierarchical structure of liposomes assembled between layers in a hollow LbL particle, so called “capsosomes” can be made.⁷⁸ By exploiting temperature-induced changes in permeability of the liposomal membranes an enzymatic reaction can be repeatedly initiated, with the enzyme remaining trapped inside the assembly, while the permeability of the substrate through the lipid membranes is controlled.⁷⁸

5. CONCLUSION AND PERSPECTIVES

In this Account, we have discussed our recent advances in the application of a range of templated polymer particles toward the study and exploitation of bio-nano interactions, including cellular binding and uptake, intracellular trafficking, and biodistribution. The study of bio-nano

interactions integrates biological and chemical knowledge, not only to help inspire new materials, but also to provide fundamentals that can revolutionize our understanding of therapeutic delivery. The goal of this area is to design and understand particle systems that can negotiate biological barriers, thus increasing therapeutic efficacy in disease processes ranging from inflammation to cancer. The interdependent, and sometimes contradictory, roles that particle size, shape, rigidity, structure, and surface chemistry have on the cellular internalization, intracellular trafficking, biocompatibility, and biodistribution behavior must also be considered (Figure 5). For example, combining stealth (reduced non-specific interactions with healthy tissues) with targeting (increased specific interactions with diseased tissues) is desirable, but particle properties responsible for these end-behaviors are often incongruous. Another example of this phenomenon is the compromise between stable drug-encapsulation in robustly assembled particles contrasted with the controlled release of drugs from easily degradable particles. Further complicating design choices is the challenge of determining which physicochemical properties are responsible for a particular *in vivo* behavior. Deliberate particle design requires a thorough understanding of the underlying bio-nano interactions. Improving our understanding in this area will therefore open new opportunities and enable innovations in the fields of material science, biology and nanobiotechnology. In addition, we contribute a free web-based tool to organize and share our on-going journey in the research of bio-nano interactions,⁷⁹ and hope that this can inspire other research groups to do something similar. We believe this to have great potential in facilitating the dissemination, organization and retrieval of information in this complex field and thus accelerate the development of new and improved polymer particles, with well-understood and controlled bio-nano interactions.

Figure 5. The interplay of physicochemical properties of polymer particles not only influences their compatibility, distribution, and ability to overcome biological barriers to delivery and effectiveness, but also determines their potential applications. Rather than attempting to solely design the ultimate ideal particle, purposeful particle design should seek to understand the nature of relevant bio-nano interactions, and account for the tradeoffs and interdependencies in particle properties. The eventual success of a particle for a particular application can be enhanced by allowing these factors to inform upstream engineering and manufacturing decisions. The examples included are intended to provide a general overview and are not exhaustive.

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The authors declare no competing financial interest.

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ACKNOWLEDGMENT

This work was supported by the ARC under the Australian Laureate Fellowship (FL120100030, F.C.) and through the ARC Centre of Excellence in Convergent Bio-Nano Science and Technology (project number CE140100036). J.J.R acknowledges the OCE Scheme.

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Title:

Nanoengineered Templated Polymer Particles: Navigating the Biological Realm

Date:

2016-06-01

Citation:

Cui, J., Richardson, J. J., Bjornmalm, M., Faria, M. & Caruso, F. (2016). Nanoengineered Templated Polymer Particles: Navigating the Biological Realm. ACCOUNTS OF CHEMICAL RESEARCH, 49 (6), pp.1139-1148. <https://doi.org/10.1021/acs.accounts.6b00088>.

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