

Nanoengineering Antibody Assemblies for Biomedical Applications

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Abstract

The clinical success of monoclonal antibody therapy has inspired research in understanding the fundamental molecular basis of antibody-antigen interactions and the engineering of antibodies with enhanced or novel properties. With the emergence of nanomedicine, antibodies have been widely applied as targeting ligands decorated on the surface of therapeutic nanostructured modalities – including liposomes, protein nanoparticles, and polymeric assemblies – for drug delivery and imaging applications. However, little is known about how antibodies assembled in a cluster or particulate form interact with antigens in a biological system, largely due to the challenge in preparing ‘pure’ antibody assemblies with controlled physicochemical properties.

In this thesis, a mesoporous silica template-mediated assembly platform was applied to fabricate well-defined nano-assemblies of therapeutic antibodies, including conventional monoclonal antibodies and antibody-drug conjugates. The antibody nano-assemblies (AbNAs), crosslinked with poly(ethylene glycol)-N-hydroxysuccinimide (PEG-NHS), preserved the selectivity of the monoclonal antibody and induced receptor-mediated internalization of antibodies to achieve enhanced intracellular response, such as growth inhibition. This strategy presents opportunities for intracellular delivery of monoclonal antibodies, as well as a versatile platform for fundamental studies on the interactions between antibody assemblies and cells.

Facile engineering of AbNAs can be achieved by leveraging the intrinsic property of the PEG crosslinker, such as chain architecture (PEG arm numbers and arm length), to regulate bio-nano interactions. As a widely recognized stealth material, PEG can prolong blood circulation time to allow the accumulation of nanoparticles in target tissues, however, it could also result in decreased targeting efficacy by blocking the antigen-binding sites. This thesis investigates the influence of PEG crosslinking, specifically the effect of using PEG crosslinkers with

different chain architecture on the formation of AbNAs and their bio-interaction with respect to specific binding and uptake by phagocytic cells. PEG crosslinkers with less arms but longer arm length were found to be more beneficial for AbNAs to achieve both minimal phagocytic capture and optimal targeting.

Furthermore, the targeting efficacy of AbNAs could be enhanced by substituting conventional monoclonal antibodies with engineered antibody fragments. Nanobodies, also known as single-domain antibodies (sdAb), are the smallest antigen-binding unit (12-15 kDa) that solely bind to the target antigen. The unique structure of nanobodies offers several desirable features, including small size, high stability, strong antigen-binding affinity and low immunogenicity, which makes nanobodies superior for antibody nano-assembly engineering. The nanobody nano-assemblies (NanoNAs), prepared via the template-mediated assembly platform, exhibited significantly enhanced selective association to target cells and reduced phagocytic association in comparison with full-sized AbNAs, owing to the unique structure of nanobodies that allowed a large amount of active binding sites to be presented on the particle surfaces and eliminated crystallisable fragment (Fc) receptor-mediated capture by phagocytic cells.

Overall, the versatile antibody nano-assembly systems expanded our understanding of antibody-antigen interactions, and provides a facile platform to engineer antibody assemblies with novel or enhanced properties for biomedical applications.

Declaration

This is to certify that:

- (i) The thesis comprises only my 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;
- (iii) The thesis is less than 100,000 words in length, exclusive of tables, bibliographies, appendices, and footnotes.

Yingjie Hu

Preface

In **Chapter 2**, Dr. Christina Cortez-Jugo, Dr. Jianhua Li, Dr. Yi Ju and Prof. Frank Caruso assisted with the project design and offered helpful advice with experimental plans. Dr. Jiajing Zhou performed the TEM measurements. Prof. Christoph C. Hagemeyer and Dr. Karen Alt provided the material Herceptin and Kadcyla.

In **Chapter 3**, Prof. Frank Caruso assisted with the project design. Dr. Christina Cortez-Jugo and Dr. Yi Ju offered helpful advice with experimental plans, and Dr. Christina Cortez-Jugo also conducted the cellular association experiments in human whole blood. Dr Jiajing Zhou and Zhixing Lin performed the TEM measurements. Dr Tian Zheng performed the AFM measurements. Prof. Christoph C. Hagemeyer and Dr. Karen Alt provided the material Herceptin.

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2. Chen, J.; Li, J.; Zhou, J.; Lin, Z.; Cavalieri, F.; Czuba-Wojnilowicz, E.; **Hu, Y.**; Glab, A.; Ju, Y.; Richardson, J. J.; Caruso, F. Metal–Phenolic Coatings as a Platform to Trigger Endosomal Escape of Nanoparticles. *ACS Nano*. **2019**, *13*, 11653-11664.
3. Yun, G.; Richardson, J. J.; Capelli, M.; **Hu, Y.**; Besford, Q. A.; Weiss, A. C. G.; Lee, H.; Choi, I. S.; Gibson, B. C.; Reineck, P.; Caruso, F. The Biomolecule Corona in 2D and Reverse: Patterning Metal-Phenolic Networks on Proteins, Lipids, Nucleic Acids, Polysaccharides, and Fingerprints. *Adv Funct Mater* **2019**, *30*, 1905805.

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5. **Hu, Y.** “Nanoengineering Antibody Assemblies for Biomedical Application”, *Particulate Fluids Processing Centre (PFPC) Seminar*, October 2019, The University of Melbourne, Australia.
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Table of Contents

Abstract	i
Declaration	iii
Preface	iv
Publications	v
Presentations	vi
Acknowledgements	vii
Table of Contents.....	ix
Table of Schemes and Figures	xiii
 Chapter 1 Introduction and Literature Review.....	 1
1.1 Introduction.....	3
1.2 Monoclonal Antibody Therapy.....	4
1.2.1 Monoclonal Antibody	5
1.2.2 Antibody-Drug Conjugates	11
1.2.3 Therapeutic Antibody Fragments	13
1.3 Antibodies in Nanomedicine.....	15
1.3.1 Surface Modification on Nanoparticles with Antibodies.....	15
1.3.2 Antibody-Based Nanoparticles	18
1.4 Assembly Strategies for Antibody Nanoparticles.....	19
1.4.1 Template-Assisted Assembly.....	20
1.4.2 PEGylation.....	24
1.5 Thesis Scope	27
1.6 References.....	29

Chapter 2 Template Assisted Antibody Assembly: A Versatile Approach for Functional Antibody Nanoparticles.....37

2.1	Aims.....	39
2.2	Introduction.....	39
2.3	Experimental Section	41
2.3.1	Materials	41
2.3.2	Preparation of Antibody Nano-Assemblies	42
2.3.3	Quantitative Analysis of Antibodies in AbNAs.....	43
2.3.4	Antibody Labelling	44
2.3.5	Particle Characterisation	44
2.3.6	Cell Culture.....	45
2.3.7	Cellular Association Assessed by Flow Cytometry.....	45
2.3.8	Cellular Internalisation Assessed by Confocal Microscopy	45
2.3.9	Internalisation in 3D Spheroids	46
2.3.10	Cell Viability.....	47
2.4	Results and Discussion	48
2.4.1	Synthesis and Characterisation	48
2.4.2	Bioactivity of HerNAs	50
2.4.3	Bioactivity of KadNAs	56
2.5	Conclusions.....	60
2.6	Supporting Information.....	61
2.7	References.....	74

Chapter 3 Influence of Poly(ethylene glycol) Crosslinking on Antibody Nano-Assemblies and Cell Interactions.....78

3.1	Aims.....	80
3.2	Introduction.....	80

3.3	Experimental Section	82
3.3.1	Materials	82
3.3.2	Preparation of Antibody Nano-Assemblies	83
3.3.3	Antibody Labelling	84
3.3.4	Particle Characterisation	84
3.3.5	Stability in GSH and Proteinase	84
3.3.6	Quantitative Degradation Profiles in Proteinase.....	85
3.3.7	Cell Culture.....	85
3.3.8	Cellular Association Assessed by Flow Cytometry.....	86
3.3.9	Cellular Internalisation Assessed by Imaging Flow Cytometry	86
3.3.10	Cellular Internalisation Assessed by Confocal Microscopy	87
3.3.11	Cell Viability Assessed by XTT assay.....	87
3.3.12	Association with Phagocytes from Human Whole Blood	87
3.4	Results and Discussion	89
3.4.1	Synthesis and Characterisation	89
3.4.2	Stability Study.....	91
3.4.3	Targeting Ability.....	94
3.4.4	Cellular Internalisation.....	97
3.4.5	Targeting Cytotoxicity	101
3.4.6	Association with Phagocytes from Human Whole Blood	103
3.5	Conclusions.....	106
3.6	Supporting Information.....	107
3.7	References.....	119

Chapter 4 Engineering Single-Domain Antibody Nano-Assemblies for Enhanced Selective Cell Association.....122

4.1	Aims.....	124
4.2	Introduction.....	124

4.3	Experimental Section	126
4.3.1	Materials	126
4.3.2	Preparation of Nano-Assemblies	126
4.3.3	Antibody Labelling	127
4.3.4	Particle Characterisation	128
4.3.5	Cell Culture	128
4.3.6	Cellular Association Assessed by Flow Cytometry	128
4.3.7	Imaging by Confocal Laser Scanning Microscopy	129
4.3.8	Cell Viability Assessed by XTT Assay	129
4.3.9	Fc Receptor Blocking	130
4.3.10	<i>Ex vivo</i> Human Whole Blood Assay	130
4.4	Results and Discussion	132
4.4.1	Synthesis and Characterisation	132
4.4.2	Cellular Targeting	133
4.4.3	Cell Growth Inhibition	137
4.4.4	Association with Immortalised Monocytic THP-1 Cells	139
4.4.5	Association with Leukocytes in Human Whole Blood	141
4.5	Conclusions	145
4.6	Supporting Information	146
4.7	References	154
Chapter 5	Conclusions and Perspectives	156

Table of Schemes and Figures

Schemes

Scheme 1.1 Forms of monoclonal antibody and antibody-derived therapeutics.....	4
Scheme 1.2 Structure of a monoclonal antibody consisting of light (L) and heavy (H) chains with variable (V_L , V_H) and constant (C_L , C_H) domains.	5
Scheme 1.3 The structure of full murine, chimeric, humanised and full human antibodies.	7
Scheme 1.4 Proposed mechanisms of action of anti-Her2 monoclonal antibody Herceptin (trastuzumab).	9
Scheme 1.5 Structure and properties of antibody-drug conjugates (ADCs) from three generations.	10
Scheme 1.6 The mechanism of action of Kadcyla- an antibody-drug conjugate.....	12
Scheme 1.7 Therapeutic antibody fragments.	13
Scheme 1.8 Schematic illustration of a camelid heavy-chain antibody and nanobody.....	14
Scheme 1.9 Schematic summary of nanoparticles systems for biomedical application, along with their physicochemical properties.	15
Scheme 1.10 Conjugation strategies for surface modification of nanoparticles with antibodies.	16
Scheme 1.11 Schematic illustration of antibody-drug conjugates and cell penetrating peptides (CPP) modified gold nanoparticles.....	18
Scheme 1.12 Schematic illustration showing the synthesis of polymer nanoparticle (A) and protein nanoparticles (B) using MS as templates.	20

Scheme 1.13 Schematic illustration of the MSN template assisted assembly of drug-loaded polymer particles and their pH-dependent drug release in an acidic endolysosomal environment.	21
Scheme 1.14 Engineering PEG particles for improved biodistribution.	25
Scheme 1.15 Schematic illustration of the fabrication and targeting ability of HA-PEG metal-phenolic capsules with three different HA:PEG ratios.	26
Scheme 2.1 AbNAs, fabricated using MSN templates, are shown to selectively recognize their target receptors and efficiently internalised via receptor-mediated endocytosis.	41
Scheme 3.1 Synthesis of PEGx-HerNAs crosslinked by various PEG linkers.	89
Scheme 3.2 Different target association patterns between linear PEG and multi-armed PEG modified particles.	96
Scheme 4.1 The synthesis of NanoNAs via the template-assisted assembly platform.	132

Figures

Figure 1.1 Preparation and characterisation of self-assembled Herceptin EGCG complexes.	19
Figure 1.2 a, Schematic illustration of the synthesis of native, reduced or oxidized ovalbumin (n-OVA, r-OVA, or o-OVA) particles using MSN templated method. b, Transmission electron microscopy images of n-OVA (b), r-OVA (c), and o-OVA particles (d).	22
Figure 1.3 A, Schematic illustration of the synthesis of MDP and CpG ODNs functionalised ovalbumin particles. B, Scanning electron microscopy image of MSN (a) and transmission electron microscopy images of MSN (b) and the functionalised ovalbumin particles (c).	23
Figure 1.4 Transmission electron microscopy and scanning electron microscopy images of dendritic MSN with large pores. (a and e) Unfunctionalized MSNs. (b and f)	

Thiolfunctionalized MSNs. (c and g) Phenyl-functionalized MSNs. (d and h) Amino-functionalized MSNs.	24
Figure 2.1 Preparation and characterisation of AbNAs (Herceptin as model IgG molecules).	49
Figure 2.2 Selective association of HerNAs with BT-474 (ErbB2+) cells.....	51
Figure 2.3 Cellular internalisation of HerNAs in BT474 cells via ErbB2 receptor-mediated endocytosis, demonstrated on 2D and 3D models.	54
Figure 2.4 Inhibition effect of HerNAs on BT-474 (ErbB2+) and MDA-MB-231 (ErbB2–) cell growth.	55
Figure 2.5 Bioactivity of KadNAs: cell association, internalisation and growth inhibition...58	
Figure S2.1 N ₂ adsorption-desorption isotherm (a) and pore size distribution (b) of the MSN templates.	61
Figure S2.2 a) Loading capacity of lysozyme (Lys), bovine serum albumin (BSA), and IgG in MSN templates at different starting concentrations of protein (0.5, 1, and 2 mg mL ⁻¹). b) Zeta potential measurements of MSN, Lys, BSA, and IgG solution in phosphate buffer (pH 7.4). c) High-resolution TEM image of a representative MSN template.	62
Figure S2.3 Particle concentration and size distribution of MSN templates (a) and AbNAs (b) using nanoparticle tracking analysis.	63
Figure S2.4 Height profile of representative IgGNAs as measured by AFM from the line indicated in the left image.....	64
Figure S2.5 FTIR spectra of the free IgG and IgGNAs measured by an FTIR spectrometer fitted with an attenuated total reflectance accessory.....	65
Figure S2.6 Zeta potential of free Herceptin, Mesoporous silica and HerNAs dispersed in 2 mM of phosphate buffer (pH 7.4).	66

Figure S2.7 Colocalization of AF488-labelled free Herceptin and HerNAs (green) with lysosomal compartments of BT-474 cells after 3 h incubation at 37 °C.	67
Figure S2.8 Association of MS@HerNAs with BT-474, MDA-MB-231 cells.....	68
Figure S2.9 Internalisation of MS@HerNAs in BT-474 cells via ErbB2 receptor-mediated endocytosis, observed by CLSM.	69
Figure S2.10 Viability of cells treated with MS@HerNAs for 48 h at a given antibody concentration of 20 $\mu\text{g mL}^{-1}$, assessed by 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay.....	70
Figure S2.11 a) TEM image of KadNAs. b) Size distribution profiles of free Kadcyta and KadNAs dispersed in DPBS, as measured by dynamic light scattering.....	71
Figure S2.12 Zeta potential of free Kadcyta, Mesoporous silica and KadNAs dispersed in 2 mM of phosphate buffer (pH 7.4).	72
Figure S2.13 Viability of cells treated with free Herceptin, HerNAs, free Kadcyta, and KadNAs for 48 h at a given protein concentration of 20 $\mu\text{g mL}^{-1}$, assessed by 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay.	73
Figure 3.1 Characterisation of HerNAs crosslinked by various PEG linkers.....	90
Figure 3.2 Stability study of PEGx-HerNAs in GSH and proteinase environment.....	93
Figure 3.3 BT-474 cell association profiles of HerNAs crosslinked by PEG with different arm length (A, B and C) and different arm numbers (D, E and F), measured by flow cytometry..	95
Figure 3.4 BT-474 internalisation profiles of PEGx-HerNAs crosslinked by PEG with different arm lengths (A) and different arm numbers (B), measured by imaging flow cytometry.	99
Figure 3.5 Confocal images BT-474 cells treated with PEGx-HerNAs crosslinked by PEG with different arm lengths (A) and different arm numbers (B).....	100

Figure 3.6 Cytotoxicity of PEGx-HerNAs crosslinked by PEG with different arm lengths (A) and different arm numbers (B) in BT-474 and MDA-MB-231 cells, as determined by XTT assay.....	102
Figure 3.7 Phagocyte association of HerNAs crosslinked with PEG with different arm lengths (A) and different arm numbers (B).	104
Figure S3.1 TEM image of mesoporous silica templates	107
Figure S3.2 Size distribution profiles of PEGx-HerNAs in DPBS solution.....	108
Figure S3.3 Height profiles of PEGx-HerNAs, measured by a Cypher ES AFM (Asylum Research, US).	109
Figure S3.4 Degradation profiles of PEGx-HerNAs after incubation in 1mg mL ⁻¹ of proteinase and DPBS.....	110
Figure S3.5 Association of PEGx-HerNAs with BT-474 cells at various time points (1, 3, 6, 12, 24h).	111
Figure S3.6 Association of PEGx-HerNAs with MDA-MB-231 cells after 24 h incubation.	112
Figure S3.7 Fluorescence intensity of BT-474 and MDA-MB-231 cells after incubation with AF488 labelled PEGx-HerNAs for 24 h.....	113
Figure S3.8 Confocal images of BT-474 cells treated with Free Herceptin and IgGNAs....	114
Figure S3.9 Cytotoxicity of PEGx-HerNAs (5 µg mL ⁻¹) crosslinked by PEG with different arm lengths and different arm numbers in BT-474 and MDA-MB-231 cells.....	115
Figure S3.10. Association of PEGx-HerNAs crosslinked by various PEG with immune cells from human blood.....	116
Figure S3.11 Association of PEGx-HerNAs crosslinked by PEG with different arm lengths (A) and different arm numbers (B) with lymphocytes from human blood.	117

Figure S3.12 Association of PEGx-HerNAs crosslinked by various PEG with macrophage cells (Raw 264.7).	118
Figure 4.1 Characterisations of NanoNAs and AbNAs.	133
Figure 4.2 Association profiles of NanoNAs and AbNAs with ErbB2+ breast cancer BT-474 cell, quantified by flow cytometry analysis.	135
Figure 4.3 Images of BT-474 (ErbB2+) cells treated with NanoNAs, AbNAs, free nanobody and free antibody, captured by CLSM.	136
Figure 4.4 Inhibition effect of NanoNAs and AbNAs on BT-474 (ErbB2+) cell growth. ...	138
Figure 4.5 Association profiles of NanoNAs and AbNAs with immortalised human monocytic THP-1 cell, quantified by flow cytometry analysis.	140
Figure 4.6 Association of NanoNAs and AbNAs with Fcγ receptors blocked THP-1 cells, quantified by flow cytometry analysis.	141
Figure 4.7 Association profiles of NanoNAs and AbNAs with B cells, granulocytes and monocytes from human whole blood (donated by donor 1), quantified by flow cytometry analysis.	143
Figure S4.1 TEM image of IgGNAs and fluorescence microscopy image of Alexa Fluor 647 labelled IgGNAs.	146
Figure S4.2 Association (%) profiles of NanoNAs and AbNAs with ErbB2– breast cancer MDA-MB-468 cells, quantified by flow cytometry analysis.	147
Figure S4.3 Association (intensity) profiles of NanoNAs and AbNAs with ErbB2– breast cancer MDA-MB-468 cells, quantified by flow cytometry analysis.	148
Figure S4.4 Images of BT-474 (ErbB2+) cells treated with IgGNAs, captured by CLSM. .	149
Figure S4.5 Images of MDA-MB-468 (ErbB2–) cells treated with NanoNAs, AbNAs, control particle IgGNAs, free nanobody and free antibody, captured by CLSM.	150

Figure S4.6 Inhibition effect of NanoNAs and AbNAs on MDA-MB-468 (ErbB2–) cell growth.....	151
Figure S4.7 Association profiles of NanoNAs and AbNAs with leucocytes from human whole blood (collected from donor 1), quantified by flow cytometry analysis.....	152
Figure S4.8 Association profiles of NanoNAs and AbNAs with leucocytes from human whole blood (collected from donor 2), quantified by flow cytometry analysis.....	153

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Chapter 1

Introduction and Literature Review

Contents

1.1	Introduction.....	3
1.2	Monoclonal Antibody Therapy.....	4
1.2.1	Monoclonal Antibody	5
1.2.2	Antibody-Drug Conjugates.....	11
1.2.3	Therapeutic Antibody Fragments	13
1.3	Antibodies in Nanomedicine.....	15
1.3.1	Surface Modification on Nanoparticles with Antibodies.....	15
1.3.2	Antibody-Based Nanoparticles	18
1.4	Assembly Strategies for Antibody Nanoparticles.....	19
1.4.1	Template-Assisted Assembly.....	20
1.4.2	PEGylation.....	24
1.5	Thesis Scope	27
1.6	References.....	29

1.1 Introduction

Monoclonal antibodies (MAbs) are considered to be the most rapidly growing class of human therapeutics owing to their high specificity and effectiveness.^{1, 2} In the past few decades, MAbs have achieved successful translation from subjects of benchtop medical research to clinically approved drugs. About 100 antibody-based drugs have been approved for clinical treatment of several serious diseases, including cancer, autoimmune diseases, chronic inflammatory diseases and virus infections.³ The clinical success of monoclonal antibodies has stimulated research in understanding the fundamental molecular basis of antibody-antigen interactions, engineering of antibodies with enhanced or novel properties, and the application of antibodies in targeted technologies.

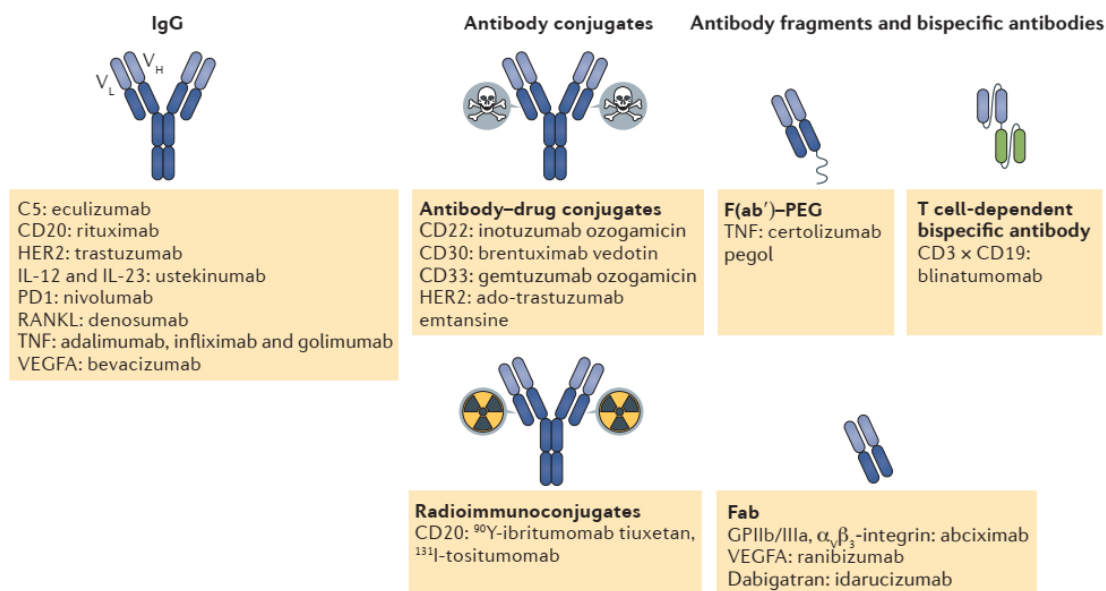
With the rise of nanomedicine, antibodies have been widely applied as targeting ligands on nanotechnology-enabled therapeutic modalities, including liposomes,⁴ protein nanoparticles,⁵ and polymeric micelles,⁶ for drug delivery and imaging applications. The attachment of antibodies to these nanoscale platforms has been typically achieved through covalent or noncovalent bioconjugation techniques.^{7, 8} The physicochemical properties of the underlying substrate, including material type, size, and shape,⁸⁻¹⁰ have been shown to greatly affect the interactions of the immobilized antibodies with their targets. However, little is known about the effects of assembled antibodies or antibody clusters themselves on antibody-antigen interactions, largely due to the challenge in preparing ‘pure’ antibody nanoparticles. Unlike heterogeneous antibody aggregates of undefined size that are formed during the manufacture of monoclonal antibodies,¹¹ the desirable antibody nanoparticles are expected to have controlled size and well-defined morphology.

This chapter will introduce monoclonal antibodies as therapeutics molecules including antibody-drug conjugates and therapeutic antibody fragments, followed by their application in

nanostructured drug delivery systems – including systems modified with antibodies on the surface, and the systems largely composed of antibodies. Furthermore, particle assembly strategies will be reviewed to explore the loading and preparation of well-controlled antibody nanostructures.

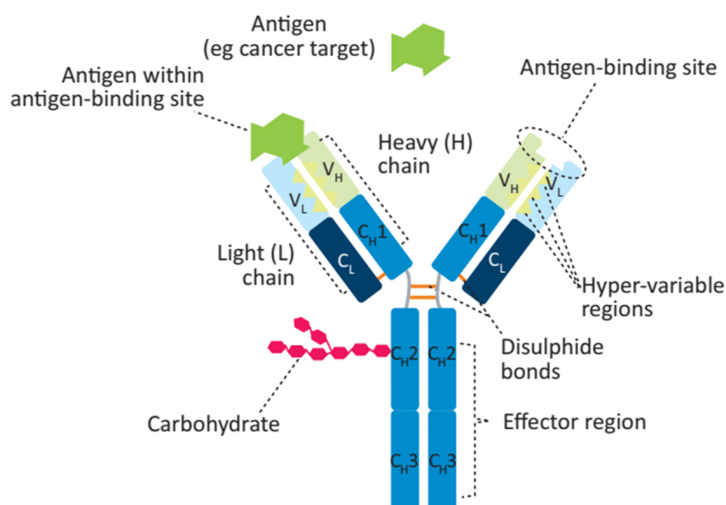
1.2 Monoclonal Antibody Therapy

Over the past few decades, monoclonal antibody therapy has developed rapidly from bench top discovery research to approved products in the market for the treatment of various human diseases.¹ With the growing knowledge of antibody-antigen interactions and the advance of antibody production technologies, monoclonal antibody derived therapeutics, such as antibody-drug conjugates and therapeutic antibody fragments, have emerged that offer improved biological function and clinical outcomes (**Scheme 1.1**). In this section, structures and properties of full monoclonal antibodies, antibody-drug conjugates and antibody fragments will be discussed, along with case studies.



Scheme 1.1 Forms of monoclonal antibody and antibody-derived therapeutics. Clinical approved examples of each format are listed in the boxes. Adapted from ref¹.

1.2.1 Monoclonal Antibody



Scheme 1.2 Structure of a monoclonal antibody consisting of light (L) and heavy (H) chains with variable (V_L , V_H) and constant (C_L , C_H) domains. Light chains and heavy chains are connected by disulphide bonds. Reproduced from ref ¹².

Structures and Properties

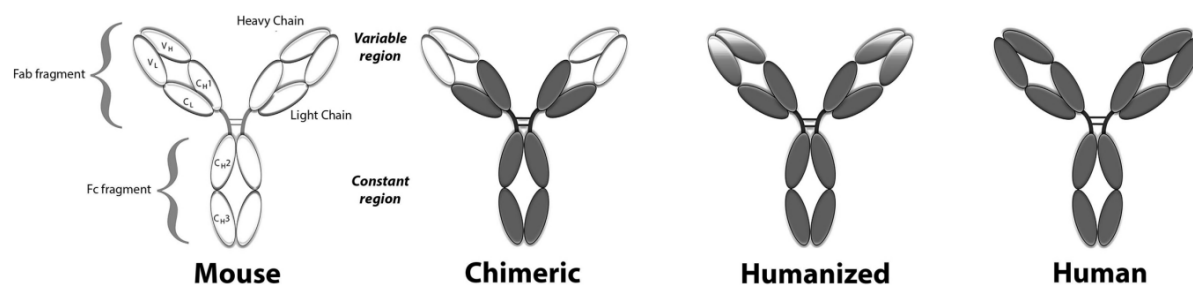
Monoclonal antibodies are large Y-shaped immunoglobulin G (IgG) protein molecules with a molecular weight of approximately 150 kDa (**Scheme 1.2**). One monoclonal antibody consists of two identical heavy chains (50 kDa each) and two identical light chains (25 kDa each). Light chains and heavy chains were connected by disulphide bonds. Each light chain consists of one constant (C_L) domain and one variable (V_L) domain, while each heavy chain consists of three constant (C_H) domains and one variable (V_H) domain. One variable and one constant domain of each of the heavy chain and the light chain form the antigen binding fragment (Fab), represented by the two arms of the ‘Y’ structure (2 Fab fragments); while the two constant domains of each of the heavy chain represent the fragment crystallizable region (Fc), located at the bottom tail of the ‘Y’ structure. Within the Fab region, two variable domains (V_L , V_H) compose the antigen-binding site, also referred to as Fv. The Fv contains hyper-variable regions (also known as complementarity-determining regions) that determine the specific binding with

antigens. The Fc region is involved in the interaction with various cell receptors such as Fc receptors and complement proteins, mediating various cellular effector functions such as antibody dependent cellular cytotoxicity (ADCC).³ Fc regions of all IgG molecules from the same species are conserved.

Brief History

In 1975, the first monoclonal antibodies were produced via hybridoma technology by Cesar Milstein and George J. F. Köhler,¹³ who later won the Nobel Prize in Physiology or Medicine in 1984 for this ground-breaking discovery that revolutionised a new age of therapeutic antibodies. However, the initial monoclonal antibodies were 100% murine (mouse-derived) immunoglobulin. Due to their murine source, these monoclonal antibodies were recognised as foreign antigens in human bodies, hence were attacked and quickly cleared by the human immune system, leading to low therapeutic efficacy. To produce more ‘human-like’ monoclonal antibodies, the chimerisation process was introduced by Morrison *et al.* (**Scheme 1.3**).¹⁴ They grafted the murine variable region onto a human IgG constant region to form the chimeric antibodies that composed of 70 % human IgG.¹⁵ Furthermore, Jones *et al.* described an antibody humanisation technique that grafted a specific murine hypervariable loop of interest onto a full human antibody to obtain a humanised antibody that composed of 85 – 90 % human molecule.^{15, 16} The chimerisation and humanisation technology initiated the advancement of monoclonal antibody therapy. Currently, the majority of clinically approved monoclonal antibodies are either chimeric or humanised.¹⁵ More recent antibody engineering technologies have been developed to generate full human antibodies that outperform the hybrid murine-human antibodies.¹⁷⁻¹⁹ One of the technologies is the creation of transgenic humanised mice, in which the whole mouse IgG gene repertoire is replaced by a human IgG repertoire.^{18,}
¹⁹ When immunised with target antigen, the transgenic humanised mice will produce full human IgG with the intact targeting function. Combined with traditional hybridoma technology,

this approach can yield a large amount of desired full human IgG with high affinity and low human immunogenicity.



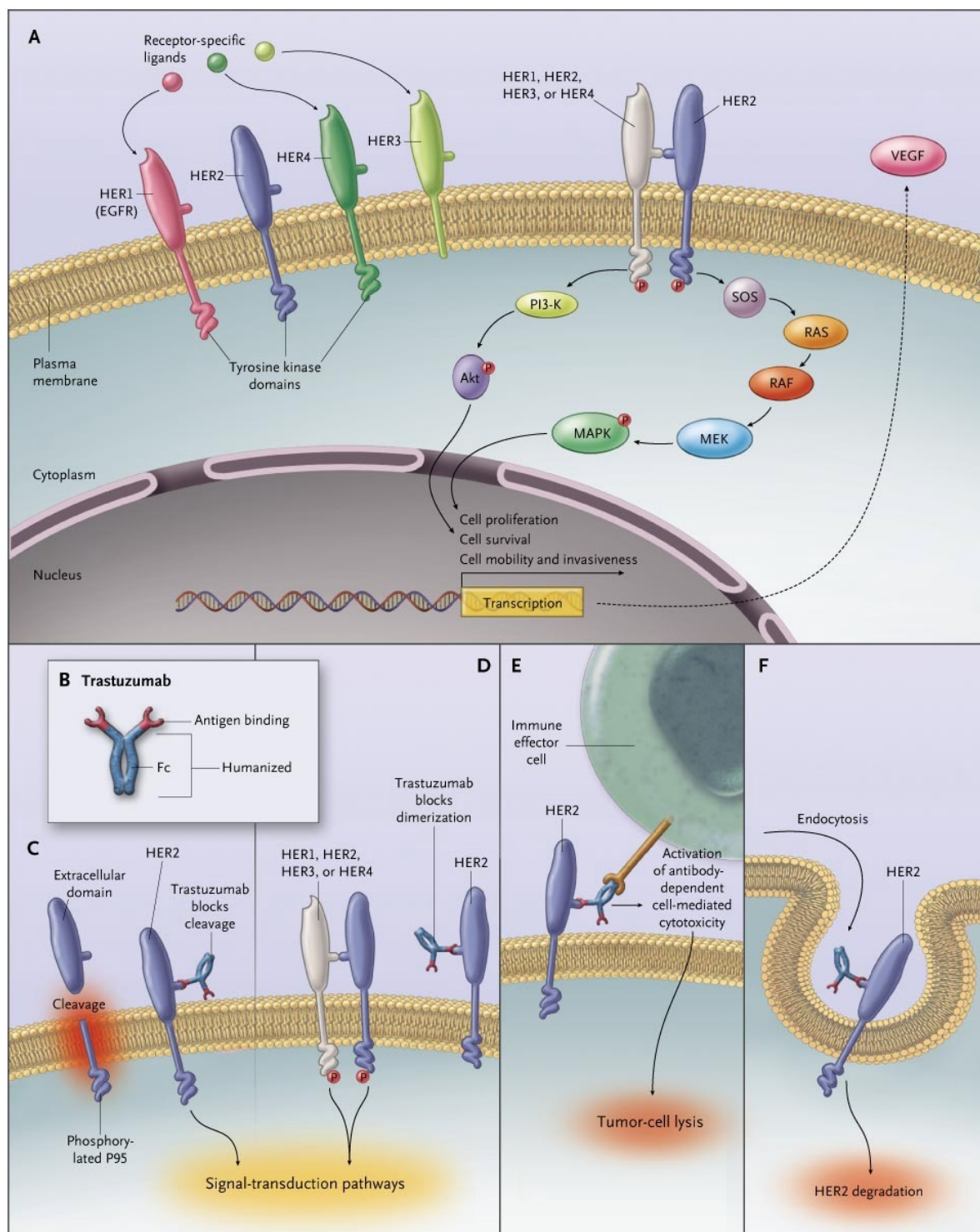
Scheme 1.3 The structure of full murine, chimeric, humanised and full human antibodies. Murine sequences are depicted in light colour and human sequences in dark. Adapted from ref 20.

Owing to research and commercial investment in antibody engineering technologies, the monoclonal antibody field has grown rapidly and achieved great success in clinical applications. About 100 therapeutic monoclonal antibodies are now available for clinical use in the treatment of cancer, autoimmune diseases, chronic inflammatory diseases and viral infections.^{1, 3} More than 550 new antibody drugs are currently in clinical trials, including over 50 antibody drugs in Phase III.²¹

A Case Study: Herceptin

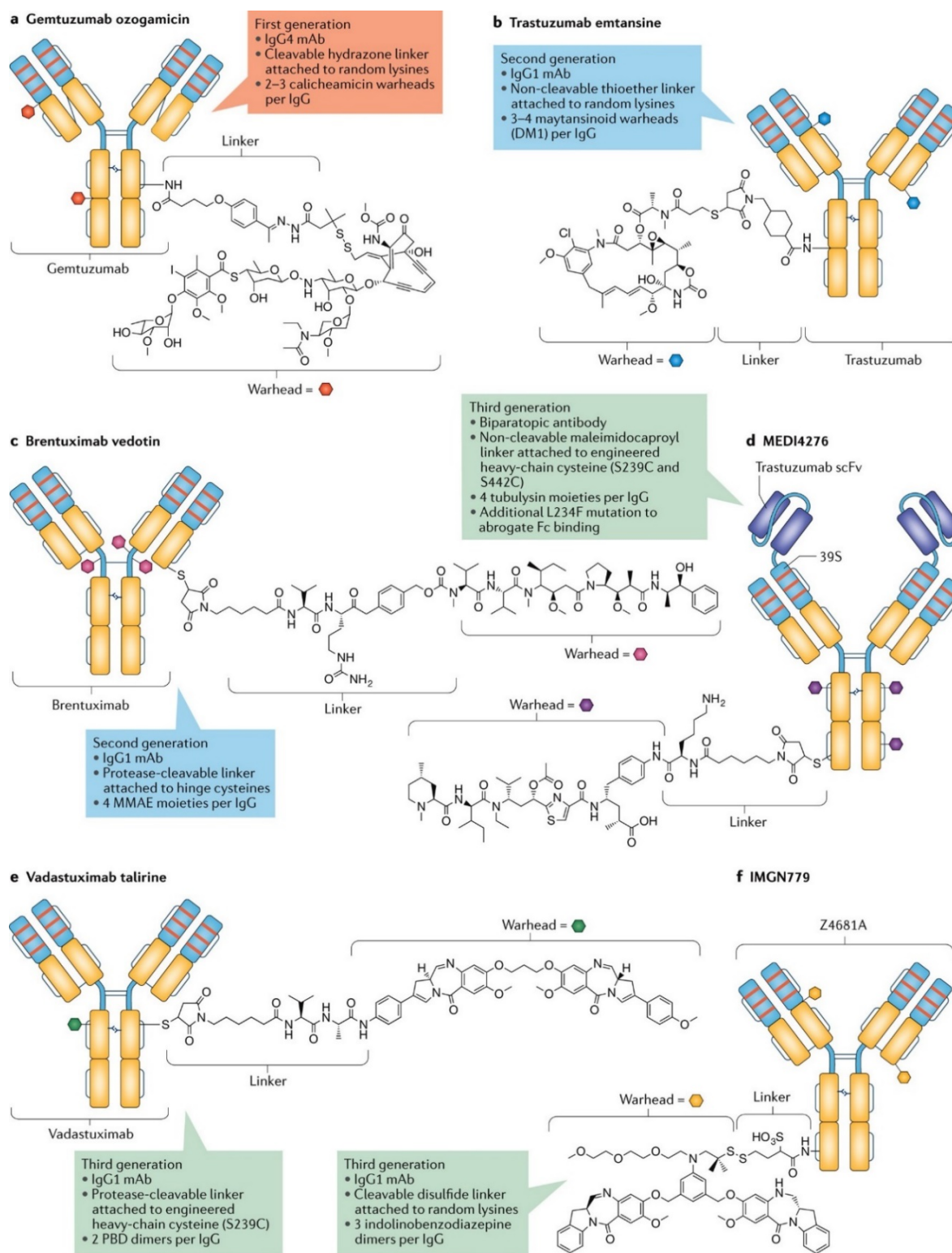
Recombinant humanised trastuzumab (Herceptin®) is the first monoclonal antibody approved for cancer therapy. Herceptin targets the human epidermal growth factor receptor 2 (Her2, encoded by the ErbB2 gene) that is overexpressed on 25 – 30 % of human breast cancers.^{22, 23} The overexpression of Her2 is activated through homodimerisation or heterodimerisation with other epidermal growth factor receptor, which triggers the activation of downstream pathways, resulting in proliferation of cancer cells and the production of vascular endothelial growth factor (VEGF), which facilitates angiogenesis (**Scheme 1.4A**).²⁴⁻²⁶ Herceptin inhibits the

growth of Her2 overexpressed cancer cells by binding to the extracellular juxtamembrane domain of Her2.^{22, 24-27} The mechanism of action is not completely clear in a molecular level. The most well accepted proposed mechanisms include: 1) reducing the shedding of the extracellular domain of Her2, which further reduces membrane-bound phosphorylated P95 and the downstream activation of signal transduction pathways; 2) blocking the homodimerisation or heterodimerisation of Her2 to inhibit downstream cell proliferation and survival related pathways; 3) triggering antibody-dependent cell-mediated cytotoxicity via the Fc domain; and 4) down-regulation of Her2 present on the membrane surface by inducing endocytosis (**Scheme 1.4B-F**).²⁸



Scheme 1.4 Proposed mechanisms of action of anti-Her2 monoclonal antibody Herceptin (trastuzumab). Adapted from ref²⁸.

Chapter 1 Introduction and Literature Review



Nature Reviews | Drug Discovery

Scheme 1.5 Structure and properties of antibody-drug conjugates (ADCs) from three generations. Cytotoxic small molecules are conjugated to monoclonal antibodies by various linkers. Adapted from ref ²⁹.

1.2.2 Antibody-Drug Conjugates

Structures and Properties

To boost the efficacy of monoclonal antibody therapy, cytotoxic small molecule drugs are conjugated on the therapeutic monoclonal antibodies via chemical linkers to obtain antibody-drug conjugates, also known as immunoconjugates.²⁹ The antibody-drug conjugates retain the specificity of the monoclonal antibody; therefore, they are able to deliver the potent cytotoxic drug payload to the target cell with high specificity to achieve effective targeted cell death.

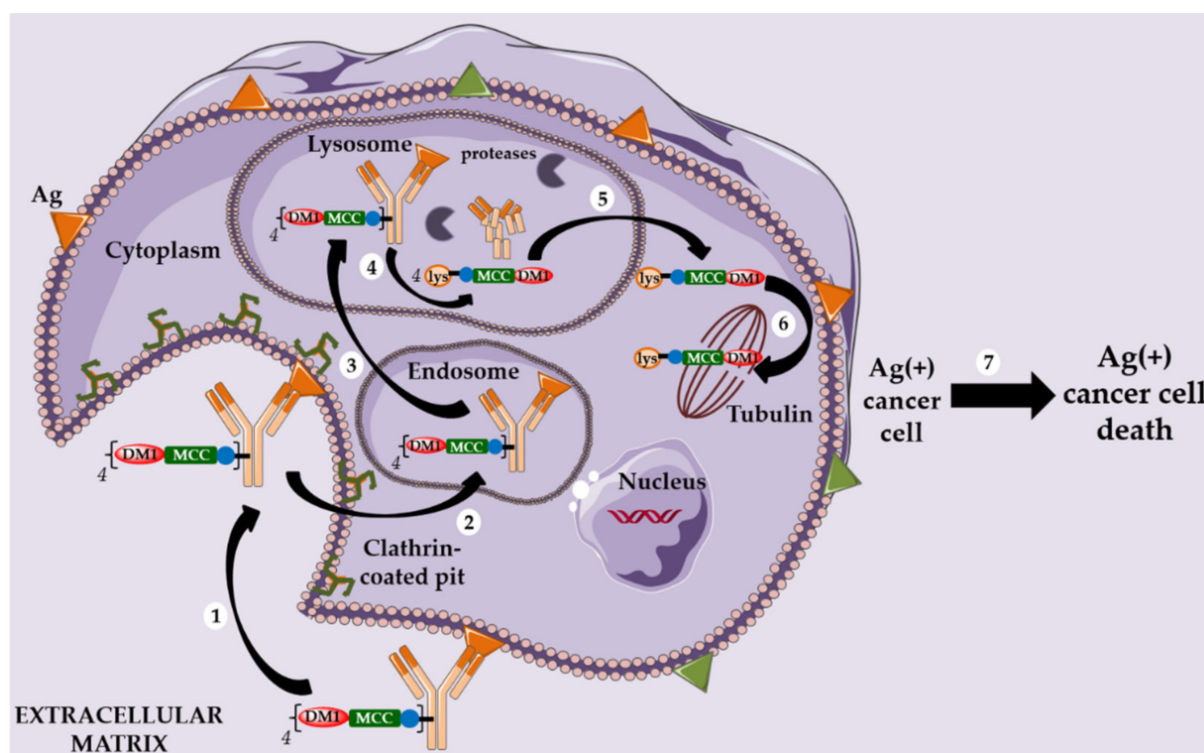
Three generations of antibody-drug conjugates have been developed using various monoclonal antibodies, linkers and cytotoxic small molecule drugs (**Scheme 1.5**).²⁹ The design of these three main components requires a fine-tuning process that considers critical factors including the antibody bioactivity, linker conjugation chemistry, and toxicity of the small molecule drug.²⁹⁻³⁴ Currently, the majority of antibody-drug conjugates incorporate 2 – 4 cytotoxic small molecule drugs on each antibody via a cleavable or non-cleavable linker. These cytotoxic small molecule drugs either target DNA to cause DNA structure damage, or inhibit microtubule polymerisation to arrest cell cycle, eventually leading to cell death.³⁴

To this day, 9 antibody-drug conjugates have been approved for the clinical treatment of leukemia, breast cancer and urothelial cancer. Over 80 conjugates are expected from clinical pipelines.^{29, 30}

A Case Study: Kadcyla

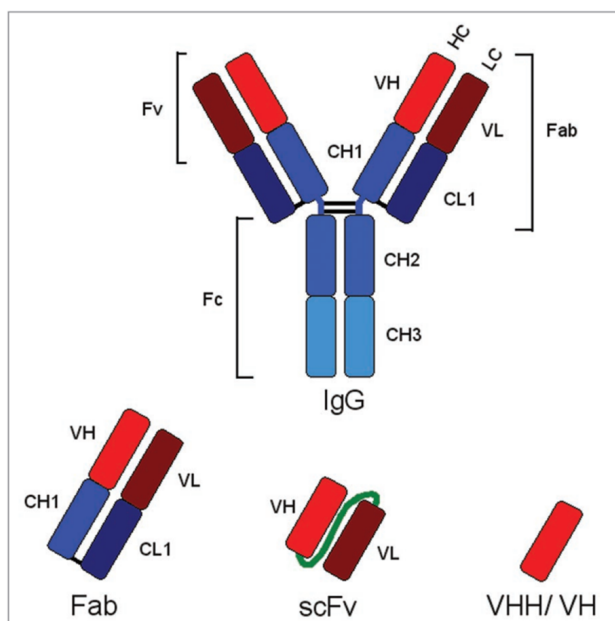
Kadcyla®, or trastuzumab emtansine, is the first antibody-drug conjugation approved for solid tumours.³⁰ Each of the trastuzumab emtansine molecule is composed of a humanised monoclonal antibody trastuzumab and 3 – 4 maytansinoid (DM1) randomly conjugated on lysine residues of the antibody via a non-cleavable thioether linker.²⁹ Kadcyla is currently used clinically for the treatment of Her2 positive metastatic breast cancer. The proposed mechanism

of action is as follows: 1) when Kadcylla molecules reach the tumour site, the antigen binding sites of trastuzumab recognise the overexpressed Her2 on the cancer cell membrane, and bind to the Her2 extracellular region to trigger trastuzumab mediated growth inhibition; 2) Kadcylla bound to the cell membrane are internalised by the cancer cells via a clathrin-dependent mechanism and are captured in endosomes; 3) endosomes containing Kadcylla later fuses with lysosomes; 4) the trastuzumab along with the Her2 are digested by proteases in lysosomes to release the cytotoxic payload DM1; 5) the DM1 molecules are released into cytoplasm and then 6) interact with tubulin to arrest cell cycle; 7) eventually, the combined action of trastuzumab and DM1 leads to the death of Her2 positive cancer cells (**Scheme 1.6**).^{30, 35, 36}



Scheme 1.6 The mechanism of action of Kadcylla- an antibody-drug conjugate. Adapted from ref³⁰.

1.2.3 Therapeutic Antibody Fragments



Scheme 1.7 Therapeutic antibody fragments. Adapted from ref³⁷.

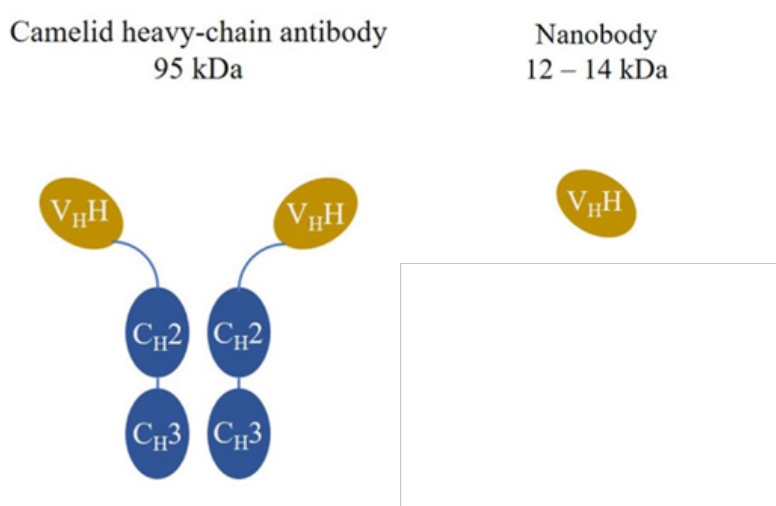
Structures and Properties

Various therapeutic antibody fragments that retain the antigen binding specificity of the full-sized monoclonal antibody molecules have been developed via antibody engineering (**Scheme 1.7**). These antibody fragments include antigen binding fragments (Fab), single-chain variable fragments (scFv), and single-domain antibodies (sdAb, also known as nanobodies).^{37, 38} One Fab (55 kDa) consists of one heavy chain and one light chain that are linked by a single disulfide bond, and each chain is composed of one variable and one constant domain. scFv (25 kDa), unlike Fab, is not a natural fragment of an antibody. It is composed of one variable heavy chain domain (VH) and one variable light chain domain (VL) that are connected by a synthetic flexible polypeptide linker.³⁹ The smallest fragment, nanobody (15 kDa), is solely composed of one heavy chain variable domain that is derived from a camelid heavy chain antibody. All formats of therapeutic antibody fragments contain at least one variable domain to bind antigen with high specificity and affinity and lack the Fc region. Compared with full-sized antibodies,

engineered antibody fragments demonstrate unique features such as improved tissue penetration, rapid clearance from the human body and low manufacturing cost,^{37, 40, 41} which are desirable for certain applications including cancer treatment and *in vivo* imaging.^{42, 43} Over 12 antibody fragments have been clinically approved and most of them are Fabs.^{20, 44} More promising antibody fragment therapeutics are expected with the extensive research and clinical studies in this area.

A Case Study: Nanobody

Naturally occurring antibodies containing only heavy chains were discovered in the serum of camelids.⁴⁵ The heavy-chain-only antibodies consist of two heavy chains, and each chain is composed of one variable domain (V_{HH}) and two constant domains (C_{H2} , C_{H3}). A nanobody, engineered from the camelid heavy-chain antibody, solely comprises of one V_{HH} domain with full antibody binding affinity and specificity (**Scheme 1.8**).⁴⁶ As the smallest therapeutic antibody fragment, nanobodies are able to efficiently penetrate solid tumour, even pass through the brain blood barrier, which make them ideal candidates for cancer therapy.^{47, 48} Currently, there is one nanobody in clinical trials (Phase I) for the treatment of solid tumours.



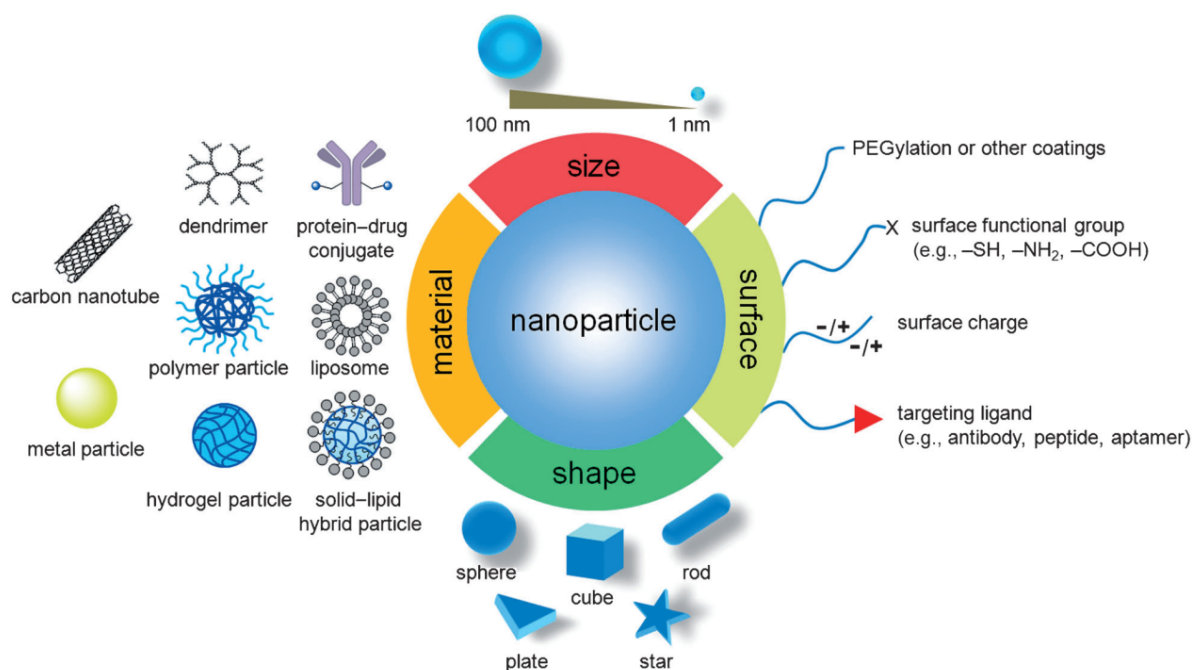
Scheme 1.8 Schematic illustration of a camelid heavy-chain antibody and nanobody.

Reproduced from ref⁴⁷.

1.3 Antibodies in Nanomedicine

Over the past a few decades, nanomedicine has attracted great attention especially in the field of cancer therapy.⁴⁹⁻⁵⁴ Antibodies, owing to their high specificity and effectiveness, have been widely applied in nanoparticle systems as targeting ligands to achieve active targeting and accumulation at desired sites, such as tumour tissues (**Scheme 1.9**).⁵⁵ This section will introduce the application of antibodies in various nanoparticle systems, including systems modified with antibodies on the surface and systems largely composed of antibodies.

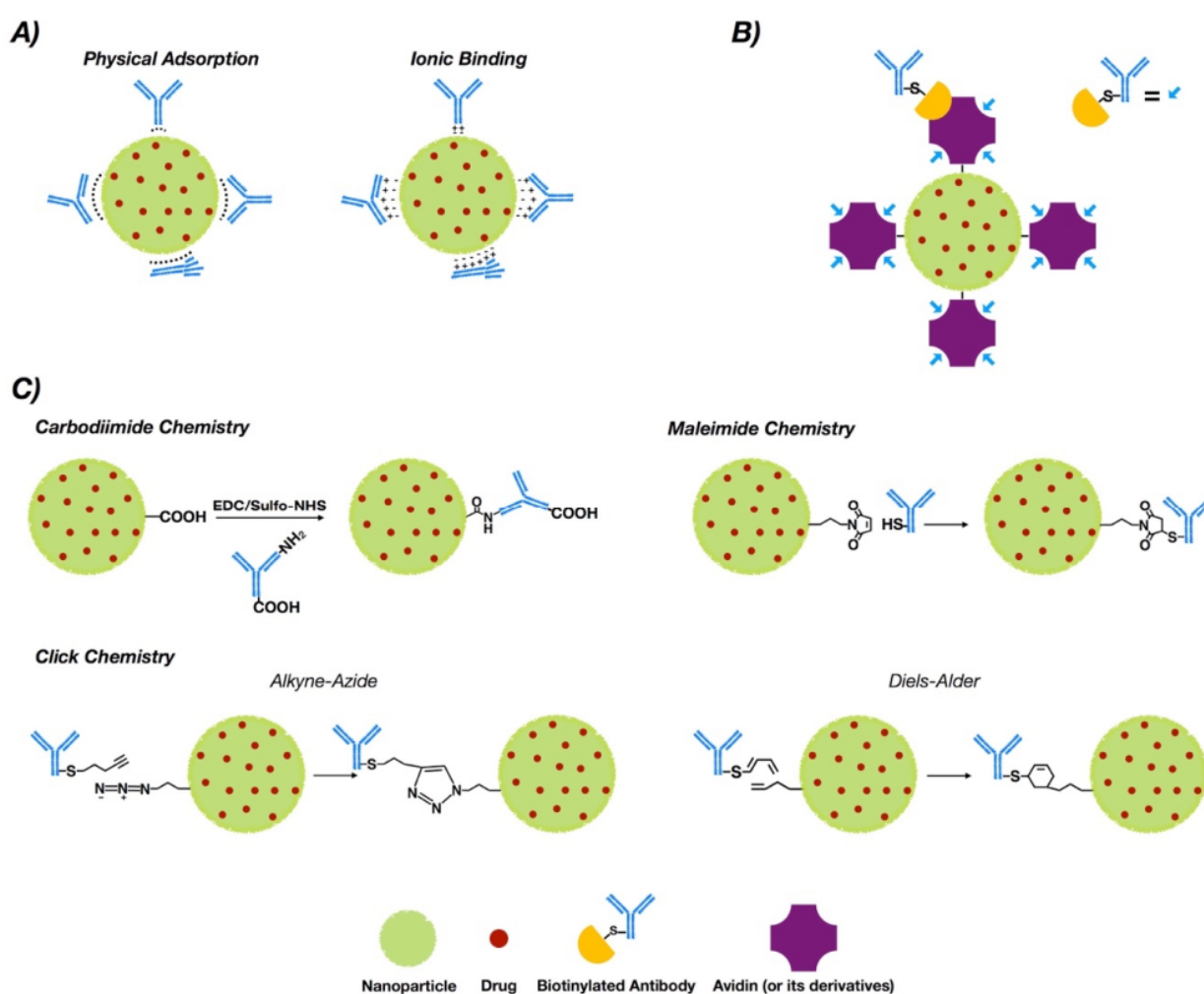
1.3.1 Surface Modification on Nanoparticles with Antibodies



Scheme 1.9 Schematic summary of nanoparticles systems for biomedical application, along with their physicochemical properties. Adapted from ref ⁴⁹.

Various nanoparticle systems, including liposomes, polymer micelles, protein particles and metal particles, have been modified or conjugated with antibodies on their surface for active targeting.⁴⁻⁶ The surface modification of nanoparticles with antibodies can be typically achieved through physical or electrostatic adsorption, adapter mediated conjugation, or

covalent binding (**Scheme 1.10**).^{7, 8, 56} For example, Acharya *et al.* modified the surface of rapamycin-loaded polymeric poly(lactide-co-glycolide) (PLGA) nanoparticles (NP) with antibodies that target the epidermal growth factor receptor (EGFR) via 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) mediated covalent crosslinking. The antibody modified PLGA NPs could effectively deliver the cytotoxic payload rapamycin to EGFR positive cancer cells *in vitro* and achieve superior anti-cancer efficacy in comparison with free drug.⁵⁷

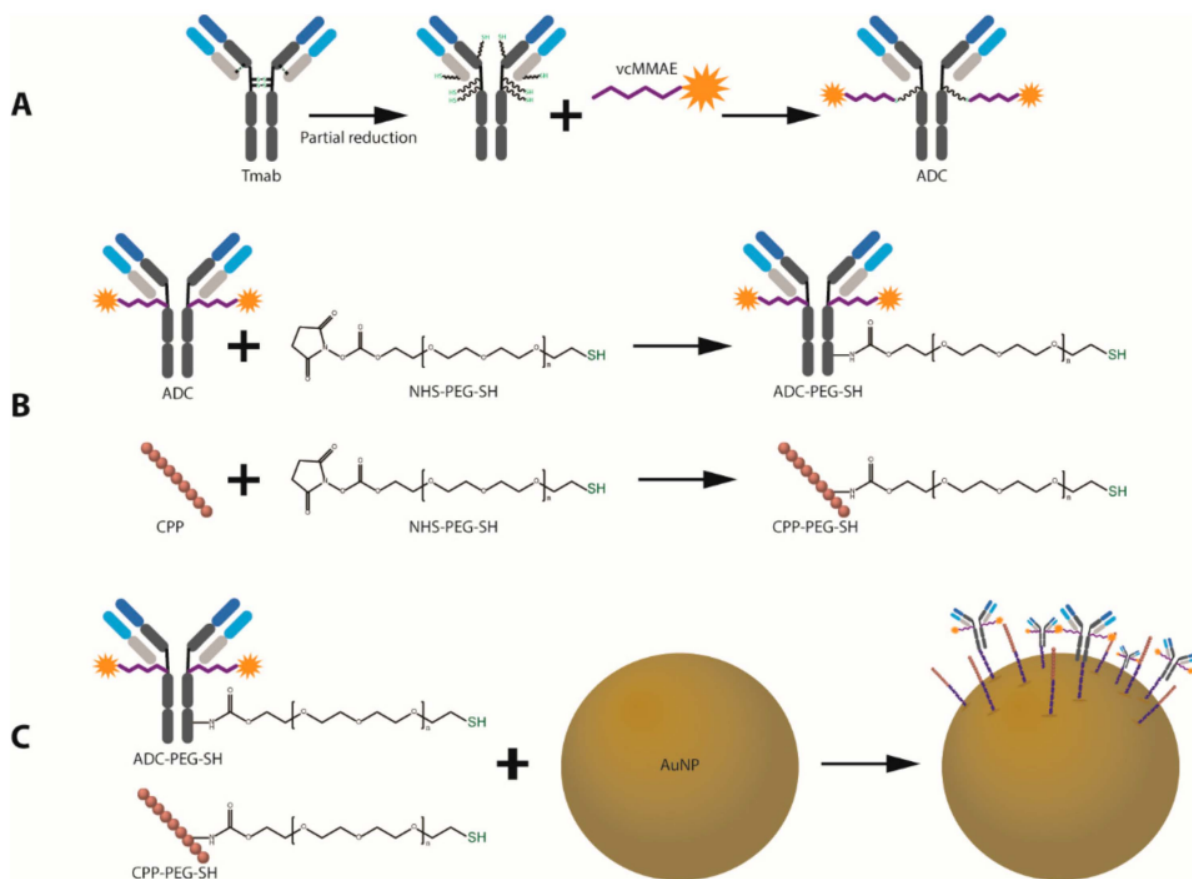


Scheme 1.10 Conjugation strategies for surface modification of nanoparticles with antibodies.

A, adsorption; B, adapters mediated conjugation; C, covalent binding. Adapted from ref⁵⁶.

The physicochemical properties of the underlying substrate, including material type, size, shape and surface properties, can greatly affect the interactions of immobilized antibodies with their target antigens or receptors (**Scheme 1.9**).^{8-10, 58} The density and orientation of the antibody on the particle surface also play an important role in the antibody-antigen interaction.⁵⁶ However, there is currently no universal rule for optimal targeting due to the diversity of nanoparticle systems and the complexity of bio-nano interactions.

Apart from the conventional whole monoclonal antibodies, antibody conjugates and antibody fragments such as nanobodies have also been applied on the surface of various nanoparticle systems for enhanced targeting efficacy.⁵⁹⁻⁶² Cruz and Kayser modified the surface of gold nanoparticles with anti-Her2 antibody-drug conjugates as well as a cell penetrating peptide via disulfide bonds to achieve active targeting to Her2 positive breast cancer cells and with enhanced cellular uptake *in vitro* (**Scheme 1.11**).⁵⁹ Altintas *et al.* developed drug-loaded albumin nanoparticles with their surface coated with nanobodies targeting epidermal growth factor receptor (EGFR) and bifunctional polyethylene glycol 3500 (PEG).⁶¹ The nanobody-coated albumin nanoparticles showed specific binding with EGFR positive cancer cells, which led to selective cellular internalisation via receptor mediated endocytosis and achieved intracellular drug release and drug-induced cell growth inhibition.



Scheme 1.11 Schematic illustration of antibody-drug conjugates and cell penetrating peptides (CPP) modified gold nanoparticles. A. preparation of antibody-drug conjugates (ADC). (B) Conjugation of ADC/CPP with NHS-PEG-SH to obtain ADC-PEG-SH and CPP-PEG-SH. (C) Conjugation of ADC-PEG-SH and CPP-PEG-SH onto the surface of AuNPs. Adapted from ref 59.

1.3.2 Antibody-Based Nanoparticles

As described above, the majority of antibody coated nanoparticle systems have a small quantity of antibodies modified or conjugated on the particle surface for active targeting. There is only one example of nanoparticle system that is largely composed of antibodies. Chung and co-workers developed self-assembled nanocomplexes comprising anti-Her2 monoclonal antibodies Herceptin and green tea catechin derivatives (–)-Epigallocatechin-3-O-gallate (EGCG).⁶³ Briefly, Herceptin antibodies were first mixed with oligomerized EGCG (OEGCG)

to form a core complex, followed by the coating of poly(ethylene glycol)–EGCG shell to stabilise the nanocomplex (**Figure 1.1**). The Herceptin EGCG complexes exhibited improved tumour selective targeting and tumour growth inhibition both *in vitro* and *in vivo*, in comparison with free Herceptin. This study showed great potential of antibody-based nanoparticles in cancer therapy.

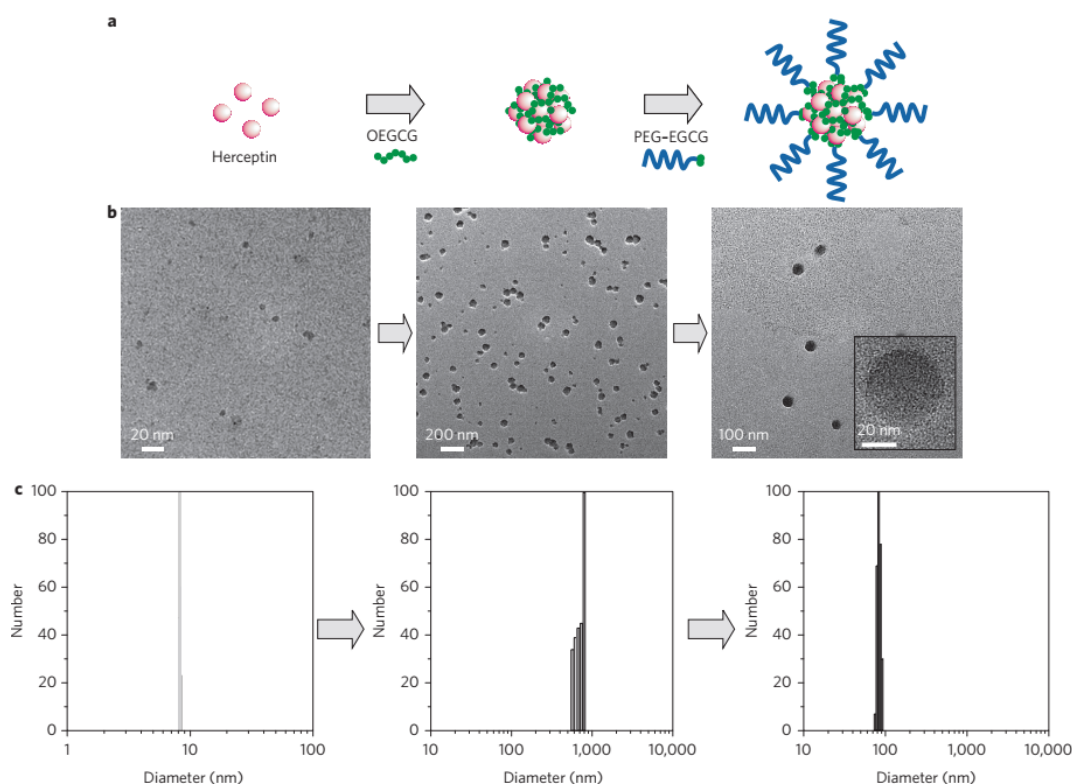
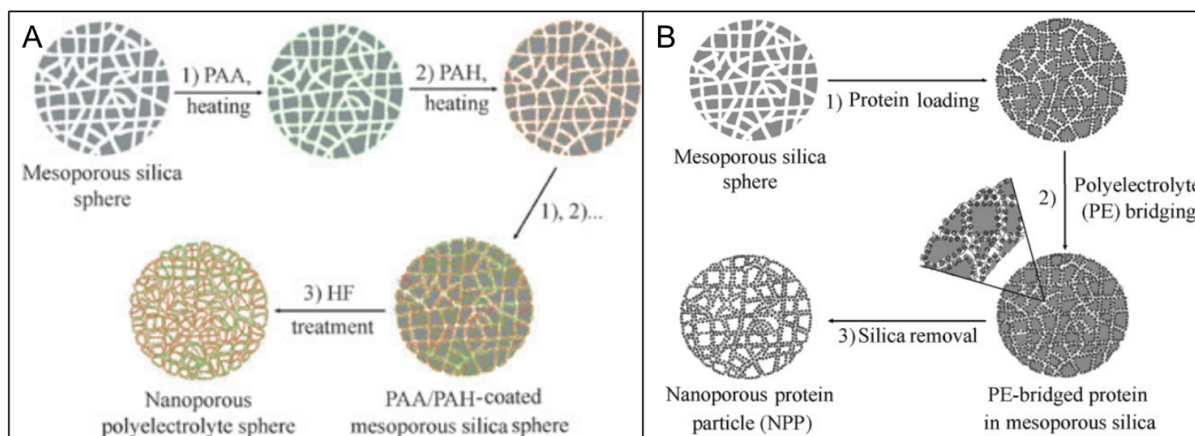


Figure 1.1 Preparation and characterisation of self-assembled Herceptin EGCG complexes. a, Schematic illustration of the self-assembly process. b, c, TEM images (b) and hydrodynamic size distributions (c) of complexes observed at each step of self-assembly. Adapted from ref⁶³.

1.4 Assembly Strategies for Antibody Nanoparticles

As mentioned above, studies on pure antibody nanoparticles and their interactions with the target antigen in biological systems are limited, largely due to limitations in the preparation of well-defined antibody nanoparticles. Antibody nanoparticle assembly strategies, including via a templated assisted method and PEGylation, will be introduced in this session.

1.4.1 Template-Assisted Assembly

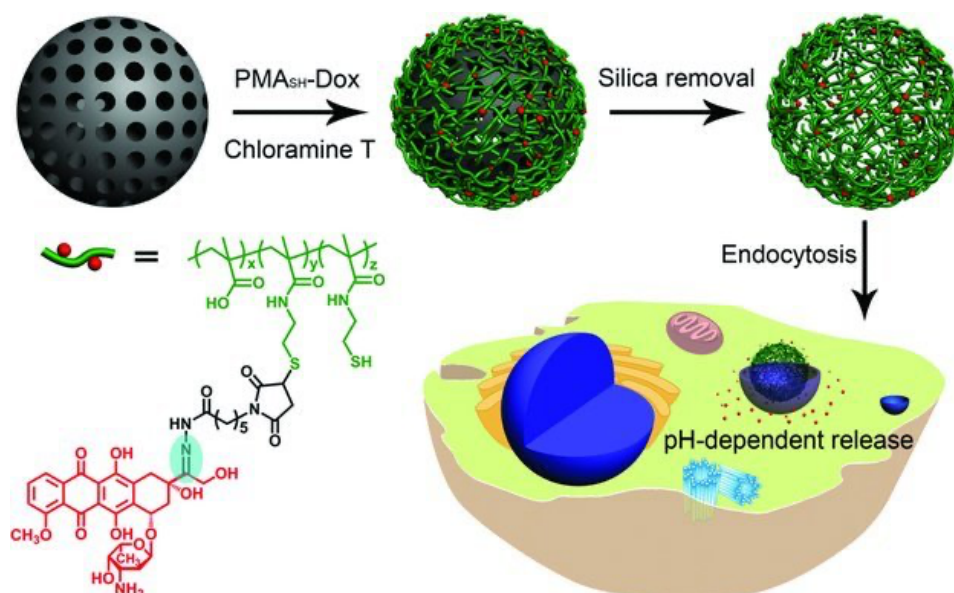


Scheme 1.12 Schematic illustration showing the synthesis of polymer nanoparticle (A) and protein nanoparticles (B) using MS as templates. Reproduced from refs ^{64, 65}.

Template assisted assembly is a method widely used in nanoparticle engineering.⁶⁶ Templates with porous structures, such as calcium carbonate particles and mesoporous silica (MS) particles, allow various soft materials – including polymers and proteins – to infiltrate into the cavities via physical or chemical interactions. Following infiltration, the soft materials inside the templates can be immobilised by covalent or noncovalent crosslinking. Subsequently, the templates are removed in an environment that is harmless to the loaded materials to obtain nanoparticles comprising only the crosslinked soft material (**Scheme 1.12**).⁶⁴⁻⁶⁹ Among all the available templates, mesoporous silica nanoparticles (MSN) are the most widely used owing to their well-controlled size, shape, structure and surface chemistry.⁶⁹⁻⁷³

Cui *et al.* synthesised a 500 nm pH-labile polymer-drug particle system using the MSN template assisted method. Firstly, the small molecule anticancer drug doxorubicin (Dox) was conjugated to a host polymer poly(methacrylic acid) (PMA_{SH}), via a labile linker that degrades at pH 5.5, to form the Dox-loaded PMA_{SH} (PMA_{SH}-Dox). Then, the PMA_{SH}-Dox was infiltrated into MSN templates, followed by covalent crosslinking by adding chloramine T that oxidises free thiol groups on PMA_{SH}-Dox into disulfide bonds. PMA_{SH}-Dox particles were obtained

after removal of the templates with hydrofluoric acid. The pH-labile PMA_{SH}-Dox particles were shown to internalise into endolysosome (pH 5.5), where the encapsulated Dox is released from the particle via degradation of the labile linker to achieve cell death (**Scheme 1.13**).⁷⁰



Scheme 1.13 Schematic illustration of the MSN template assisted assembly of drug-loaded polymer particles and their pH-dependent drug release in an acidic endolysosomal environment. Adapted from ref ⁷⁰.

Gause *et al.* applied the MSN templated method to synthesise pure protein particles. They incubated ovalbumin under three different redox states (native, reduced or oxidized) with MSN templates, and crosslinked the protein via amide bond coupling. Native, reduced or oxidized ovalbumin particles were obtained following silica removal. These ovalbumin particles exhibited well-controlled morphology and could trigger redox-state-dependent immunogenicity *in vivo* (**Figure 1.2**).⁷¹ Furthermore, Gause *et al.* functionalised the ovalbumin protein particles with agonists for nucleotide-binding oligomerization domain-like receptor 2 and toll-like receptor 9 (muramyl dipeptide (MDP) and CpG oligonucleotides (ODNs), respectively) to achieve improved immune response. The same MSN templated method was used for the synthesis of the MDP and CpG ODNs functionalised ovalbumin particles (**Figure**

1.3).⁷² These studies demonstrate that protein nanoparticles synthesised by a MSN template assisted method possess well-controlled physical chemical properties, which presents a promising strategy for the assembly of antibodies nanoparticles.

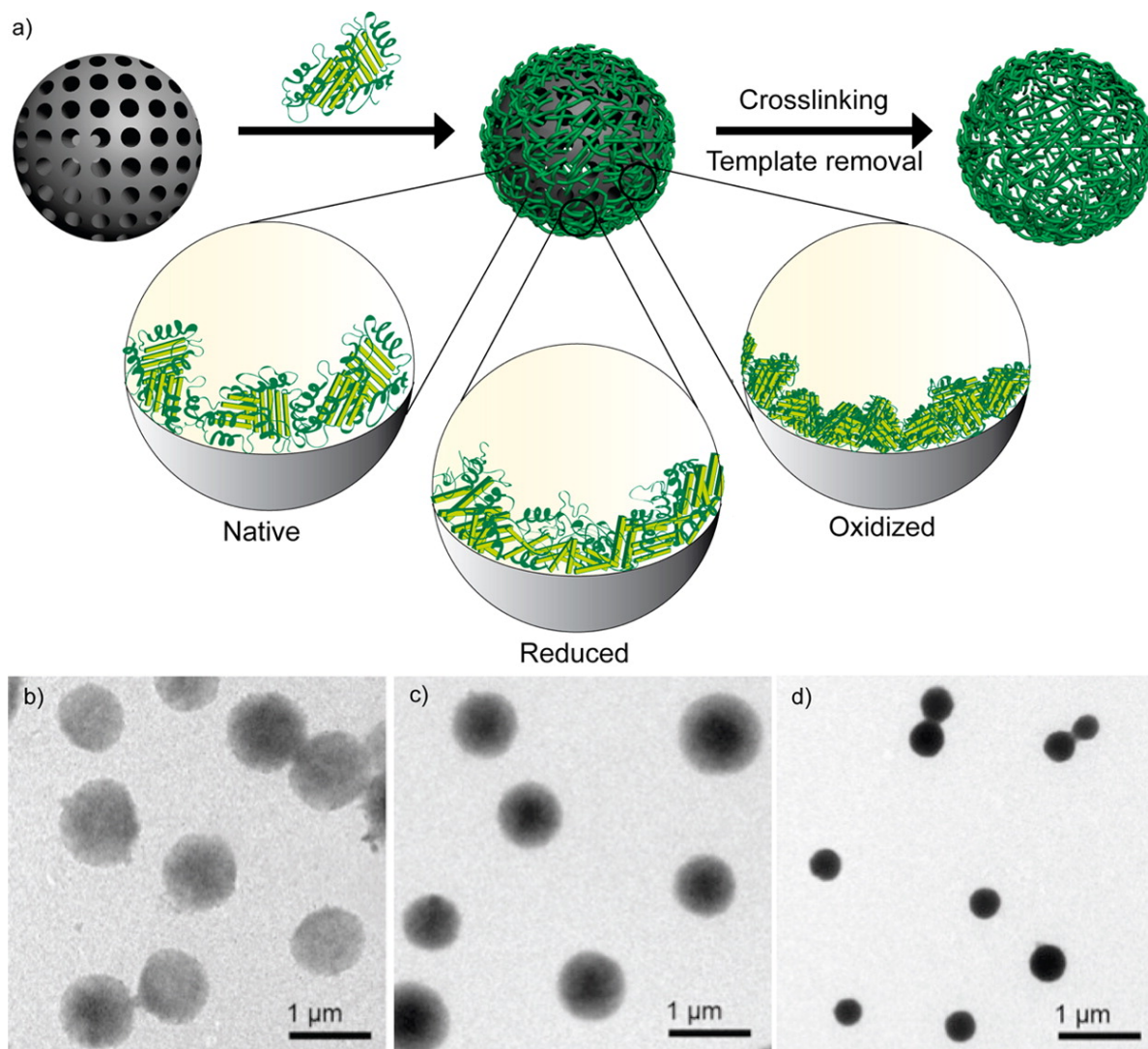


Figure 1.2 a, Schematic illustration of the synthesis of native, reduced or oxidized ovalbumin (n-OVA, r-OVA, or o-OVA) particles using MSN templated method. b, Transmission electron microscopy images of n-OVA (b), r-OVA (c), and o-OVA particles (d). Adapted from ref ⁷¹.

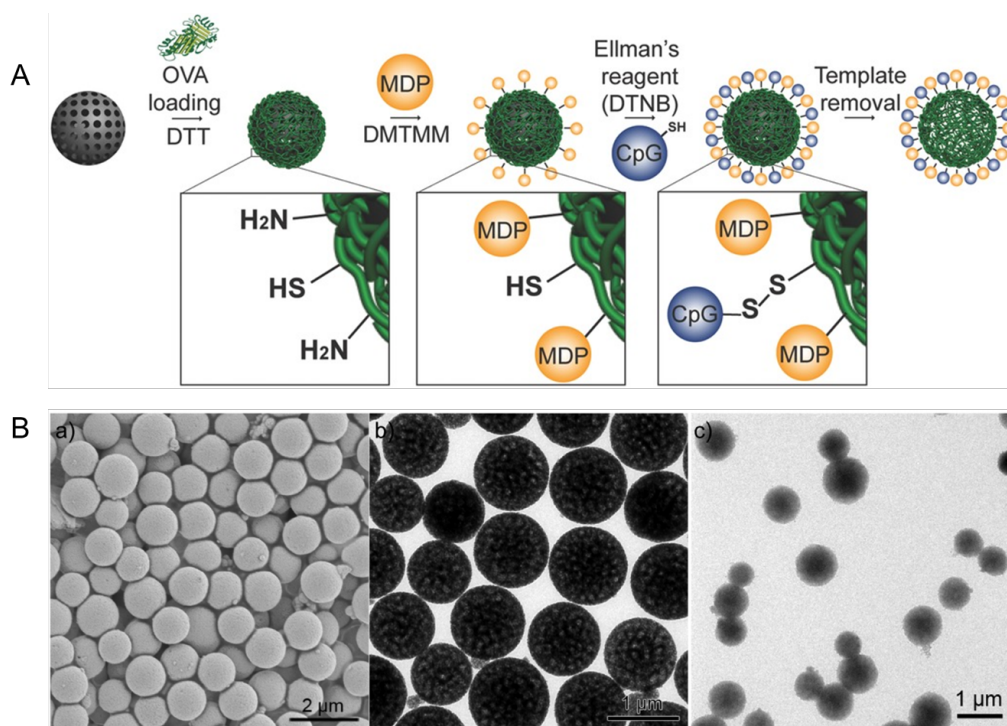


Figure 1.3 A, Schematic illustration of the synthesis of MDP and CpG ODNs functionalised ovalbumin particles. B, Scanning electron microscopy image of MSN (a) and transmission electron microscopy images of MSN (b) and the functionalised ovalbumin particles (c). Reproduced from ref ⁷².

Even though MSN templates have been used to prepare various functional protein particles, the conventional mesoporous silica templates with a pore size of 2 nm are not suitable for antibody assembly due to the large size of antibody molecules (~10 nm). Recently, Chiu *et al.* developed a dendrite MSN with well controlled particle size (< 200 nm) and large pore sizes (20 – 30 nm).⁷⁴ The MSN can be easily modified with various functional groups to achieve optimal loading of various materials (Figure 1.4). The success in the synthesis of 200 nm MSNs with large pores has enabled their use as templates for the assembly of molecules with large molecular weights.⁷⁴⁻⁷⁶

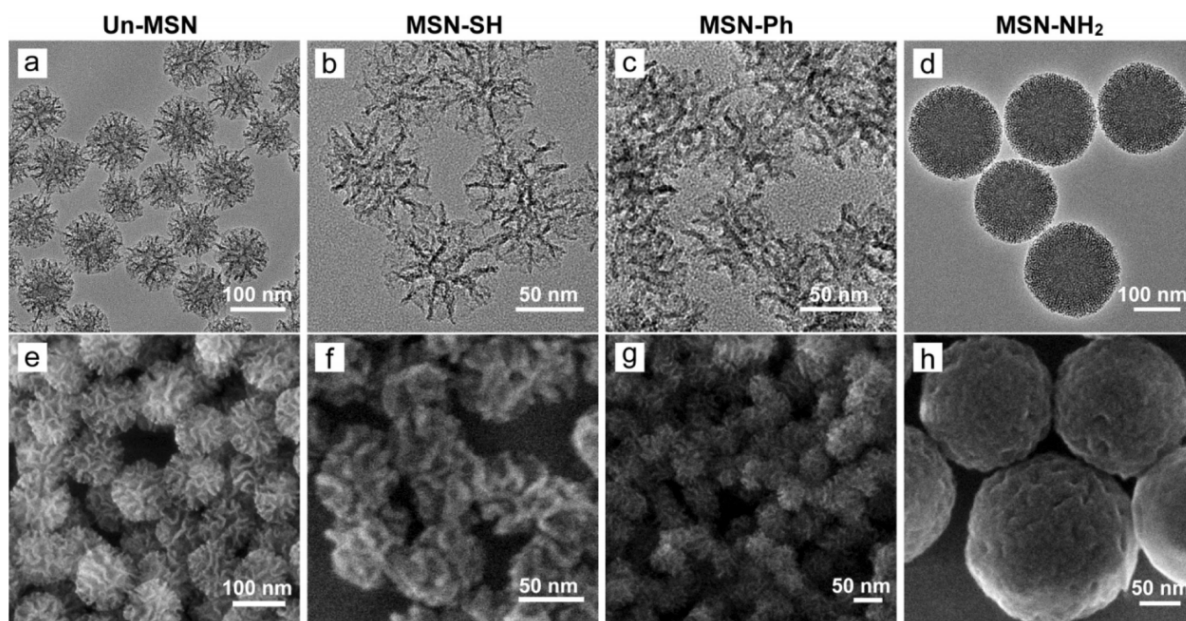
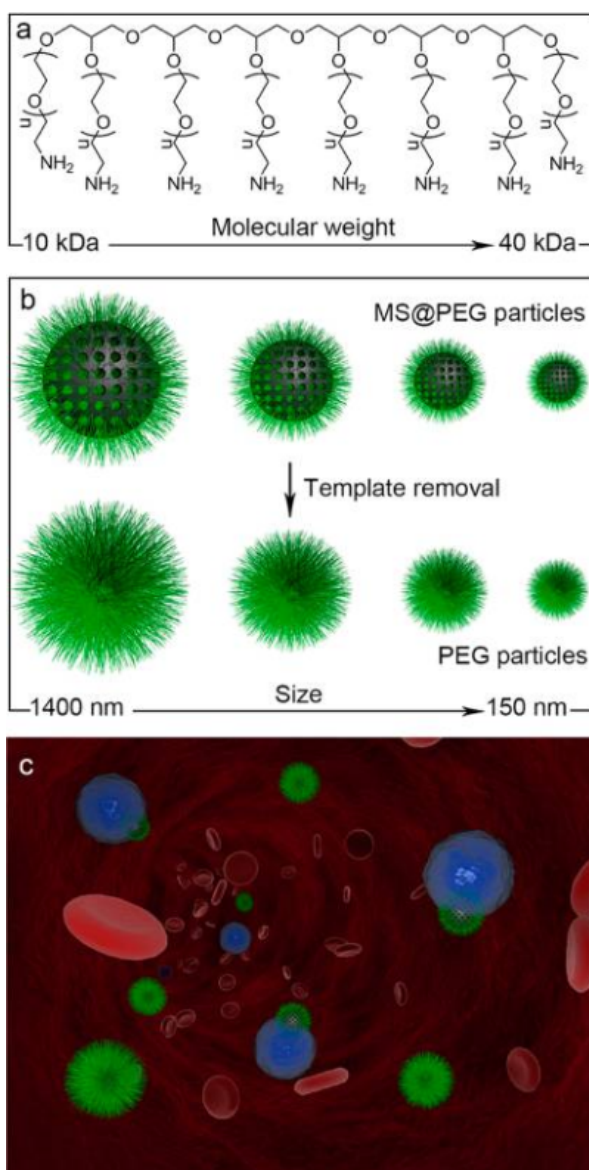


Figure 1.4 Transmission electron microscopy and scanning electron microscopy images of dendritic MSN with large pores. (a and e) Unfunctionalized MSNs. (b and f) Thiolfunctionalized MSNs. (c and g) Phenyl-functionalized MSNs. (d and h) Amino-functionalized MSNs. Adapted from ref ⁷⁴.

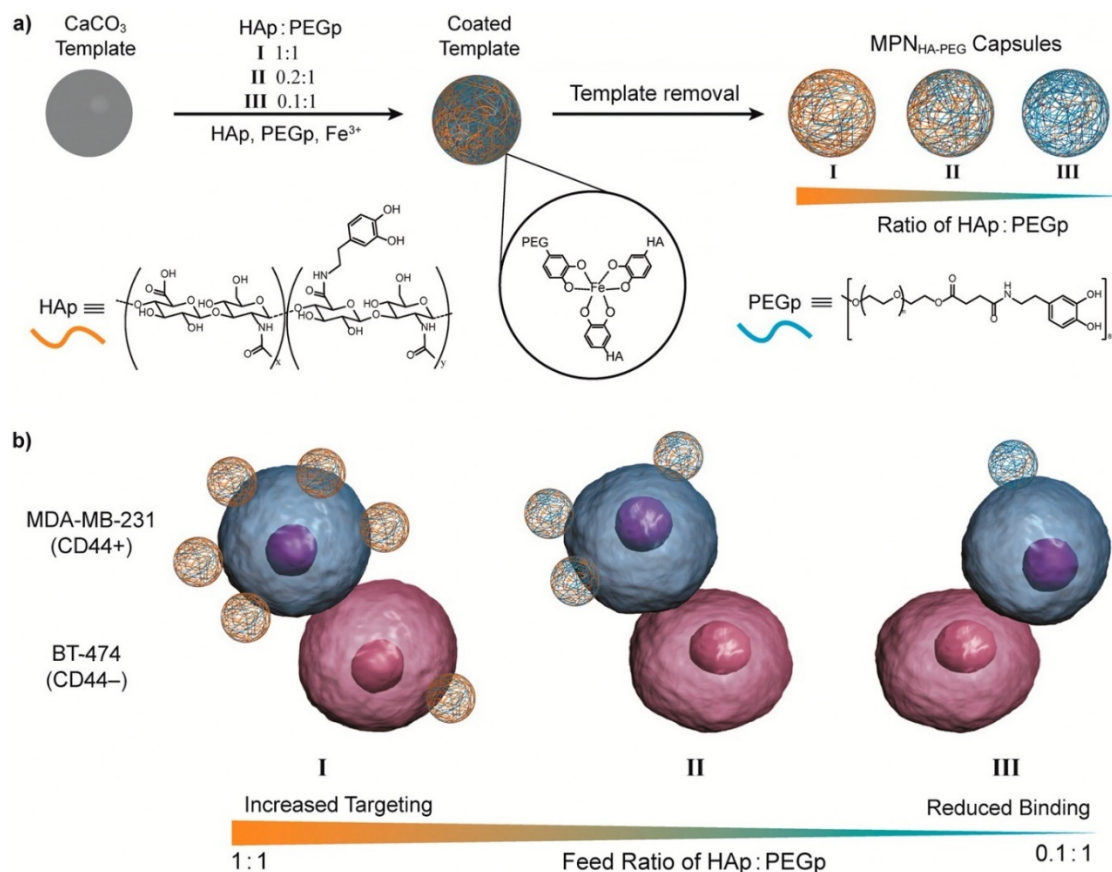
1.4.2 PEGylation

Poly(ethylene glycol) (PEG) is an FDA approved polymer that is commonly used as an excipient in pharmaceutical products to improve their surface hydrophilicity and solubility.⁷⁷⁻
⁷⁹ Functionalizing nanoparticles with PEG, or PEGylation, has been widely applied as an effective strategy to prolong the blood circulation time by the formation of a hydrating layer to hinder protein adsorption and subsequent mononuclear phagocyte system clearance.⁸⁰⁻⁸⁴ The stealth properties of PEGylated nanoparticles are largely related to the PEG chain architecture (such as PEG arm numbers and arm length) and surface density.^{77, 85-87} Cui *et al.* reported PEG particles prepared via a mesoporous silica templated method using PEG with different molecular weights (10, 20, and 40 kDa). The PEG nanoparticles comprising of higher

molecular weight PEG had lower association with phagocytes in human whole blood, and also displayed longer *in vivo* blood circulation time in a mouse model (Scheme 1.14).⁷⁷



Scheme 1.14 Engineering PEG particles for improved biodistribution. a, 8-arm-PEG-NH₂ with various molecular weight used for particle synthesis. B, Schematic illustration of the MS@PEG (template present) and PEG particles (template absent) with different sizes. C, Schematic illustration of the retention of PEG particles in the circulation system with low leucocyte association, where MS@PEG are largely associated with phagocytic cell (blue). Adapted from ref ⁷⁷.



Scheme 1.15 Schematic illustration of the fabrication and targeting ability of HA-PEG metal-phenolic capsules with three different HA:PEG ratios. a, Fabrication of metal-phenolic capsules using hyaluronic acid--polyphenol (HAp) and PEG-polyphenol (PEGp) at three different ratio. b, The CD44 targeting ability of HA-PEG metal-phenolic capsules with different HA:PEG ratios. Adapted from ref ⁸⁸.

Even through the presence of PEG provides favourable stealth properties, it can also reduce the biological activities of functional molecules (such as enzymes, antibodies and hyaluronic acid) by limiting the interactions between the functional molecules and corresponding receptors, possibly due to the low-fouling nature of PEG and the steric crowding at the active or binding site.⁸⁸⁻⁹¹ Specifically, when modified on the antibodies or antibody-based nanoparticles, PEG can block the antigen-binding fragment and hinder specific targeting.⁹⁰ Ju *et al.* studied the effect of PEGylation on the CD44 targeting ability of hyaluronic acid (HA)-based capsules.

HA-PEG capsules were fabricated by incubating synthetic HA-polyphenol (HAp) and PEG-polyphenol (PEGp) with Fe^{III} via a calcium carbonate templated method. With the increase of PEG component in the capsule system, the HA-PEG metal-phenolic capsules exhibited decreased association with CD44⁺ cancer cells, indicating that over PEGylation could hinder the active targeting of functional molecules (**Scheme 1.15**).⁸⁸ Despite the extensive studies on PEGylated nanoparticles, there is no general criterion as to what is essential to balance the stealth and biological activities due to the physiochemical differences among various particle systems.^{86-88, 91-94}

1.5 Thesis Scope

The clinical success of monoclonal antibody therapy has inspired research in understanding the fundamental molecular basis of antibody-antigen interactions and the engineering of antibodies and antibody assemblies with enhanced or novel properties. In the context of nanomedicine, antibodies have been widely applied as targeting ligands coated on the surface of nanoparticles for drug delivery and imaging applications. However, there have been limited studies on pure antibody nanoparticles and how they might interact with antigens in complex biological systems, largely due to the challenge in preparing well-defined antibody nanoparticles.

This thesis will describe the synthesis of antibody nanoparticles with controlled physicochemical properties, using an MSN template assisted method (Chapter 2). Herceptin and the antibody-drug conjugate Kadcyla, were used as model therapeutic antibodies to demonstrate this antibody assembly platform. Briefly, antibodies were infiltrated into 250 nm MSN templates, followed by crosslinking with PEG-NHS and removal of the templates. The AbNAs preserved the targeting function of the antibody and induced internalisation of the assemblies to enhance the subsequent intracellular response, which presents opportunities for the delivery of monoclonal antibodies at high concentrations and/or against intracellular targets,

as well as providing a versatile platform for fundamental studies on antibody cluster–cell interactions.

Prolonged blood circulation is essential for AbNAs to reach target sites and achieve desirable therapeutic efficacy *in vivo*. Functionalisation with PEG, or PEGylation has been proven to be an effective strategy to achieve this goal. However, PEGylation can also reduce the targeting efficacy by blocking the complementary-determining regions of antibodies from antigen binding. To thoroughly understand the effect of PEGylation on the antibody assemblies, the effect of the molecular architecture of the PEG crosslinker on the targeting ability and stealth property of AbNAs was examined (Chapter 3). Chapter 3 reports a fundamental study investigating the bio-nano interactions of PEGylated antibody nano-assemblies (PEGx-AbNAs) prepared using PEG crosslinkers with different arm lengths and arm numbers. Their targeting ability and stealth properties were explored in *in vitro* cell models and *ex vivo* human whole blood.

A novel antibody fragment – a nanobody that comprises solely of one variable domain – presents several desirable properties such as small size, high stability, strong antigen-binding affinity and low immunogenicity. These properties make nanobody a suitable candidate for antibody nano-assembly engineering. Chapter 4 extends antibody assemblies to nanobodies to prepare nanobody nano-assemblies (NanoNAs) via the mesoporous silica template assisted method. The targeting ability and stealth properties of NanoNAs are explored in *in vitro* cell models and *ex vivo* human whole blood. In comparison with full-sized AbNAs, the NanoNAs exhibited enhanced antigen targeting efficacy and reduced immunogenicity owing to the unique structure of the nanobody, potentially providing opportunities for the application of NanoNAs in cancer therapy.

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Chapter 2

Template Assisted Antibody Assembly: A Versatile Approach for Functional Antibody Nanoparticles

Contents

2.1	Aims.....	39
2.2	Introduction.....	39
2.3	Experimental Section.....	41
2.3.1	Materials	41
2.3.2	Preparation of Antibody Nano-Assemblies	42
2.3.3	Quantitative Analysis of Antibodies in AbNAs.....	43
2.3.4	Antibody Labelling	44
2.3.5	Particle Characterisation	44
2.3.6	Cell Culture.....	45
2.3.7	Cellular Association Assessed by Flow Cytometry.....	45
2.3.8	Cellular Internalisation Assessed by Confocal Microscopy	45
2.3.9	Internalisation in 3D Spheroids	46
2.3.10	Cell Viability.....	47
2.4	Results and Discussion	48
2.4.1	Synthesis and Characterisation	48
2.4.2	Bioactivity of HerNAs	50
2.4.3	Bioactivity of KadNAs	56
2.5	Conclusions.....	60
2.6	Supporting Information.....	61
2.7	References.....	74

2.1 Aims

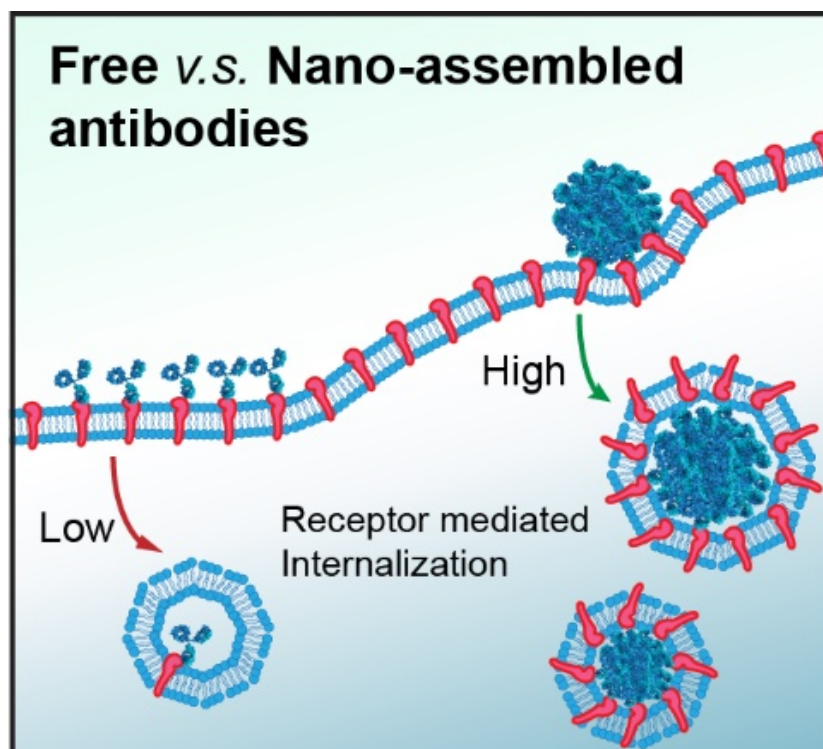
The clinical success of monoclonal antibody therapy has inspired research in understanding the fundamental molecular basis of antibody-antigen interactions and the engineering of antibodies and antibody assemblies with enhanced or novel properties. In this chapter, the aims are to synthesise 250 nm nano-assemblies of therapeutic antibodies (AbNAs) with controlled physicochemical properties using a versatile template-mediated assembly method and to understand how these novel AbNAs interact with targeted receptors and their potential effects on cellular activities. Herceptin and the antibody-drug conjugate Kadcyla were used as model antibody molecules. The bioactivities of AbNAs were investigated on human breast cancer BT-474 and MDA-MB-231 cells.

2.2 Introduction

Monoclonal antibodies are the fastest growing class of human therapeutics and have a proven clinical track record for the treatment and diagnosis of several serious diseases, including cancer and inflammatory conditions, owing to their high specificity and effectiveness.¹⁻³ For cellular targets, antibodies bind to their specific antigen, which could be receptors present on the cellular membrane that trigger a cascade of intracellular signalling events, or intracellular targets that affect various cellular activities.^{4, 5} Many FDA-approved monoclonal antibodies, and their drug conjugates, bind to specific receptors overexpressed on tumour cells, enabling the delivery of therapeutic or imaging agents in addition to the antibody itself into tumours.⁶ Given their significance in medical research and in the clinic, it is important to understand the fundamental molecular basis of antibody-antigen interactions, find new antibody targets, and design antibodies with enhanced or novel functions.

Antibodies have been widely applied as targeting ligands on nanotechnology-enabled therapeutic modalities, including liposomes,⁷ protein nanoparticles,⁸ and polymeric micelles,⁹ for drug delivery and imaging applications. The attachment of antibodies to these nanoscale platforms has been typically achieved through surface assembly of the antibody molecules using covalent or noncovalent bioconjugation techniques.^{10, 11} The physicochemical properties of the underlying substrate, including material type, size, and shape,^{10, 12-14} have been shown to affect the interactions of the immobilized antibodies with their targets. However, little is known about the effect of assembled antibodies or antibody clusters themselves on antibody-antigen interactions, mainly because of the challenge in preparing ‘pure’ antibody nanoparticles with controlled size despite limited attempts made to fabricate antibody nano-assemblies via self-assembly methods including copolymer conjugation and antibody complexation with catechin derivatives.^{15, 16} These clusters are a separate class to heterogeneous antibody aggregates of undefined size that are formed during the manufacture of monoclonal antibodies.^{17, 18}

Pure protein particles (diameter 930 nm) have previously been prepared by a template-mediated method using mesoporous silica nanoparticles (MSNs).¹⁹ The synthesis of protein replica particles (i.e., with the template removed) with sizes around 200 nm has remained a challenge, especially from proteins with high molecular weights. The recent success in the synthesis of 200 nm MSNs²⁰⁻²² with large pores, however, has enabled their use as templates for the assembly of larger biomolecules. Herein, we use a facile template-mediated approach to fabricate amide-stabilized antibody nanoparticles with controlled physicochemical properties. The versatility of the approach was demonstrated using the therapeutic antibody Herceptin and its drug conjugate Kadcyla. We evaluate how these novel antibody nano-assemblies (AbNAs) interact with targeted receptors and their potential effects on cellular activities (**Scheme 2.1**).



Scheme 2.1 AbNAs, prepared using MSN templates, are shown to selectively recognize their target receptors and efficiently internalised via receptor-mediated endocytosis. These nanoparticles represent a new platform for engineering nanomaterials and understanding bio-nano interactions.

2.3 Experimental Section

2.3.1 Materials

Cetyltrimethylammonium bromide (CTAB), tetraethyl orthosilicate (TEOS), triethanolamine (TEA), sodium salicylate (NaSal), hydrofluoric acid (HF, 48 wt%), ammonium fluoride (NH_4F), sodium carbonate (Na_2CO_3), dimethyl sulfoxide (DMSO), phenazine methosulfate (PMS), and immunoglobulin G (IgG) from sheep serum were purchased from Sigma-Aldrich. Polyethylene glycol N-hydroxysuccinimide (PEG-NHS, MW 600) were purchased from Creative PEGworks. Pierce BCA protein assay kit, Alexa Fluor 488 carboxylic acid

succinimidyl ester (AF488), Alexa Fluor 647 carboxylic acid succinimidyl ester (AF647), RPMI 1640 medium, Dulbecco's modified Eagle's medium (DMEM), Fetal bovine serum (FBS), Dulbecco's phosphate-buffered saline (DPBS) buffer, ErbB2 monoclonal antibody, Alexa Fluor 594-conjugated rabbit anti-mouse IgG (H+L) Superclonal™ secondary antibody, Alexa Fluor 594-wheat germ agglutinin conjugate (AF594-WGA), trihydrochloride (Hoechst 33342), 2,3-Bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide inner salt (XTT) were purchased from Thermo Fisher Scientific. BioRad 4–20% Mini-PROTEAN TGX stain-free protein Bis-Tris gels and Tris/glycine/sodium dodecyl sulfate running buffer were purchased from BioRad. NuPAGE LDS sample buffer, SeeBlue Plus2 prestained protein standard, SimplyBlue safe stain, and Dead cell apoptosis kit with Alexa Fluor 488 Annexin V & Propidium Iodide (PI) were purchased from Thermo Fisher Scientific. Herceptin and Kadcyra antibodies were from F. Hoffmann-La Roche Ltd. Ultrapure water with a resistance of greater than 18 MΩ cm (Milli-Q water) was obtained from a three-stage Millipore Milli-Q Plus 185 purification system.

2.3.2 Preparation of Antibody Nano-Assemblies

MSN templates with large pores were synthesised via the anion-assisted method using CTAB as a cationic surfactant, NaSal as a structure-directing agent, TEOS as a silica source, and TEA as a catalyst.²³ Briefly, 68 mg TEA, 380 mg CTAB, and 168 mg NaSal were successively added to 25 mL Milli-Q water, and the mixture was stirred at 80 °C in an oil bath for 1 h. Afterwards, 4 mL TEOS was slowly added to the above solution and stirred at 80 °C for 2 h. The products were collected by centrifugation at 10000 g and washed three times with ethanol. The products were then purified by extraction with ~1 M HCl in methanol at 60 °C for 6 h; the extraction

process was repeated three times. The MSNs were washed with ethanol and finally oven-dried at 80 °C overnight.

Subsequently, 500 μL of IgG solution (2 mg mL^{-1}) was added to 500 μL of MSN solution (2 mg mL^{-1}) in sodium acetate buffer (0.1 M, pH 5.0). The particle/protein dispersion was allowed to incubate for 2 h at room temperature on an Eppendorf thermomixer to allow the IgG molecules to infiltrate the pores of the template via electrostatic adsorption. Excess IgG was removed by centrifugation ($\sim 5000\text{ g}$, 5 min, $\times 3$), and the IgG-loaded nanoparticles were dispersed in DPBS (pH 7.4). The loading capacity was determined by measuring the protein concentration before and after protein incubation with the MSN template using a Pierce BCA protein assay kit according to the manufacturer's protocol. Crosslinking of the IgG molecules within the MSN template was achieved by adding PEG-NHS crosslinker (50 μL of 20 mg mL^{-1} in DMSO) to the IgG-loaded MSNs in DPBS buffer and allowed to incubate for 4 h at room temperature with constant agitation. AbNAs (IgGNAs in this case) were finally obtained by dissolving the MSN template with 1 M HF/3 M NH_4F solution (pH ~ 5), followed by washing with DPBS buffer three times. *Caution! HF is highly toxic. Extreme care should be taken when handling HF solution, and only small quantities should be prepared.* Herceptin nano-assemblies (HerNAs) and Kadcykla nano-assemblies (KadNAs) were prepared using the same procedure.

2.3.3 Quantitative Analysis of Antibodies in AbNAs

The AbNAs protein concentration was determined using a Pierce BCA protein assay kit; particle concentration was measured by nanoparticle tracking analysis using a 405 nm laser. At a protein concentration of 0.02 mg mL^{-1} , the particle concentration is 2.23×10^9 particles mL^{-1} (**Figure S2.3, b**). Thus, 1 mg of AbNAs (4.01×10^{15} IgG molecules) contains 1.12×10^{11}

particles. Therefore, one AbNA contains approximately 3.58×10^4 IgG molecules ($4.01 \times 10^{15}/1.12 \times 10^{11}$).

2.3.4 Antibody Labelling

For the antibody labelling, 5 μL AF488 or AF647 (1 mg mL⁻¹) in DMSO was added to 200 μL of antibody solution (5 mg mL⁻¹) in DPBS buffer containing 0.1 M Na₂CO₃ (pH 8.3) for 2 h at room temperature with constant agitation. Excess free fluorophores were removed using ZebaTM spin desalting columns (40 K molecular weight cut-off, 2 mL). The labelled antibodies were then used to prepare AbNAs for imaging.

2.3.5 Particle Characterisation

Transmission electron microscopy (TEM) images were taken on a FEI Tecnai Spirit microscope operated at 120 kV. Samples were prepared by placing a drop of a sample suspension (suspended in deionized water) on a carbon-coated copper grid and drying under ambient conditions. Particle concentration was determined by nanoparticle tracking using a Nanosight NS300 (Malvern Panalytical, UK). The surface area and the porosity of the silica templates were determined from N₂ adsorption and desorption isotherms measured on an ASAP 2010 System (Micromeritics, US). Hydrodynamic size distribution and Zeta potentials were measured using a Zetasizer Nano ZS (Malvern Instruments, UK). Atomic force microscopy (AFM) images were acquired by a Cypher AFM (Asylum, US). Circular dichroism (CD) spectra were recorded on a circular Dichroism spectrometer (Model 401, AVIV Biomedical, US). Fourier transform infrared (FTIR) spectra in attenuated total reflectance mode were obtained using an FTIR spectrometer (TENSOR II, Bruker, Germany).

2.3.6 Cell Culture

Human breast cancer BT-474 cells were maintained in RPMI medium 1640 supplemented with 10% (v/v) FBS. Human breast cancer MDA-MB-231 cells were incubated in DMEM with 10% (v/v) FBS. All cultures were maintained at 37 °C in a 5% CO₂-humidified atmosphere.

2.3.7 Cellular Association Assessed by Flow Cytometry

Cells were seed at a density of 10⁵ per well in 24-well plates and incubate at 37 °C overnight. Then the culture medium was removed and replaced with fresh medium containing AF488-labelled free antibody and AbNAs (20 µg mL⁻¹). Cells were further incubated at 37 °C for 24h. To assess receptor binding specificity, BT-474 were blocked by pre-incubating cells with non-fluorescent free Herceptin (20 µg mL⁻¹) at 37 °C for 3 h before the incubation with the aforementioned AF488 labelled samples. The sample incubation was conducted under dynamic flow condition using a self-contained device to maintain a well-mixed environment.²⁴ Subsequently, cells were gently washed with DPBS to remove unassociated nanoparticles, followed by treatment with 0.25% trypsin / ethylenediaminetetraacetic acid solution at 37 °C for 5 min. The cells were collected via centrifugation at 300 g for 5 min and the resulting pellet was then resuspended in DPBS and analysed by an Apogee A50-Micro flow cytometer. At least 10⁴ cells were analysed for each sample.

2.3.8 Cellular Internalisation Assessed by Confocal Microscopy

Cells were seeded in an eight-well Lab-Tek II chambered slide at a density of 5 × 10⁴ cells per well for 24h. After incubation, cells were treated with AF488-labelled free antibody and AbNAs (20 µg mL⁻¹) for 24h. Incubation was conducted under dynamic flow condition using

a self-contained device to maintain a well-mixed environment.²⁴ Then the cells were washed with DPBS, fixed with 4% paraformaldehyde for 10 min at room temperature, stained with WGA-594 for 5 min (optional), and counterstained with Hoechst 33342 for another 5 min. The cells were washed with DPBS after each staining step and finally immersed in DPBS before imaging, which was performed on a Nikon A1R+ confocal microscope.

For BT-474 cells, ErbB2 receptors were visualized by immunofluorescence staining. After treated with free antibody and AbNAs, Cells were fixed with 4% paraformaldehyde, permeabilized with ice-cold ethanol for 10 min, and blocked with 2% bovine serum albumin (BSA) for 30 min. Then cells were incubated with mouse anti-ErbB2 monoclonal antibody at a dilution of 1:100 ($1 \mu\text{g mL}^{-1}$) for 1 h at room temperature, and further incubated with Alexa Fluor 594-conjugated rabbit anti-mouse IgG (H+L) Superclonal™ at a concentration of $1 \mu\text{g mL}^{-1}$ for 1 h. Washing with DPBS was performed between steps.

2.3.9 Internalisation in 3D Spheroids

To construct 3D spheroids model, BT-474 cells were seeded in a Perfecta3D hanging drop plate at a concentration of 1×10^4 cells/well in 40 μL of medium containing 0.075 mg mL^{-1} collagen. Cells were incubated for 24h to allow the formation of spheroids in each droplet. Free antibody and AbNAs were gently added into the droplets at a concentration of $200 \mu\text{g mL}^{-1}$. After 48h incubation, spheroids were harvested from the plates, and then fixed with 4% paraformaldehyde for 20 min at room temperature. Finally, spheroids were stained with WGA-594 and Hoechst 33342 for 1h before imaged by a Nikon A1R+ confocal microscope. Washing with DPBS was performed between steps.

2.3.10 Cell Viability

Cells were seeded at a density of 1×10^4 cells per well in triplicate in 96-well microplates and allowed to adhere overnight. Free antibody and AbNAs were added and incubated with cells for 48 h. Incubation was conducted under dynamic flow condition using a self-contained device to maintain a well-mixed environment.²⁴ For all samples, the concentration of antibody was kept constant at $20 \mu\text{g mL}^{-1}$. After incubation, the culture medium was replaced by fresh medium containing 0.2 mg mL^{-1} of activated XTT (10 ml of 0.2 mg mL^{-1} XTT in complete medium were activated by adding $20 \mu\text{L}$ of 1mM PMS in DPBS), and cells were allowed to incubate further at 37°C for 3 h. Cell viability was measured under the Infinite M200 microplate reader (Tecan, Switzerland) at 475 nm , with 647 nm as reference wavelength. Cell viability was expressed as a percentage by normalizing absorbance to untreated cells.

2.4 Results and Discussion

2.4.1 Synthesis and Characterisation

The AbNAs were fabricated by loading antibodies (immunoglobulin G or IgG) in MSNs templates, followed by crosslinking with poly(ethylene glycol)-N-hydroxysuccinimide (PEG-NHS) and removal of the templates (**Figure 2.1A**). The resulting AbNAs formed from IgG are referred to as IgGNAs. IgG is the most abundant type of antibody, with a molecular weight (M_w) of ~150 kDa. To ensure a high loading of IgG in the templates, MSNs with pore sizes as large as 20 nm, surface area up to 624 m² g⁻¹ (**Figure S2.1**), and an average diameter of 250 nm (**Figure 2.1B, 2.1D and S2.3a**) were prepared. In physiological buffer (pH 7.4), the loading capacity of sheep serum IgG (M_w ~150 kDa; Stokes radius (r_s) 6.4 nm; isoelectric point (pI) 11) into negatively charged MSNs could reach as high as ~700 µg protein per 1 mg MSNs, which was significantly higher than the loading achieved for positively charged lysozyme (M_w 14.3 kDa; r_s 1.8 nm; pI 11) or negatively charged bovine serum albumin (M_w 66.5 kDa; r_s 3.48 nm; pI 4.8) (**Figure S2.2a**). The loading capacity of IgG could be increased to ~900 µg protein per 1 mg of MSNs by incubating the templates with IgG solution at a lower pH (sodium acetate buffer, pH 5). The results showed that the MSNs favoured the loading of IgG molecules, which is mainly due to the strong electrostatic interactions between the silica nanoparticles and IgG (**Figure S2.2b**) and the size-matching effect that allows the IgG molecules to be effectively entrapped in the pores of the templates (**Figure S2.2c**).

Freestanding AbNAs were obtained after the removal of the silica templates by HF treatment (pH ~5). It is worth noting that IgG proteins are robust, and exposure of these antibodies to acidic pH induced only limited conformational changes,²⁵ which is beneficial for retaining their bioactivity. The transmission electron microscopy (TEM) image showed that AbNAs deposited on a carbon film had an average diameter of 250 nm (**Figure 2.1C**), which corresponded with

the original size of the MSN templates and was consistent with the hydrodynamic size measured by dynamic light scattering and nanoparticle tracking system (**Figure 2.1D** and **S2.3b**). The thickness of air-dried AbNAs measured by atomic force microscopy (AFM) was approximately 80nm (**Figure S2.4**), which indicates that the assembled antibody nanoparticles were highly hydrated in aqueous solution.²⁶ Circular dichroism (CD) spectroscopy (**Figure 2.1E**) showed that the formation of AbNAs did not affect the molecular conformation of the antibody, as the AbNAs maintained the same secondary structures as free antibodies. Fourier transform infrared spectroscopy measurements (**Figure S2.5**) confirmed that the AbNAs retained the main characteristic bands of free antibody, including the amide I band at 1640 cm^{-1} , the amide II band at 1540 cm^{-1} , and amines at 3400 cm^{-1} .²⁷

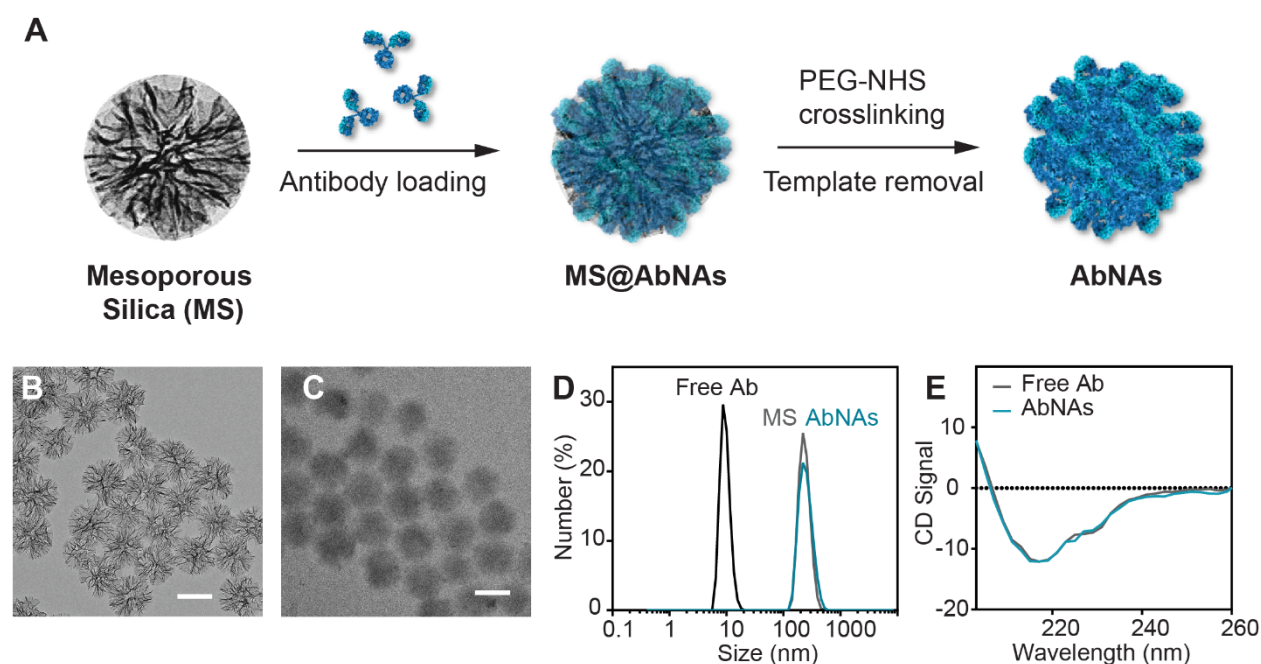


Figure 2.1 Preparation and characterisation of AbNAs (Herceptin as model IgG molecules).

A, Schematic illustration of the template-assisted assembly of antibody molecules into antibody nano-assemblies (AbNAs). B, TEM image of mesoporous silica nanoparticle templates with large pores. C, TEM image of AbNAs after template removal. Scale bars: 200

nm. D, Size distribution of free antibody (Ab), mesoporous silica template and AbNAs by dynamic light scattering. E, Circular dichroism spectra of free Ab and AbNAs.

2.4.2 Bioactivity of HerNAs

To investigate the antigen recognition ability of AbNAs, Herceptin was used as a model IgG molecule. The resulting AbNAs formed from Herceptin were denoted as HerNAs. Herceptin, or ‘Trastuzumab’, is an FDA-approved monoclonal antibody that targets the ErbB2 receptor, which is overexpressed in ~25% of invasive breast carcinomas.²⁸ Herceptin nano-assemblies (HerNAs) were prepared and (**Figure 2.1C, 2.1D, and S2.6**) evaluated for their ability to specifically bind to the overexpressed ErbB2 receptors in ErbB2 positive (ErbB2+) BT-474 breast cancer cells. ErbB2 negative (ErbB2-) MDA-MB-231 breast cancer cells were used as control cells (**Figure 2.2A**). The nanoparticles formed from generic IgG from sheep serum were denoted as IgGNAs and used as control nanoparticles.

The cell targeting ability of HerNAs mediated by ErbB2 recognition was investigated by flow cytometry analysis and confocal laser scanning microscopy (CLSM) (**Figure 2.2 and 2.3**). Conjugation of Alexa Fluor 488 (AF488) dye to the Herceptin molecules aided the detection of antibodies after exposure to cells for 24 h in culture medium at 37 °C. The IgGNAs have negligible associations with BT-474 cells, while both free Herceptin and HerNAs bound to almost all BT-474 cells (98% cell association). In contrast, neither the free Herceptin nor the HerNAs were associated with the ErbB2- MDA-MB-231 cells (less than 5%). Blocking the HER2 receptors by pre-incubating cells with free Herceptin could significantly reduce the association of HerNAs in ErbB2+ cells by over 90% (**Figure 2.2B**). The above results suggest that HerNAs reserved the antigen recognition ability of Herceptin to specifically recognize ErbB2+ cells (BT-474) by interacting with ErbB2 receptors. Furthermore, the AF488

fluorescence intensity of the treated BT-474 cells was quantified as an indicator of the number of antibodies associated with each cell. The intensity of cells treated with HerNAs increased by 45% compared with cells treated with free Herceptin, which suggests that high concentration of HerNAs associated with target cells (**Figure 2.2C**). This finding presents a potential strategy to achieve antibody accumulation in target cells.

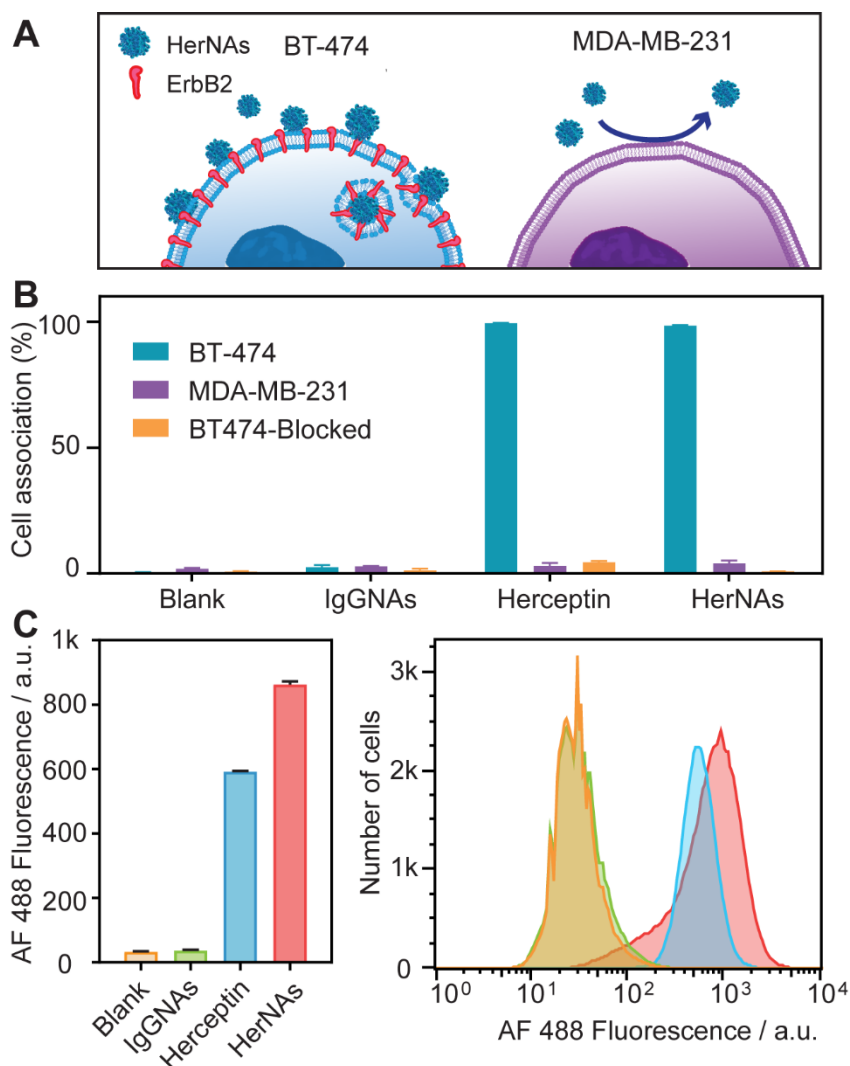


Figure 2.2 Selective association of HerNAs with BT-474 (*ErbB2*+) cells. A, Schematic illustration of selective cell association of HerNAs with *ErbB2*+ cells. B, Cell association (%) profile of HerNAs, free Herceptin and control particle IgGNAs quantified by flow cytometry analysis. Antibodies/AbNAs were labelled with Alexa Fluor 488, incubated with BT-474, MDA-MB-231 and *ErbB2*-blocked BT-474 cells for 24 h. BT-474 were blocked by pre-

incubating cells with non-fluorescent free Herceptin ($20\ \mu\text{g mL}^{-1}$) at $37\ ^\circ\text{C}$ for 3 h to assess receptor binding specificity. Dosage was $20\ \mu\text{g mL}^{-1}$ protein for all samples. C, Alexa Fluor 488 fluorescence intensity of BT-474 cells treated with HerNAs, free Herceptin or control particle IgGNAs at the same protein concentration ($20\ \mu\text{g mL}^{-1}$). The intensity profile indicated the number of antibodies associated with each cell.

The internalisation and intracellular distribution of Herceptin and HerNAs in BT-474 cells were examined by CLSM. Following 24h of incubation with AF488-labelled free Herceptin and HerNAs at 37°C , BT-474 cells were fixed, and the nuclei were stained with Hoechst 33342, while the ErbB2 receptors were stained by immunofluorescence to visualize the antibody-receptor interactions. As shown in **Figure 2.3A and 2.3B**, free Herceptin antibodies were co-localised with the ErbB2 receptors and mostly distributed on the cell membrane with limited internalisation observed. This is consistent with previous studies that demonstrated the fast internalisation and recycling of Herceptin upon binding with ErbB2 receptors on the cell membrane.^{29, 30} In contrast, the nanostructured HerNAs not only distributed on the cell membrane but largely accumulated in the cytoplasm with the ErbB2 receptors (**Figure 2.3B**). The HerNAs were localized in the endolysosomal compartments after internalisation via receptor-mediated endocytosis (**Figure S2.7**), whereas significantly lower levels of endolysosomal colocalization were found for the free Herceptin. Stronger AF488 fluorescence signal was also observed in the microscopy images of cells treated with HerNAs compared with cells treated with free Herceptin, which is consistent with the mean cell fluorescence intensity as determined by flow cytometry (**Figure 2.2C**). The above results suggest that HerNAs could specifically recognize the ErbB2⁺ cells by interacting with the ErbB2 receptors, which is prerequisite for the internalisation of high concentration of HerNAs via receptor-mediated endocytosis.

Moreover, the internalisation behaviour of HerNAs was demonstrated on a 3D tumour model comprising BT-474 cells. The spheroid tumour model was established by incubating cells in a Perfecta3D hanging drop plate with the presence of collagen. The matured spheroids (~300 μm in diameter) were treated with free Herceptin and HerNAs for 48h, then visualized by membrane staining and imaged by CLSM. Results showed that both free Herceptin and HerNAs penetrated the 3D spheroids, but free Herceptin was largely localized on the cell membrane while HerNAs were mostly internalised inside the cells (**Figure 2.3C**). The results in the 3D model were consistent with the findings from the 2D internalisation study (**Figure 2.3B**) and indicate the feasibility of AbNAs to permeate through solid tumour tissue.

Subsequently, the effect of HerNAs on ErbB2⁺ and ErbB2[−] cancer cell growth was explored *in vitro*. As reported previously, ErbB2 signalling promotes cell proliferation and Herceptin interferes with ErbB2-triggered signalling by preventing ErbB2 receptor dimerization.^{31, 32} To investigate if HerNAs retained the cell growth inhibition function of Herceptin, the proliferation of ErbB2⁺ BT-474 and ErbB2[−] MDA-MB-231 cells were examined by XTT cell viability assay after treatment with HerNAs, free Herceptin or controls (IgGNAs) for 48h. As shown in **Figure 2.4**, ~50% reduction in cell viability was observed in BT-474 cells after treatment with HerNAs when compared with the cell viability of the untreated cells (blank), whereas no reduction in cell viability was found in MDA-MB-231 cells after the treatment. Importantly, HerNAs reduced cell viability to the same extent as free Herceptin, which suggests that HerNAs preserved the ability of free Herceptin to selectively inhibit cancer cell growth or proliferation via interacting with ErbB2 receptors.

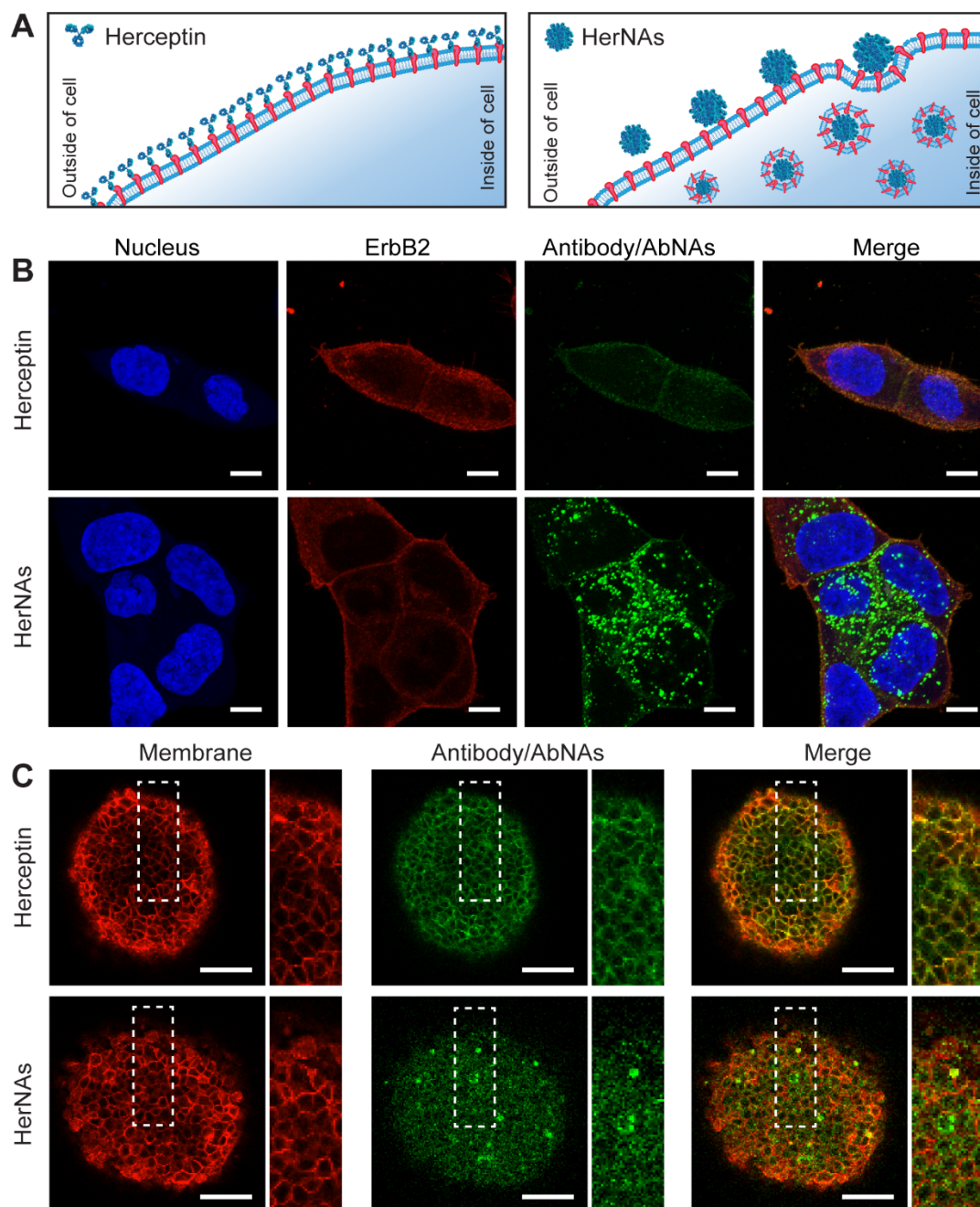


Figure 2.3 Cellular internalisation of HerNAs in BT474 cells via ErbB2 receptor-mediated endocytosis, demonstrated on 2D and 3D models. A, Schematic illustration of cellular internalisation of HerNAs comparing with free Herceptin. B, Internalisation of HerNAs in BT-474 cells via ErbB2 receptor-mediated endocytosis, observed by CLSM. BT-474 cells were treated with free Herceptin or HerNAs at a dosage of $20 \mu\text{g mL}^{-1}$ for 24h. Nuclei were stained with Hoechst 33342 dye (blue); ErbB2 receptors were immunostained with anti-ErbB2 monoclonal antibody followed by Alexa Fluor 594-conjugated secondary antibody (red);

Herceptin antibodies were labelled with Alexa Fluor 488 (green). Scale bars: 10 μm . C, Internalisation of HerNAs in BT-474 spheroids, observed by CLSM. The spheroids were formed by incubating cells in a Perfecta3D hanging drop plate with the presence of collagen. Matured spheroids were treated with free Herceptin and HerNAs a dosage of 20 $\mu\text{g mL}^{-1}$ for 48h. Cell membrane was stained with WGA-594 (red); Herceptin antibodies were labelled with Alexa Fluor 488 (green); Enlarged images of regions indicated by dashed lines were showed on the right side of each original image. Scale bars: 100 μm .

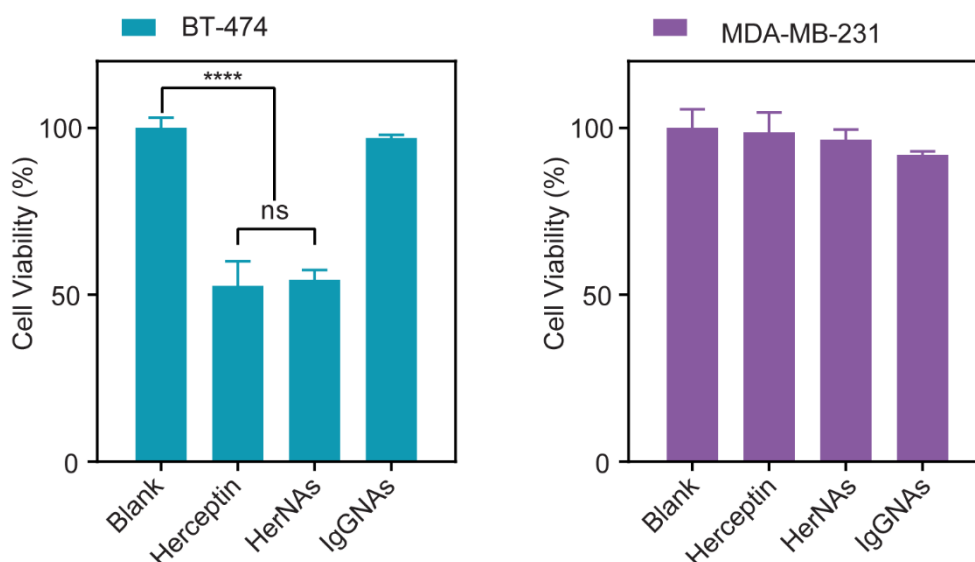


Figure 2.4 Inhibition effect of HerNAs on BT-474 (ErbB2+) and MDA-MB-231 (ErbB2-) cell growth. Cells were treated with free Herceptin, HerNAs, and IgGNAs for 48 h at a protein concentration of 20 $\mu\text{g mL}^{-1}$. The cell viability was then examined by 2,3-bis-(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay. **** Indicates significant difference ($p < 0.0001$, $n = 3$) between cells treated with free Herceptin/HerNAs and untreated cells; *ns* Indicates there is no significant difference ($p > 0.05$, $n = 3$) between cells treated with free Herceptin and cells treated with HerNAs.

Interestingly, HerNAs with the mesoporous silica core intact (MS@HerNAs) were less selective with regards to the association, internalisation ability, and cytotoxicity as compared with freestanding HerNAs (without MSN core inside) (**Figure S2.8, S2.9, and S2.10**). MS@HerNAs unselectively bound to the same percentage (~ 60%) of BT-474 cells and MDA-MB-231 cells (**Figure S2.8**). No significant cytotoxicity was induced by MS@HerNAs on either cell lines (**Figure S2.10**). The reduction of targeting specificity is likely due to the presence of MS cores which dominated the surface properties of nanoparticles, leading to the formation of biomolecular corona and nonspecific cellular interactions as we previously observed on other polymer nanoparticles.^{33, 34} The formation of biomolecular coronas on nanoparticles could also hinder binding of the antibody to its receptor.³⁵

2.4.3 Bioactivity of KadNAs

After demonstrating the selectivity and the uptake of HerNAs via receptor-mediated endocytosis, a Herceptin-drug conjugate, Kadcyla, was applied to test the versatility of the AbNA platform. Kadcyla (also known as ado-trastuzumab emtansine) is the first FDA-approved antibody-drug conjugate for clinical ErbB2+ breast cancer treatment.³⁶ It is composed of the ErbB2 receptor targeting monoclonal antibody, Herceptin (trastuzumab), and a cytotoxic agent emtansine (DM1) that inhibits microtubules polymerization to cause cell-cycle arrest and cell death.^{37, 38} On binding to subdomain IV of the ErbB2 receptor, Kadcyla undergoes receptor-mediated internalisation and lysosomal degradation, resulting in the intracellular release of DM1.³⁹ KadNAs were prepared via the template-assisted antibody assembly method as described for HerNAs (**Figure S2.11 and S2.12**). The selective cell association of KadNAs was evaluated on ErbB2+ BT-474 cells and ErbB2– MDA-MB-231 by flow cytometry, which showed that KadNAs preserved the targeting ability of free Kadcyla to

selectively associate with ErbB2⁺ BT-474 cells (**Figure 2.5B**). To assess whether the intracellular effect of Kadcyta is enhanced, cellular internalisation was investigated using CLSM and the cell growth inhibition was examined. KadNAs were largely internalised inside the target cells while free KadNAs appear mostly distributed on the membrane (**Figure 2.5C**). Moreover, KadNAs exhibited improved cytotoxicity in BT-474 cells compared with free Kadcyta (**Figure 2.5D**), likely owing to the enhanced internalisation and accumulation of DM1 inside cells (**Figure 2.5A**). Under the same incubation conditions, KadNAs also appeared more cytotoxic than HerNAs (**Figure 2.4, 2.5D and S2.13**).

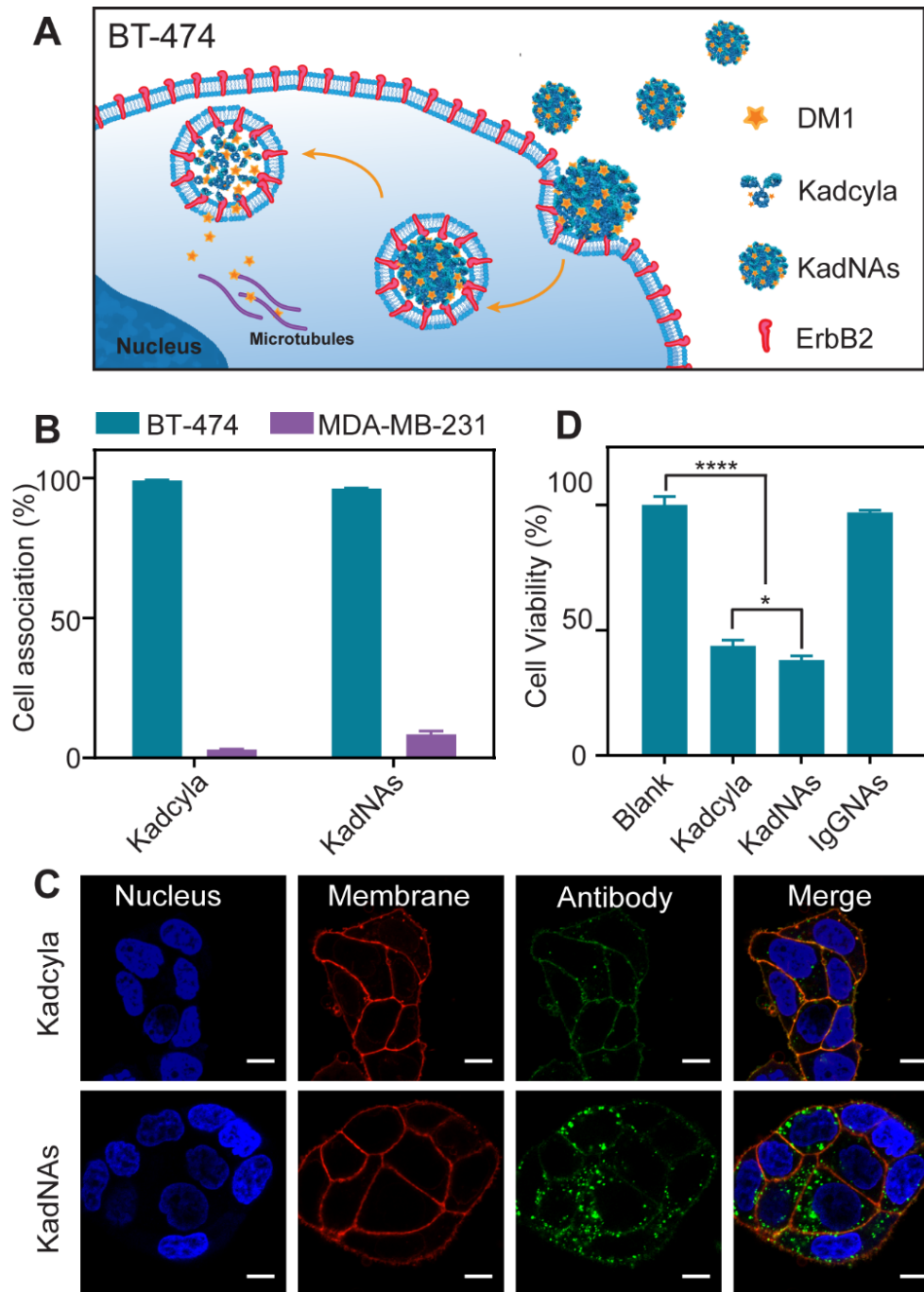


Figure 2.5 Bioactivity of KadNAs: cell association, internalisation and growth inhibition. A, Schematic illustration of interactions between KadNAs and cells. KadNAs selectively bind to the ErbB2 receptor on cell membrane, and internalised via ErbB2-mediated endocytosis. Lysosomal degradation releases DM1 into cytoplasm causing cell-cycle arrest and cell death. B, Association of KadNAs and free Kadcyla with BT-474 (ErbB2+) and MDA-MB-231 (ErbB2-) cells after 24 h incubation, quantified by flow cytometry analysis. C, Internalisation

of KadNAs in BT-474 cells after 24 h treatment, observed by CLSM. Nuclei were stained with Hoechst 33342 dye (blue); Cell membranes were stained with WGA-594 (red); Kadcyta antibodies were labelled with Alexa Fluor 488 (green). Scale bars: 10 μm . D, Viability of cells treated with free Kadcyta, KadNAs, and IgGNAs for 48 h at a given protein concentration of 20 $\mu\text{g mL}^{-1}$, assessed by 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay. **** Indicates significant difference ($p < 0.0001$, $n = 3$) between cells treated with free Kadcyta/KadNAs and untreated cells; * Indicates significant difference ($p < 0.05$, $n = 3$) between cells treated with free Kadcyta and cells treated with KadNAs.

2.5 Conclusions

In summary, this study demonstrates the template-assisted antibody assembly platform that widely applies to therapeutic antibodies and antibody-drug conjugates. The AbNAs prepared by the assembly method exhibited well-defined morphology and retained the antigen specificity of the free antibody. Importantly, the particulate nature of the AbNAs significantly induced antibody internalisation and accumulation, and enhanced subsequent intracellular responses such as cell growth inhibition. These findings provide evidence that AbNAs may mediate cell processes in a way different from free antibodies. The template-assisted assembly of antibodies provides a platform for exploring and understanding the unique biological behaviour of well-defined antibody nanostructures and can facilitate the engineering of novel multifunctional diagnostics and antibody therapeutics. Our future work is focused on examining the effect of antibody nanoparticle engineering on their *in vivo* behaviour, targeting ability and therapeutic efficacy.

2.6 Supporting Information

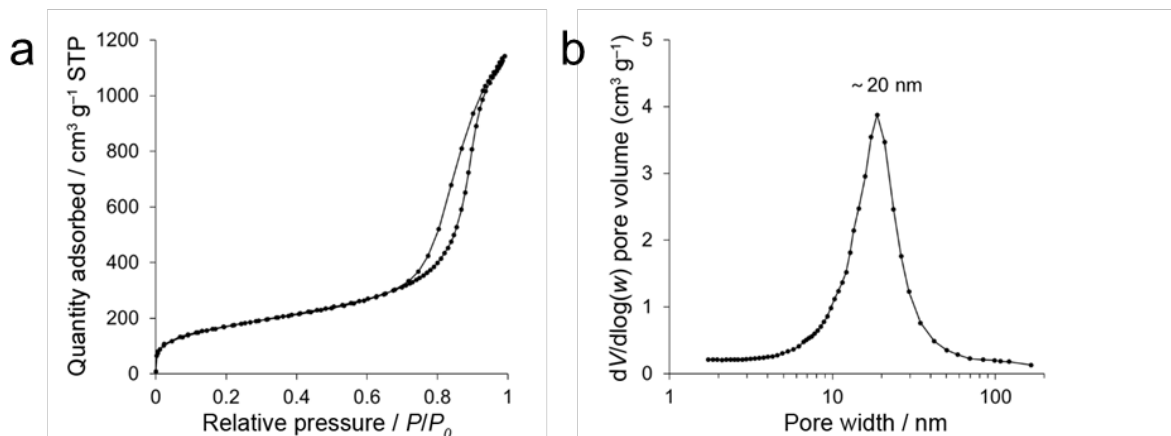


Figure S2.1 N₂ adsorption-desorption isotherm (a) and pore size distribution (b) of the MSN templates. The large pore size (~20 nm), pore volume (1.76 cm³ g⁻¹), and large surface area (624.1 m² g⁻¹) of the templates are expected to facilitate the loading of large molecular weight molecules.

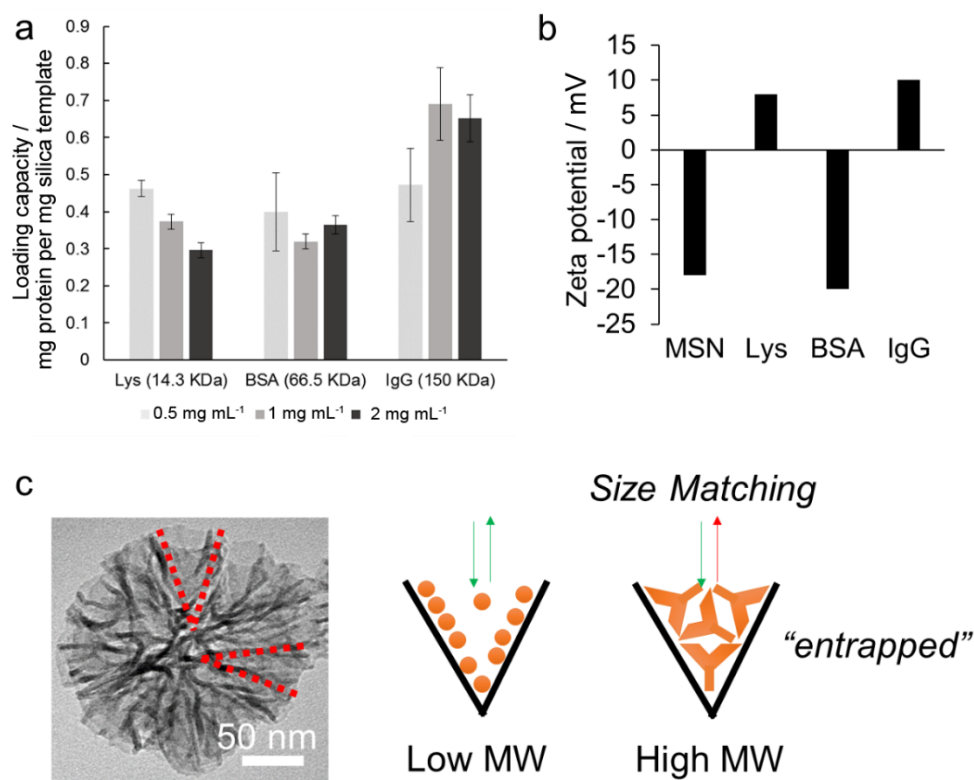


Figure S2.2 a) Loading capacity of lysozyme (Lys), bovine serum albumin (BSA), and IgG in MSN templates at different starting concentrations of protein (0.5, 1, and 2 mg mL⁻¹). The loading capacity was estimated from the protein concentration before and after incubation, and was measured using a BCA protein assay kit. b) Zeta potential measurements of MSN, Lys, BSA, and IgG solution in phosphate buffer (pH 7.4). c) High-resolution TEM image of a representative MSN template. The large pores in the template provide adequate space for proteins to infiltrate and for the IgG molecules to be effectively entrapped in the pores owing to size-matching effects. In contrast, proteins with lower molecular weights (MWs) (Lys and BSA) may move more freely in and out of the pores of the template as illustrated in the scheme.

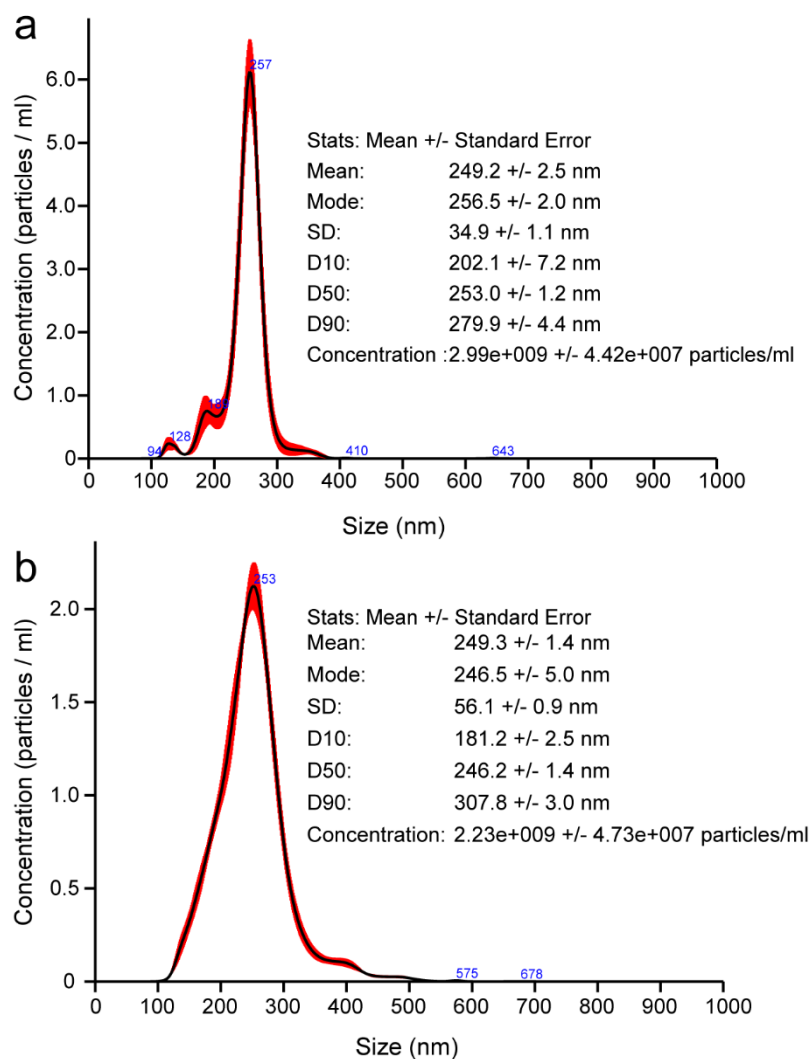


Figure S2.3 Particle concentration and size distribution of MSN templates (a) and AbNAs (b) using nanoparticle tracking analysis. Averaged data from triplicate measurements of particle solution diluted 100 times from the stock solution (2 mg mL^{-1}) are shown. The mean size of MSN and AbNAs were $(249.2 \pm 2.5) \text{ nm}$, $(249.3 \pm 1.4) \text{ nm}$, respectively, which was consistent with TEM observation.

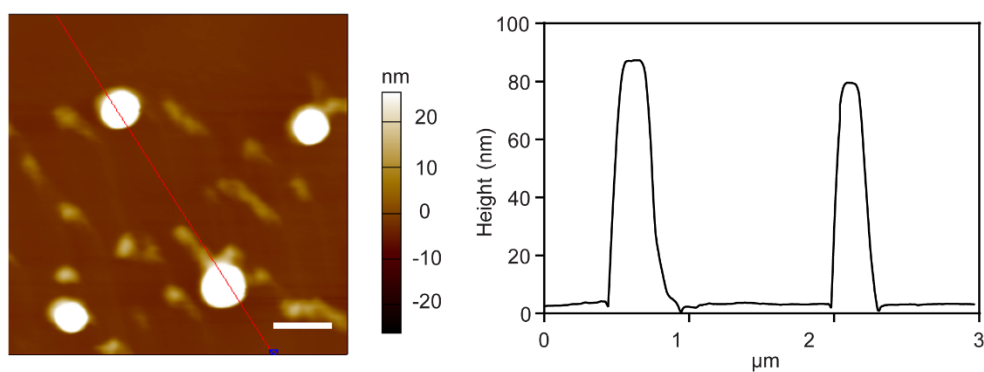


Figure S2.4 Height profile of representative IgGNAs as measured by AFM from the line indicated in the left image. Scale bar: 1 μm . Diluted IgGNAs suspensions were air-dried onto mica substrates, which were pre-cleaned with methanol/acetone and pre-coated with polyethylenimine.

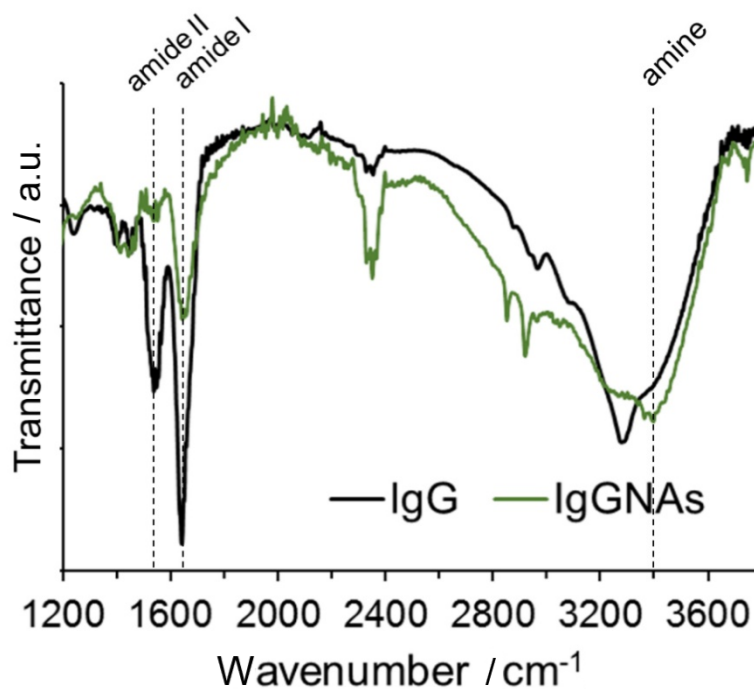


Figure S2.5 FTIR spectra of the free IgG and IgGNAs measured by an FTIR spectrometer fitted with an attenuated total reflectance accessory. A drop ($\sim 3 \mu\text{L}$) of the sample solution (2 mg mL^{-1}) was placed on the diamond window and allowed to dry before measurement. The spectra of IgG and IgGNAs showed peaks at the same locations but with different peak intensities, suggesting that the IgGNAs retained the chemical structure of IgG following crosslinking.

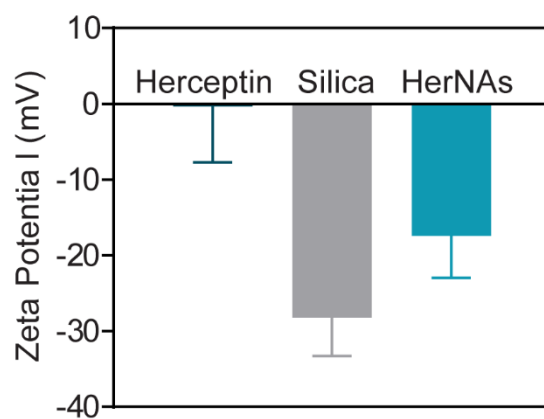


Figure S2.6 Zeta potential of free Herceptin, Mesoporous silica and HerNAs dispersed in 2 mM of phosphate buffer (pH 7.4).

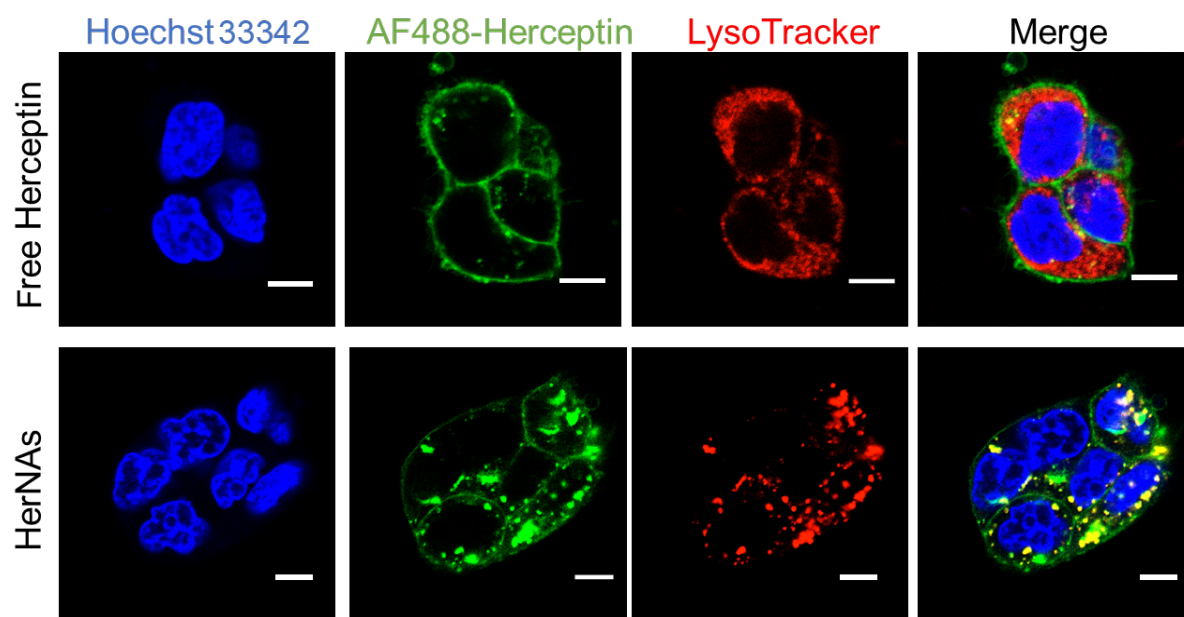


Figure S2.7 Colocalization of AF488-labelled free Herceptin and HerNAs (green) with lysosomal compartments of BT-474 cells after 3 h incubation at 37 °C. The lysosomes (red) were stained by LysoTracker Red DND-99. Cell nuclei were stained with Hoechst 33342 dye (blue). Scale bars: 10 μm .

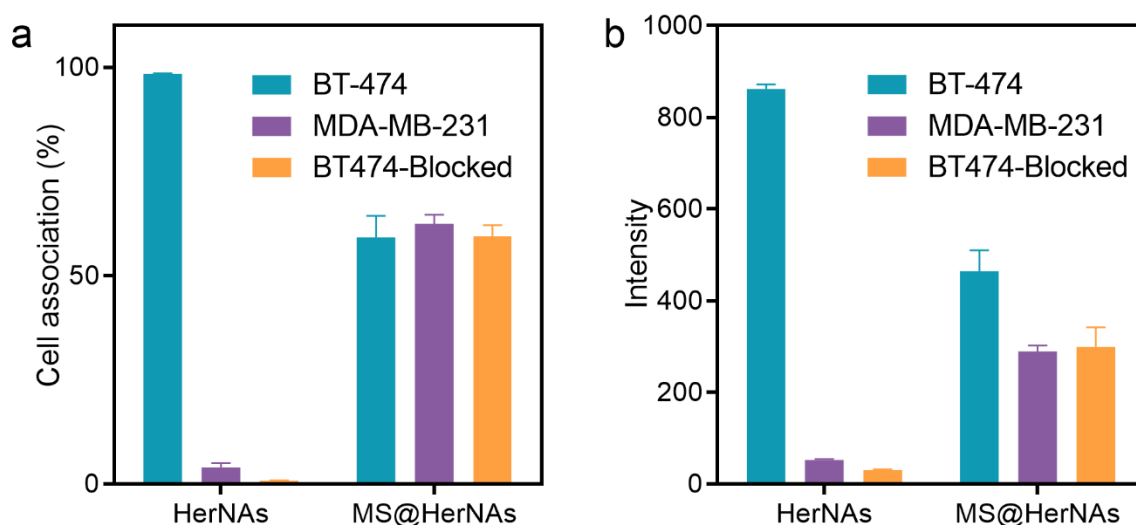


Figure S2.8 Association of MS@HerNAs with BT-474, MDA-MB-231 cells. a), Cell association (%) of HerNAs and MS@HerNAs with BT-474, MDA-MB-231 or blocked BT-474 cells after 24 h treatment, quantified by flow cytometry analysis. Antibodies were labelled with Alexa Fluor 488. BT-474 cells were pre-blocked by pre-incubating cells with non-fluorescent free Herceptin to assess receptor binding specificity. b), Alexa Fluor 488 fluorescence intensity of cells treated with HerNAs and MS@HerNAs for 24 h. The intensity profile is an indication of the number of antibodies associated with each cell.

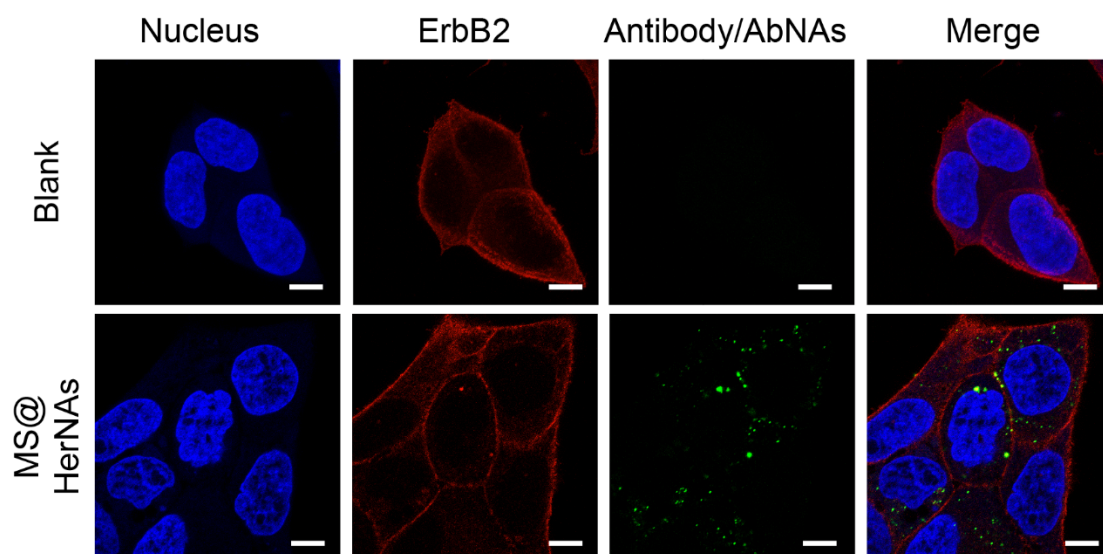


Figure S2.9 Internalisation of MS@HerNAs in BT-474 cells via ErbB2 receptor-mediated endocytosis, observed by CLSM. BT-474 cells were treated with MS@HerNAs for 24 h. Nuclei were stained with Hoechst 33342 dye (blue); ErbB2 receptors were immunostained with anti-ErbB2 monoclonal antibody followed by Alexa Fluor 594-conjugated secondary antibody (red); Herceptin antibodies were labelled with Alexa Fluor 488 (green). Scale bars: 10 μm .

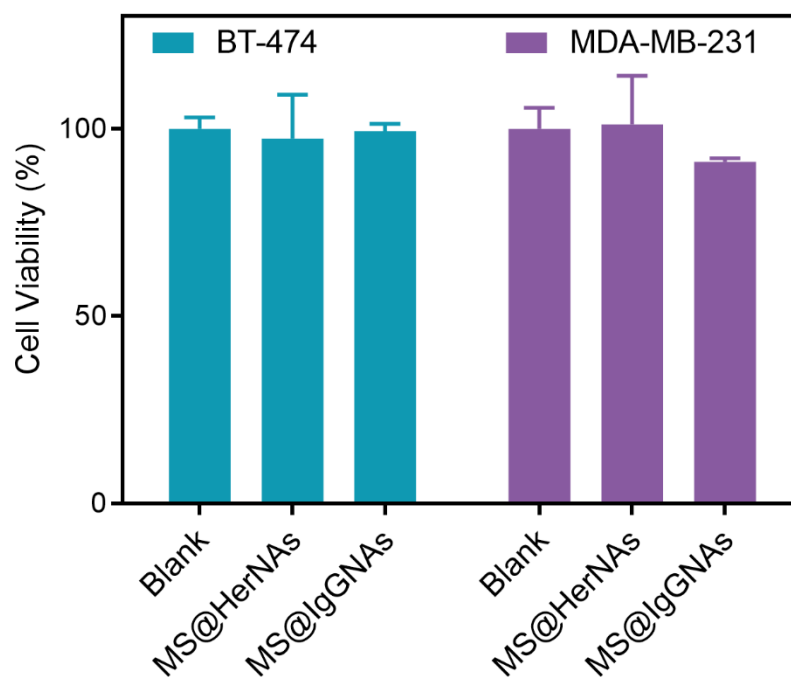


Figure S2.10 Viability of cells treated with MS@HerNAs for 48 h at a given antibody concentration of $20 \mu\text{g mL}^{-1}$, assessed by 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay.

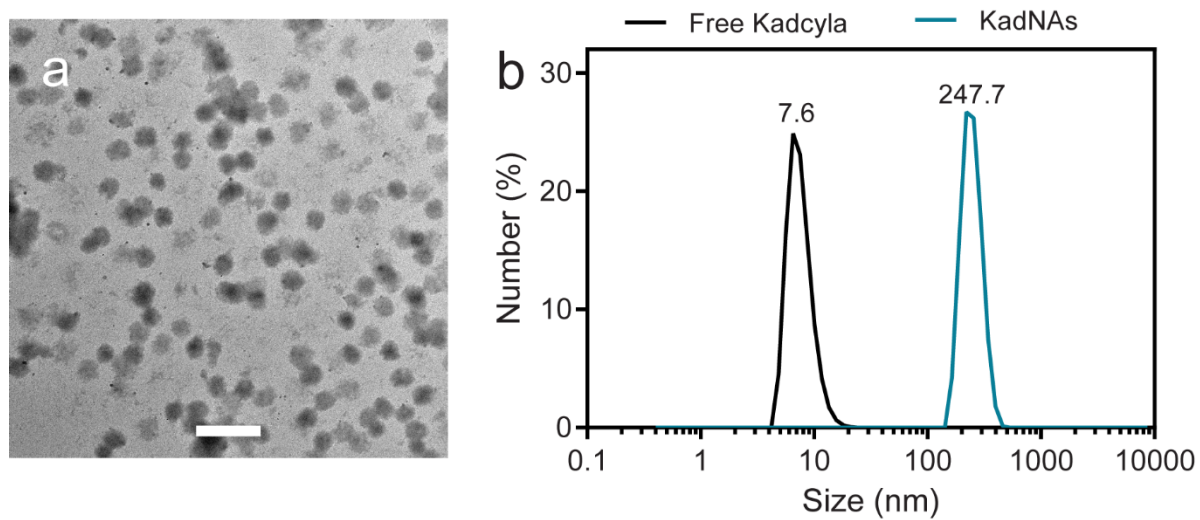


Figure S2.11 a) TEM image of KadNAs. b) Size distribution profiles of free Kadcyta and KadNAs dispersed in DPBS, as measured by dynamic light scattering.

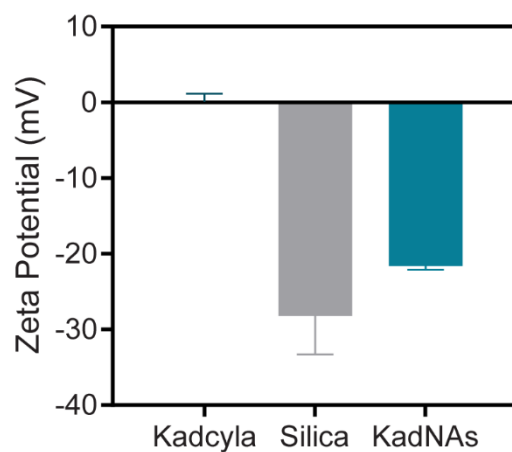


Figure S2.12 Zeta potential of free Kadcyta, Mesoporous silica and KadNAs dispersed in 2 mM of phosphate buffer (pH 7.4).

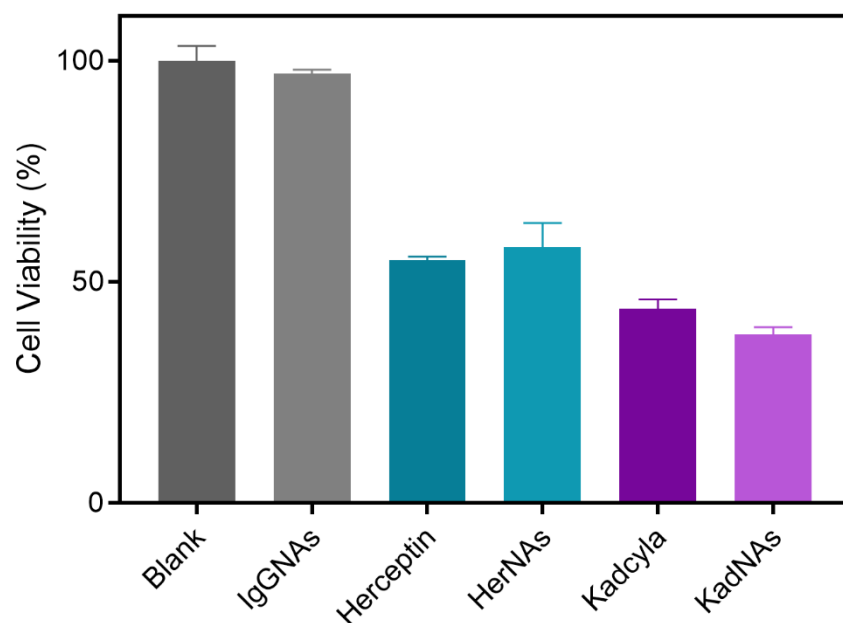


Figure S2.13 Viability of cells treated with free Herceptin, HerNAs, free Kadcyla, and KadNAs for 48 h at a given protein concentration of $20 \mu\text{g mL}^{-1}$, assessed by 2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay.

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Chapter 3

Influence of Poly(ethylene glycol)

**Crosslinking on Antibody Nano-Assemblies
and Cell Interactions**

Contents

3.1	Aims.....	80
3.2	Introduction.....	80
3.3	Experimental Section.....	82
3.3.1	Materials	82
3.3.2	Preparation of Antibody Nano-Assemblies	83
3.3.3	Antibody Labelling	84
3.3.4	Particle Characterisation	84
3.3.5	Stability in GSH and Proteinase	84
3.3.6	Quantitative Degradation Profiles in Proteinase.....	85
3.3.7	Cell Culture.....	85
3.3.8	Cellular Association Assessed by Flow Cytometry.....	86
3.3.9	Cellular Internalisation Assessed by Imaging Flow Cytometry	86
3.3.10	Cellular Internalisation Assessed by Confocal Microscopy	87
3.3.11	Cell Viability Assessed by XTT assay.....	87
3.3.12	Association with Phagocytes from Human Whole Blood	87
3.4	Results and Discussion	89
3.4.1	Synthesis and Characterisation	89
3.4.2	Stability Study.....	91
3.4.3	Targeting Ability.....	94
3.4.4	Cellular Internalisation.....	97
3.4.5	Targeting Cytotoxicity	101
3.4.6	Association with Phagocytes from Human Whole Blood	103
3.5	Conclusions.....	106
3.6	Supporting Information.....	107
3.7	References.....	119

3.1 Aims

Functionalisation with poly(ethylene glycol) (PEG), or PEGylation is a well-accepted as strategy to improve the stealth ability of nanoparticles in blood circulation, which increase the chances of nanoparticles reaching their desired action site. However, PEGylation could also result in decreased targeting efficacy by blocking the binding of targeting ligands to their target. For AbNAs, PEG could block the complementary-determining regions of antibodies from binding to its antigen or receptor. In this chapter, the aims are to investigate the effect of PEGylation, specifically the molecular architecture of the PEG crosslinker, on the assembly, targeting ability and stealth property of antibody nano-assemblies. Herceptin was used as a model antibody molecule, and the bioactivities of PEGylated Herceptin nano-assemblies were investigated on BT-474, MDA-MB-231, RAW 267.4 cell lines and human blood cells.

3.2 Introduction

Poly(ethylene glycol) (PEG) is an FDA approved polymer that is commonly used as an excipient in pharmaceutical products to improve their surface hydrophilicity and solubility.¹⁻³ Functionalizing nanoparticles with PEG, or PEGylation, has been widely acknowledged as an effective strategy to prolong circulation time by the formation of a hydrating layer to hinder protein adsorption and subsequent mononuclear phagocyte system clearance.⁴⁻⁷ The stealth properties of PEGylated nanoparticles are largely related to the PEG chain architecture (such as PEG arm numbers and arm length) and surface density.⁸⁻¹⁰

However, the presence of PEG can reduce the biological activities of functional molecules (such as enzymes, antibodies and hyaluronic acid) by limiting the interactions between the functional molecules and corresponding receptors, possibly due to the low-fouling nature of

PEG and the steric crowding at the active or binding site.¹¹⁻¹⁴ Specifically, when modified on the antibodies or antibody-based nanoparticles, PEG could block the antigen-binding fragment and further hinder the specific targeting.¹³ Despite the extensive studies on PEGylated nanoparticles, there is no general criterion as to what is essential to balance the stealth and biological activities due to the physiochemical differences among the particles systems.^{9-11, 14-17} Therefore, it is important to understand how the PEGylation of antibody-based nanoparticles affects the specific antibody-receptor binding to achieve optimal targeting and stealth ability.

In the previous chapter, the mesoporous silica template-assisted assembly of antibody nano-assemblies (AbNAs) was described. Briefly, the AbNAs were fabricated by loading antibodies in mesoporous silica templates, followed by crosslinking with poly(ethylene glycol)-N-hydroxysuccinimide (PEG-NHS) and removal of the templates. The synthesised AbNAs exhibited promising features for biomedical applications, including the selectivity of the monoclonal antibody to recognize target molecules, which triggered downstream cellular events. However, AbNAs showed increased uptake by phagocytic cells when exposed to whole blood *ex vivo*, resulting in rapid clearance by phagocytic blood cells. To achieve the desired targeting action, it is essential to improve the stealth properties of AbNAs in blood circulation.

In this chapter, various PEG crosslinkers with different arm lengths and arm numbers were used to prepare PEGylated antibody nano-assemblies (PEGx-AbNAs) via the mesoporous silica template-assisted assembly platform. The assembly, selective targeting, phagocytic association and biodistribution in mice of PEGx-HerNAs was investigated to fully understand how arm length and arm numbers of the PEG crosslinker affect the bio-nano interactions of antibody nano-assemblies.

3.3 Experimental Section

3.3.1 Materials

Cetyltrimethylammonium bromide (CTAB), tetraethyl orthosilicate (TEOS), triethanolamine (TEA), sodium salicylate (NaSal), hydrofluoric acid (HF, 48 wt%), ammonium fluoride (NH₄F), sodium carbonate (Na₂CO₃), dimethyl sulfoxide (DMSO), phenazine methosulfate (PMS), Glutathione (GSH) and immunoglobulin G (IgG) from sheep serum were purchased from Sigma-Aldrich. Polyethylene glycol N-hydroxysuccinimide (2-arm-PEG-NHS, MW 600, 2.5k and 5k) were purchased from Creative PEGworks. Multi-arm polyethylene glycol N-hydroxysuccinimide (4-arm-PEG-NHS, MW 5k, and 8-arm-PEG-NHS, MW 10k) were purchased from Jenkem. Alexa Fluor 488 carboxylic acid succinimidyl ester (AF488), Alexa Fluor 647 carboxylic acid succinimidyl ester (AF647), RPMI 1640 medium, Dulbecco's modified Eagle's medium (DMEM), Fetal bovine serum (FBS), Dulbecco's phosphate-buffered saline (DPBS) buffer, Alexa Fluor 594-wheat germ agglutinin conjugate (AF594-WGA), trihydrochloride (Hoechst 33342), 2,3-Bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide inner salt (XTT) were purchased from Thermo Fisher Scientific. BioRad 4–20% Mini-PROTEAN TGX stain-free protein Bis-Tris gels and Tris/glycine/sodium dodecyl sulfate running buffer were purchased from BioRad. NuPAGE LDS sample buffer and SeeBlue Plus2 prestained protein standard were purchased from Thermo Fisher Scientific. Herceptin antibodies were from F. Hoffmann-La Roche Ltd. Ultrapure water with a resistance of greater than 18 MΩ cm (Milli-Q water) was obtained from a three-stage Millipore Milli-Q Plus 185 purification system.

3.3.2 Preparation of Antibody Nano-Assemblies

Mesoporous silica nanoparticle (MSN) templates with large pores were synthesised via the anion-assisted method using CTAB as a cationic surfactant, NaSal as a structure-directing agent, TEOS as a silica source, and TEA as a catalyst, as described in the last chapter. Briefly, 68 mg TEA, 380 mg CTAB, and 168 mg NaSal were successively added to 25 mL Milli-Q water, and the mixture was stirred at 80 °C in an oil bath for 1 h. Afterwards, 4 mL TEOS was slowly added to the above solution and stirred at 80 °C for 2 h. The products were collected by centrifugation at 10000 g and washed three times with ethanol. The products were then purified by extraction with ~1 M HCl in methanol at 60 °C for 6 h; the extraction process was repeated three times. The MSNs were washed with ethanol and finally oven-dried at 80 °C overnight.

Subsequently, 500 µL of Herceptin solution (2 mg mL⁻¹) was added to 500 µL of MSN solution (2 mg mL⁻¹) in sodium acetate buffer (0.1 M, pH 5.0). The particle/protein dispersion was allowed to incubate for 2 h at room temperature on an Eppendorf thermomixer to allow the antibodies molecules to infiltrate the pores of the template via electrostatic adsorption. Excess antibodies were removed by centrifugation (~5000 g, 5 min, ×3), and the Herceptin-loaded nanoparticles were dispersed in DPBS (pH 7.4). The PEG-NHS crosslinker (2-arm-PEG(600)-NHS, 2-arm-PEG(2.5k)-NHS, 2-arm-PEG(5k)-NHS, 4-arm-PEG(5k)-NHS or 8-arm-PEG(10k)-NHS) was added to the Herceptin-loaded MSNs in PBS buffer and allowed to incubate for 4 h at room temperature with constant agitation. PEGx-HerNAs were finally obtained by dissolving the MSN template with 1 M HF/3 M NH₄F solution (pH ~5), followed by washing with DPBS buffer three times. *Caution! HF is highly toxic. Extreme care should be taken when handling HF solution, and only small quantities should be prepared.* Non-specific control particles IgGNAs were prepared following the same methods but IgG from sheep serum instead of Herceptin was used.

3.3.3 Antibody Labelling

For antibody labelling, 5 μL AF488 or AF647 (1 mg mL^{-1}) in DMSO was added to 200 μL of antibody solution (5 mg mL^{-1}) in DPBS buffer containing $0.1\text{ M Na}_2\text{CO}_3$ (pH 8.3) for 2 h at room temperature with constant agitation. Excess free fluorophores were removed using ZebaTM spin desalting columns (40 K molecular weight cut-off, 2 mL). The labelled antibodies were then used to prepare fluorescently labelled AbNAs for imaging.

3.3.4 Particle Characterisation

Transmission electron microscopy (TEM) images were taken on an FEI Tecnai Spirit microscope operated at 120 kV. Samples were prepared by placing a drop of a sample suspension (suspended in deionized water) on a carbon-coated copper grid and drying under ambient conditions. Hydrodynamic size distribution and zeta potentials were measured using a Zetasizer Nano ZS (Malvern Instruments, UK). Atomic force microscopy (AFM) images were acquired using a Cypher ES AFM (Asylum Research, US). Circular dichroism (CD) spectra were recorded on a circular Dichroism spectrometer (Model 401, AVIV Biomedical, US).

3.3.5 Stability in GSH and Proteinase

Free Herceptin and PEGx-HerNAs were incubated in 5 mM GSH or 1 mg mL^{-1} protease for 24 h under constant agitation. After incubation, the degradation profiles of PEGx-HerNAs were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Briefly, each sample was added to NuPAGE LDS sample buffer and heated to $70\text{ }^\circ\text{C}$ for 10 min. An aliquot of $15\text{ }\mu\text{L}$ per sample was loaded on a BioRad 4–20% Mini-PROTEAN TGX

stain-free gel to run at 120 V for 30 min. Gels were imaged under UV radiation in a Bio-Rad Gel documentation XR system. SeeBlue Plus2 prestained protein standards were loaded in the gels and visualized using SimplyBlue safe stain. Images of gels were cropped and presented with their respective standard molecular weight ladder.

3.3.6 Quantitative Degradation Profiles in Proteinase

AF488-labelled free Herceptin antibody or PEGx-HerNAs were dispersed in 1 mL protease solution (1 mg mL⁻¹) and contained in a dialysis tubing with molecular weight-cutoff of 100k. The dialysis tubing was further incubated in an external chamber filled with 9 mL of protease solution (1 mg mL⁻¹) under constant agitation. The same protocol was repeated in DPBS as control. After incubation for 2, 4, 6, 8, 10, 24 or 48 h, the AF488 fluorescence intensity in the external chamber solution (I_{degraded}) was determined using the Infinite M200 microplate reader (Tecan, Switzerland). The same amount of AF488-labelled Herceptin and PEGx-HerNAs were dispersed in 10 mL of protease solution (1 mg mL⁻¹) and their corresponding AF488 fluorescence intensity (I_{total}) was measured using the microplate reader. The degradation percentage of each sample at different time points was calculated as follows: Degradation (%) = $I_{\text{degraded}} / I_{\text{total}} * 100\%$

3.3.7 Cell Culture

ErBb2+ human breast cancer BT-474 cells were maintained in RPMI medium 1640 supplemented with 10% (v/v) FBS. ErbB2- human breast cancer MDA-MB-231 cells and immortal macrophage Raw 267.4 cells were incubated in DMEM with 10% (v/v) FBS. All cultures were maintained at 37 °C in a 5% CO₂-humidified atmosphere.

3.3.8 Cellular Association Assessed by Flow Cytometry

Cells were seeded at a density of 10^5 per well in 24-well plates and incubated at 37 °C overnight. Then the culture medium was removed and replaced with fresh medium containing AF488-labelled free Herceptin antibody or PEGx-HerNAs ($20 \mu\text{g mL}^{-1}$). After incubation for various time periods, cells were gently washed with DPBS to remove unassociated nanoparticles, followed by treatment with 0.25% trypsin at 37 °C for 5 min. The cells were collected via centrifugation at 300 g for 5 min and the resulting pellet was then resuspended in DPBS. Finally, the cells were analysed by an Apogee A50-Micro flow cytometer. At least 10^4 cells were analysed for each sample.

3.3.9 Cellular Internalisation Assessed by Imaging Flow Cytometry

Cells were seeded in a 6-well plate at a density of 5×10^5 cells per well and incubated overnight. Then the culture medium was removed and replaced with fresh medium containing AF488-labelled free Herceptin and PEGx-HerNAs ($20 \mu\text{g mL}^{-1}$). After 24 h of incubation, cells were washed gently with DPBS, harvested with 0.25% trypsin and collected via centrifugation at 300 g for 5 min. The resulting pellet was then resuspended and fixed in 200 μL 4% paraformaldehyde for 15 min at room temperature. Following this, cells were incubated with AF594-WGA ($5 \mu\text{g mL}^{-1}$) for 5 min at room temperature to stain the membrane, then further washed twice with DPBS and resuspended in 100 μL DPBS. Finally, cells were analysed using AMNIS ImageStream^{®X} MarkII imaging flow cytometer (Amnis Corporation, USA). At least 5000 cells were analysed for each sample. The built-in internalisation wizard of Amnis ImageStream IDEAS software was used to calculate the internalisation percentage on focused single cells statistically.

3.3.10 Cellular Internalisation Assessed by Confocal Microscopy

Cells were seeded in an eight-well Lab-Tek II chambered slide at a density of 5×10^4 cells per well for 24h. After incubation, cells were treated with AF488-labelled free Herceptin and PEGx-HerNAs ($20 \mu\text{g mL}^{-1}$) for 24h. Then the cells were washed with DPBS, fixed with 4% paraformaldehyde for 15 min at room temperature, stained with WGA-594 for 5 min, and counterstained with Hoechst 33342 for another 5 min. The cells were washed with DPBS after each staining step and finally immersed in DPBS before imaging, which was performed on a Nikon A1R+ confocal microscope.

3.3.11 Cell Viability Assessed by XTT assay

Cells were seeded at a density of 1×10^4 cells per well in triplicate in 96-well microplates and allowed to adhere overnight. Free Herceptin or PEGx-HerNAs were added and incubated with cells for 72 h. For all samples, the concentration of antibody was kept constant at $20 \mu\text{g mL}^{-1}$. After incubation, the culture medium was replaced by fresh medium containing 0.2 mg mL^{-1} of activated XTT (10 ml of 0.2 mg mL^{-1} XTT in complete medium were activated by adding $20 \mu\text{L}$ of 1mM PMS in DPBS), and cells were allowed to incubate further at 37°C for 3 h. Cell viability was measured under the Infinite M200 microplate reader (Tecan, Switzerland) at 475 nm, with 675 nm as the reference wavelength. Cell viability was expressed as a percentage by normalizing against the absorbance of untreated cells.

3.3.12 Association with Phagocytes from Human Whole Blood

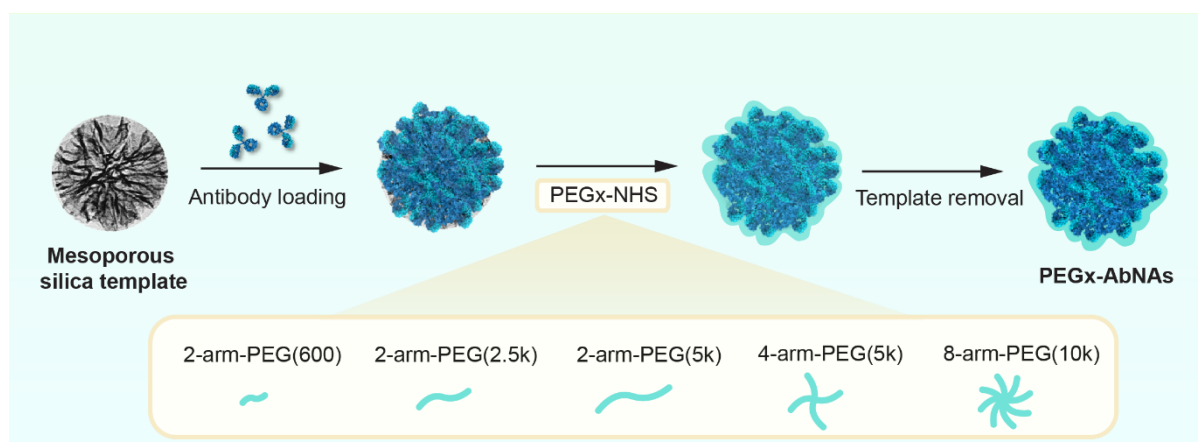
Fresh blood was collected from healthy human volunteers into VACUETTE sodium heparin tubes (Greiner Bio-One) after obtaining informed consent in accordance with The University

of Melbourne Human Ethics Approval#1443420 and the Australian National Health and Medical Research Council Statement on Ethical Conduct in Human Research. AF647 labelled Free Herceptin and PEGx-HerNAs (2 µg) were incubated in 100 µL of whole blood at 37 °C for 1 h. Subsequently, the samples were treated with lysing buffer (BD Pharm Lyse) following the manufacturer's protocol to lyse red blood cells. The remaining blood cells were further washed with flow cytometry staining (FCS) buffer at 500 g, 7 min and resuspended in 100 µL of FCA buffer. Titrated concentration of fluorophore-conjugated antibodies against CD56, CD19, CD66b, HLA-DR, CD3 or CD14 were added and incubated for 1 h on ice. Unbound antibodies were removed by washing twice with cold (4 °C) FBS buffer (500 g, 7 min). Cells were fixed with 2% w/v formaldehyde in PBS and directly analysed by flow cytometry (CytoFLEX, Beckman Coulter Life Sciences), and data were processed using FlowJoV10.

3.4 Results and Discussion

3.4.1 Synthesis and Characterisation

The PEGx-HerNAs were synthesised following an MSN template-assisted method reported in Chapter 2. Briefly, Herceptin antibodies were loaded in MSN followed by crosslinking with various PEG-NHS (PEGx-NHS) and removal of the MSN template (**Scheme 3.1**). In this study, PEG-NHS with different arm lengths (2-arm-PEG-NHS, MW 600, 2.5k and 5k) and PEG-NHS with different arm numbers (2-, 4- and 8-arm-PEG-NHS, MW 2.5k, 5k and 10k) were used to prepare the PEGx-HerNAs.



Scheme 3.1 Synthesis of PEGx-HerNAs crosslinked by various PEG linkers. Briefly, Herceptin antibodies were loaded in MSN followed by crosslinking with various PEG-NHS (PEGx-NHS) and removal of MSN template.

Transmission electron microscopy (TEM) images show that PEGx-HerNAs deposited on a carbon film had a diameter range from 200 to 400 nm (**Figure 3.1A and S3.1**). Hydrodynamic size distribution measured by dynamic light scattering in DPBS solution showed that with the increase in PEG arm length and arm numbers, the size of PEGx-HerNAs increased from 250 to 350 nm despite the same MSN template used for all PEGx-HerNAs (**Figure 3.1B and S3.2**). The thickness of AbNAs measured by atomic force microscopy (AFM) varied from 80nm –

150nm, which followed the same trend as size distribution (**Figure S3.3**). This is consistent with previous studies that have reported that increasing PEG molecular weight increases the size of nanoparticles.^{18, 19} However a different trend was observed in the measurement of zeta potentials, which is related to the particle surface charge. With the increase of PEG arm length, the zeta potential of HerNAs increased from -17.4 to -3.87 mV (**Figure 3.1C**). This indicated that the use of PEG with longer armlengths result in particles with near-neutral zeta potential. No significant difference in the zeta potential was found with the increase of PEG arm numbers. Circular dichroism (CD) spectroscopy (**Figure 3.1D**) suggested that the PEGylation regardless of arm length or arm number did not affect the molecular conformation of the antibody, as the PEGx-HerNAs reserved the same secondary structures as free antibodies.

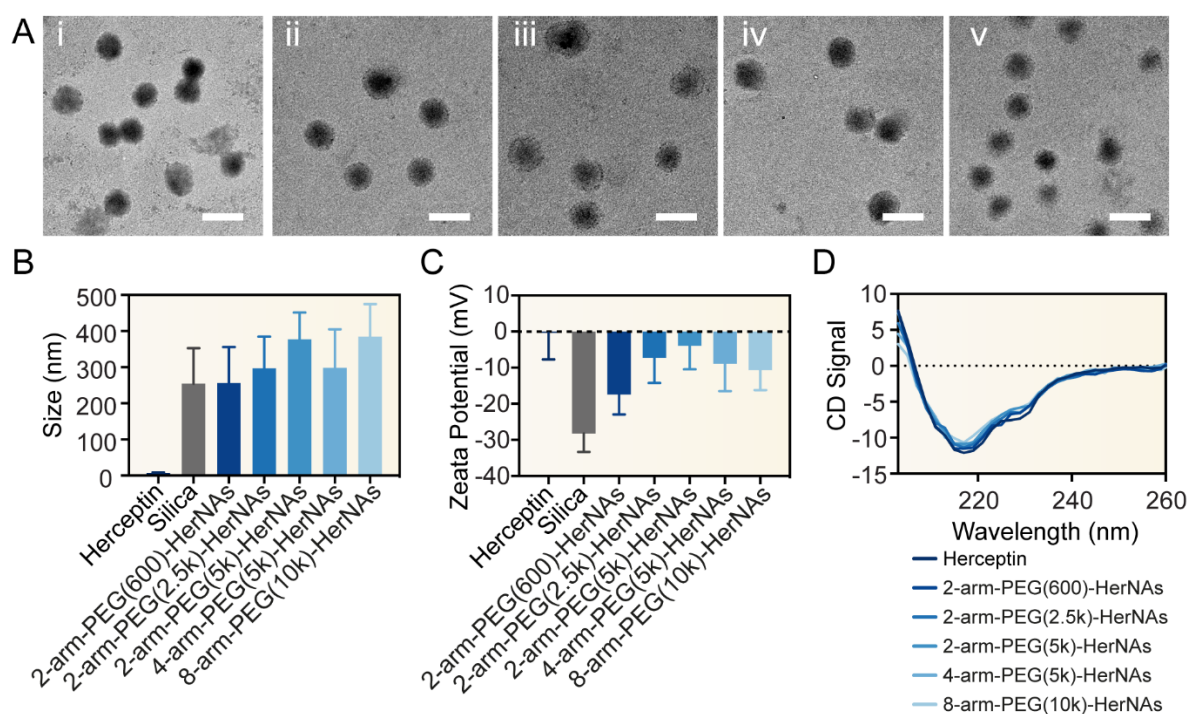


Figure 3.1 Characterisation of HerNAs crosslinked by various PEG linkers. A, TEM images of PEGx-HerNAs crosslinked with i, 2-arm-PEG(600)-HerNAs; ii, 2-arm-PEG(2.5k)-HerNAs; iii, 2-arm-PEG(5k)-HerNAs; iv 4-arm-PEG(5k)-HerNAs; v, 8-arm-PEG(10k)-HerNAs. Scale

bars: 500nm. B, Size distribution of PEGx-HerNAs in DPBS solution. C, Zeta potential of PEGx-HerNAs in what solution. D, CD spectrum of PEGx-HerNAs.

3.4.2 Stability Study

PEGylation of nanoparticles provides a steric barrier to prevent access to proteolytic enzymes and protect the functional protein in the nanoparticle from degradation.¹² To investigate how PEGylation affect the degradation of nanoparticles, the stability of PEGx-HerNAs in GSH and proteinase rich environment was studied using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE, [Figure 3.2A and 3.2B](#)). After incubation in 5 mM of GSH for 48 h, PEGx-HerNAs remained intact while free Herceptin was digested into fragments via the cleavage of disulfide bonds. This result indicates that PEGylation may shelter Herceptin antibodies from digestion by GSH in a cellular environment. However, when incubated in 1 mg mL⁻¹ of proteinase for 48 h, PEGx-HerNAs and free Herceptin were all digested into smaller fragments, showing limited protection provided by PEG when exposed to a harsh proteinase rich environment.

To further understand the stability of PEGx-HerNAs in a proteinase rich environment, the degradation percentage of PEGx-HerNAs was quantified using AF488 labelled Herceptin antibodies. The AF488 labelled free Herceptin and PEGx-HerNAs were contained in a dialysis tubing with molecular weight-cutoff of 100k and incubated in 1mg mL⁻¹ of proteinase under gentle agitation. The initial total antibody amount of each sample was quantified by measuring their AF488 fluorescence intensity using a microplate reader. After incubation for 2, 4, 6, 8, 10, 24 or 48 h, AF488 labelled antibody fragments that penetrated out of the dialysis tubing were quantified using the same method. The percentage of degraded antibody fragments relative to the corresponding total antibody amount at various time points was calculated. The

same experiment was conducted in DPBS as control. The results showed that PEGx-HerNAs were more stable in proteinase as compared with free Herceptin and the degradation percentage decreased with the increase of PEG arm length and arm numbers (**Figure 3.2C and 3.2D**). Among all PEG crosslinkers studied, 8-arm-PEG (10k) provided the most effective protection to antibodies from proteinase induced degradation, resulting in only 30% of degradation after exposure to proteinase for 48 h, while over 90% of 2-arm-PEG (600) HerNAs has been degraded under the same condition. Under the same incubation condition, all PEGx-HerNAs remained stable and intact in DPBS (**Figure S3.4**). As indicated in the above results, different PEG crosslinkers provided protection to antibodies in the nano-assemblies from proteolytic enzymes to varying degrees; PEG crosslinkers with longer arm lengths and greater arm numbers provided better protection possibly due to the thicker hydrated layer formed on the surface of nanoparticles that sterically precluded the approach of proteolytic enzymes.^{19, 20}

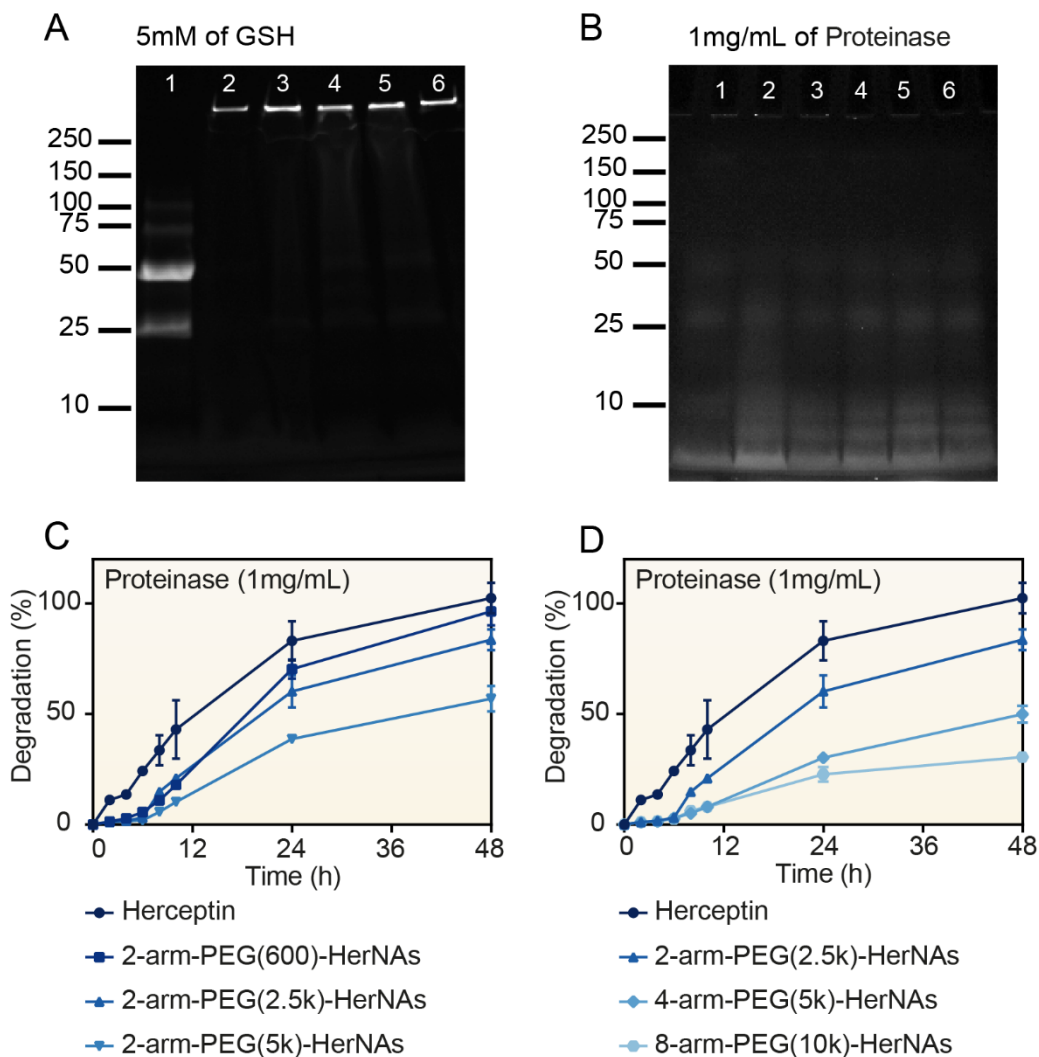


Figure 3.2 Stability study of PEGx-HerNAs in GSH and proteinase environment. A and B, Stability of HerNAs in 5 mM of GSH and 1mg mL⁻¹ of proteinase for 48h, determined by SDS-PAGE. Lane 1, Heceptin; 2, 2-arm-PEG(600)-HerNAs; 3, 2-arm-PEG(2.5k)-HerNAs; 4, 2-arm-PEG(5k)-HerNAs; 5, 4-arm-PEG(5k)-HerNAs; 6, 8-arm-PEG(10k)-HerNAs. C and D, Quantitative degradation profiles of PEGx-HerNAs crosslinked by PEG with different arm lengths (C) and different arm numbers (D) in 1mg mL⁻¹ of proteinase. Herceptin antibodies were labelled with AF488 for quantification.

3.4.3 Targeting Ability

A commercial monoclonal antibody, Herceptin, that targets the ErbB2 receptor overexpressed in ~25% of invasive breast carcinomas,²¹ was used as a model antibody to synthesise PEGx-HerNAs. The specific association to (ErbB2+) BT-474 breast cancer cells which overexpress ErbB2 was investigated by flow cytometry analysis, while ErbB2 negative (ErbB2-) MDA-MB-231 breast cancer cells were used as control cells. PEGx-HerNAs were incubated with ErbB2+ and ErbB2- cells in culture medium at 37° C for 1, 3, 6, 12 or 24h. Nonspecific IgGNAs crosslinked using 2-arm-PEG(600) (2-arm-PEG(600)-IgGNAs) were also included as control particles. Conjugation of AF488 dye to the antibody molecules facilitated the detection.

The results showed that free Herceptin associated with BT-474 cells rapidly while PEGx-HerNAs associated with BT-474 cells in a time-dependent manner (**Figure 3.3A, 3.3D and S3.5**). More interestingly, it was found that PEG arm length and arm numbers affected the association to various degrees. With the increase in PEG arm length, the percentage of cells associated with PEGx-HerNAs decreased slightly (**Figure 3.3A**). After 24 h of incubation, 2-arm-PEG(600)-HerNAs, 2-arm-PEG(2.5k)-HerNAs and 2-arm-PEG(5k)-HerNAs all associated with over 90% of BT-474 cells, with insignificant difference between each other (**Figure 3.3B**). But, with the increase of PEG arm numbers, a distinguishable reduction in cell association percentage was observed (**Figure 3.3D**). After 24 h of incubation, 2-arm-PEG(2.4)-HerNAs, 4-arm-PEG(5k)-HerNAs and 8-arm-PEG(10k)-HerNAs associated to 92.8% , 82.65% and 73.75% of BT-474 cells, respectively (**Figure 3.3E**). The above results suggest that increasing PEG arm length limited the targeting ability slightly, but increasing PEG arms numbers significantly hindered targeting. One potential explanation is that the linear 2-arm-PEG is more flexible to expose the binding sites while 4-arm and 8-arm-PEG form a more rigid shield on the particle surface (**Scheme 3.2**). Further characterisation will be conducted in the future to better understand the mechanism.

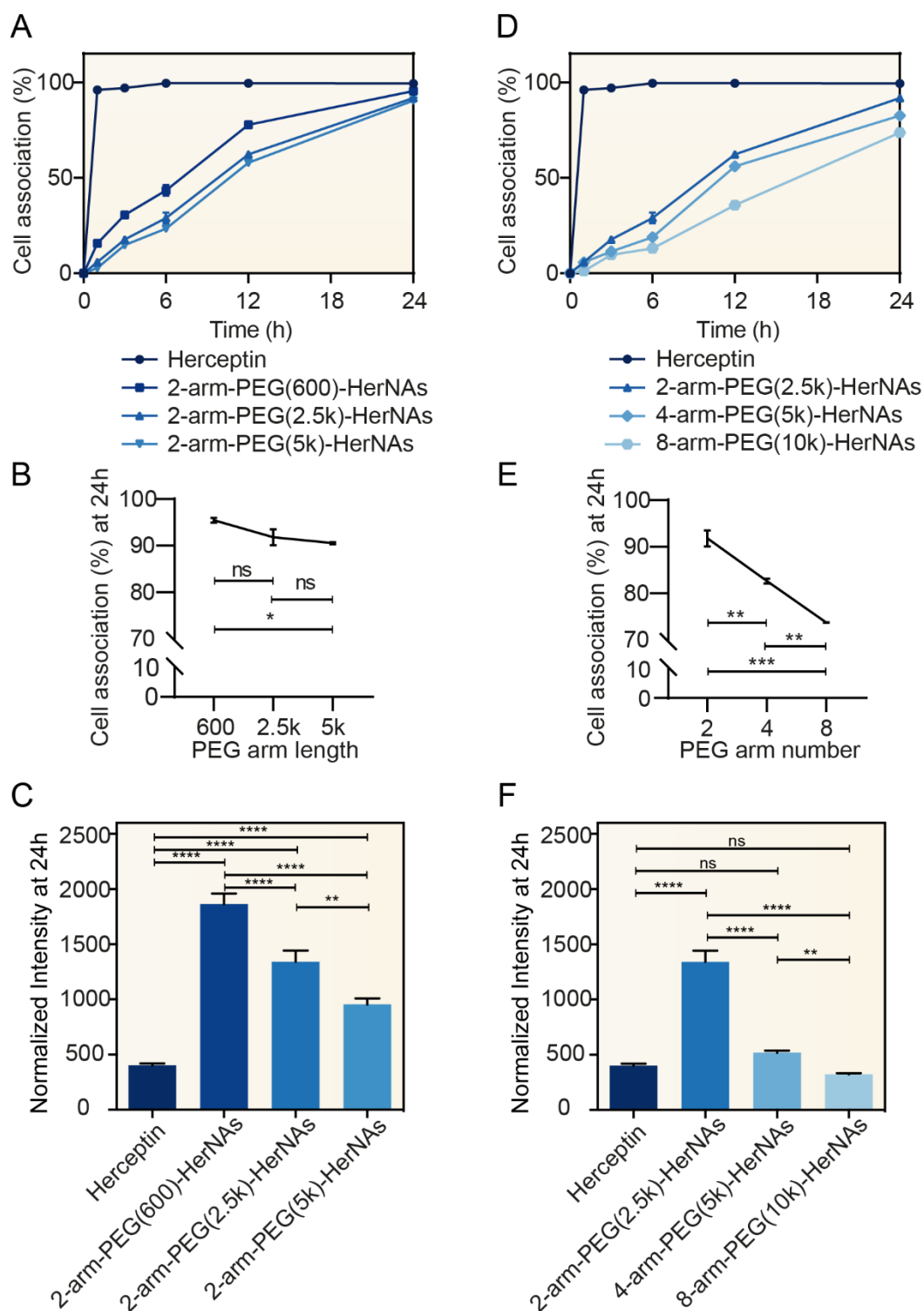
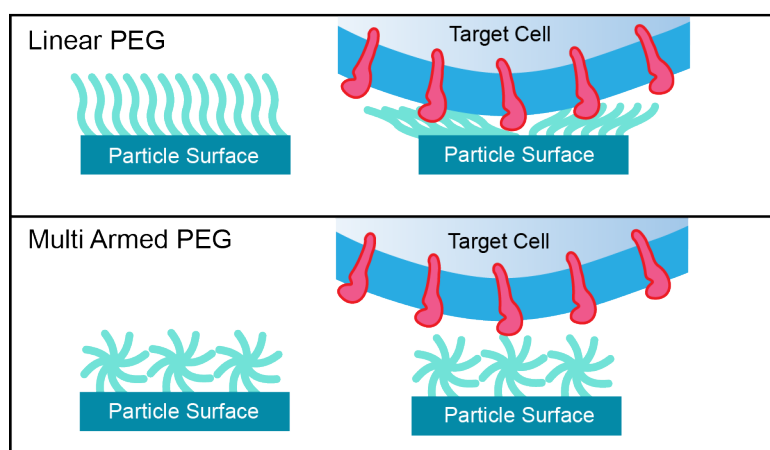


Figure 3.3 BT-474 cell association profiles of HerNAs crosslinked by PEG with different arm length (A, B and C) and different arm numbers (D, E and F), measured by flow cytometry. Herceptin antibodies were labelled with AF488 for detection. BT-474 cells were treated with the labelled Herceptin and PEGx-HerNAs at a dosage of $10 \mu\text{g mL}^{-1}$. A and D, Dynamic Cell

association (%) profiles of PEGx-HerNAs. B and E, Cell association (%) after 24 h treatment with PEGx-HerNAs. The X-axis represents the properties (arm length or arm number) of PEG used to prepare the corresponding PEGx-HerNAs. C and F, Alexa Fluor 488 fluorescence intensity of BT-474 cells after treated with PEGx-HerNAs for 24 h. The intensity profile indicated the number of antibodies associated with each cell. Notes: ***** Indicates significant difference, $p < 0.0001$, $n = 3$; *** indicates significant difference, $p < 0.001$, $n = 3$; ** indicates significant difference, $p < 0.01$, $n = 3$; * indicates significant difference, $p < 0.05$, $n = 3$; ns indicates no significant difference.



Scheme 3.2 Different target association patterns between linear PEG and multi-armed PEG modified particles.

In contrast to BT-474 cells, neither the free Herceptin nor PEGx-HerNAs associated with the ErbB2–MDA-MB-231 cells (less than 5%, [Figure S3.6](#)), which suggested that PEGx-HerNAs retained the selective targeting ability of Herceptin antibody molecules.

Furthermore, the normalized AF488 fluorescence intensity of the treated BT-474 cells was quantified as an indicator of the number of antibodies associated with each cell. The intensity

of cells treated with PEGx-HerNAs increased significantly compared to cells treated with free Herceptin, which suggested that PEGx-HerNAs allowed a substantial number of antibodies to associate to each target cell (**Figure 3.3C and 3.3F**). Moreover, with the increase of PEG arm length, the association intensity decreased gradually (**Figure 3.3C**), and a more dramatic decreasing trend was observed with the increase of PEG arm numbers (**Figure 3.3F**). The intensity of BT-474 cells after 24 h treatment with 2-arm-PEG(600)-HerNAs, 2-arm-PEG(2.5k)-HerNAs and 2-arm-PEG(5k)-HerNAs was 1863, 1342 and 955.18 respectively; while the intensity cells treatment with 2-arm-PEG(2.5k)-HerNAs, 4-arm-PEG(5k)-HerNAs and 8-arm-PEG(10k)-HerNAs was 1342, 519 and 322 respectively. Namely, the number of Herceptin molecules associated with each target cell decrease as the PEG arm length and arm numbers increased in the PEGx-HerNAs. This finding provides a potential strategy to achieve tunable antibody accumulation in specific cells for therapeutic dosage engineering.

In contrast, negligible fluorescence was detected in the treated ErbB2–MDA-MB-231 cells (**Figure S3.7**), which further confirmed the specific targeting ability of PEGx-HerNAs.

3.4.4 Cellular Internalisation

To investigate the effect of PEGylation on cellular uptake, the internalisation of PEGx-HerNAs in BT-474 cells was examined by imaging flow cytometry. After incubation with AF488 labelled PEGx-HerNAs for 24 h, cell membranes were stained with AF594-WGA and analysed with an imaging flow cytometer that captures individual images of each cell when conducting flow cytometry. Based on the acquired images, the internalisation of PEGx-HerNAs was quantified by an internalisation factor (IF) using the built-in internalisation wizard of Amnis ImageStream IDEAS software based on the spatial relationship between the fluorescent nanoparticles and the cell membrane. Positive IF indicates internalised PEGx-HerNAs, while

a negative IF indicates PEGx-HerNAs attached to the cell membrane.²² The internalisation percentage was calculated accordingly using the following formula: frequency of positive IF / frequency of both positive and negative IF x 100%. As showed in **Figure 3.4**, the internalisation percentage of PEGx-HerNAs in BT-474 cells were all above 96%. 2-arm-PEG-HerNAs exhibited high internalisation percentage (> 99%) regardless of PEG arm length (**Figure 3.4A**). While the PEG arm number increased from 2 to 8, the internalisation percentage dropped slightly from 99.2% to 96%, but the green AF488 fluorescence intensity observed from the images decreased significantly (**Figure 3.4B**). The above results indicate that after association with target cells, PEGx-HerNAs were mostly internalised inside cells instead of remaining on the surface despite their different PEGylation. Nevertheless, the number of internalised antibodies decreased slightly with the increase in PEG arm length but reduced significantly with the increase in PEG arm number.

The internalisation study was further confirmed by confocal laser scanning microscopy (CLSM). The cells were incubated with AF488 labelled PEGx-HerNAs for 24 h and then fixed with paraformaldehyde. AF488 labelled free Herceptin and IgGNAs was used as controls. Before imaging, the nuclei were stained with Hoechst 33342 and cell membranes were stained with AF594-WGA. CLSM images were captured using a Nikon A1R+ confocal microscope. The results showed that PEGx-HerNAs were internalised into target cells to different extents. A significant amount of 2-arm-PEG(600)-HerNAs were observed inside cells while only a few of 8-arm-PEG(10k)-HerNAs were spotted (**Figure 3.5**). The decreasing trend of internalised PEGx-HerNAs was consistent with the result from imaging flow cytometry. The control IgGNAs were not observed inside BT-474 cells and free Herceptin was largely distributed on the cell membrane as reported previously (**Figure S3.8**).

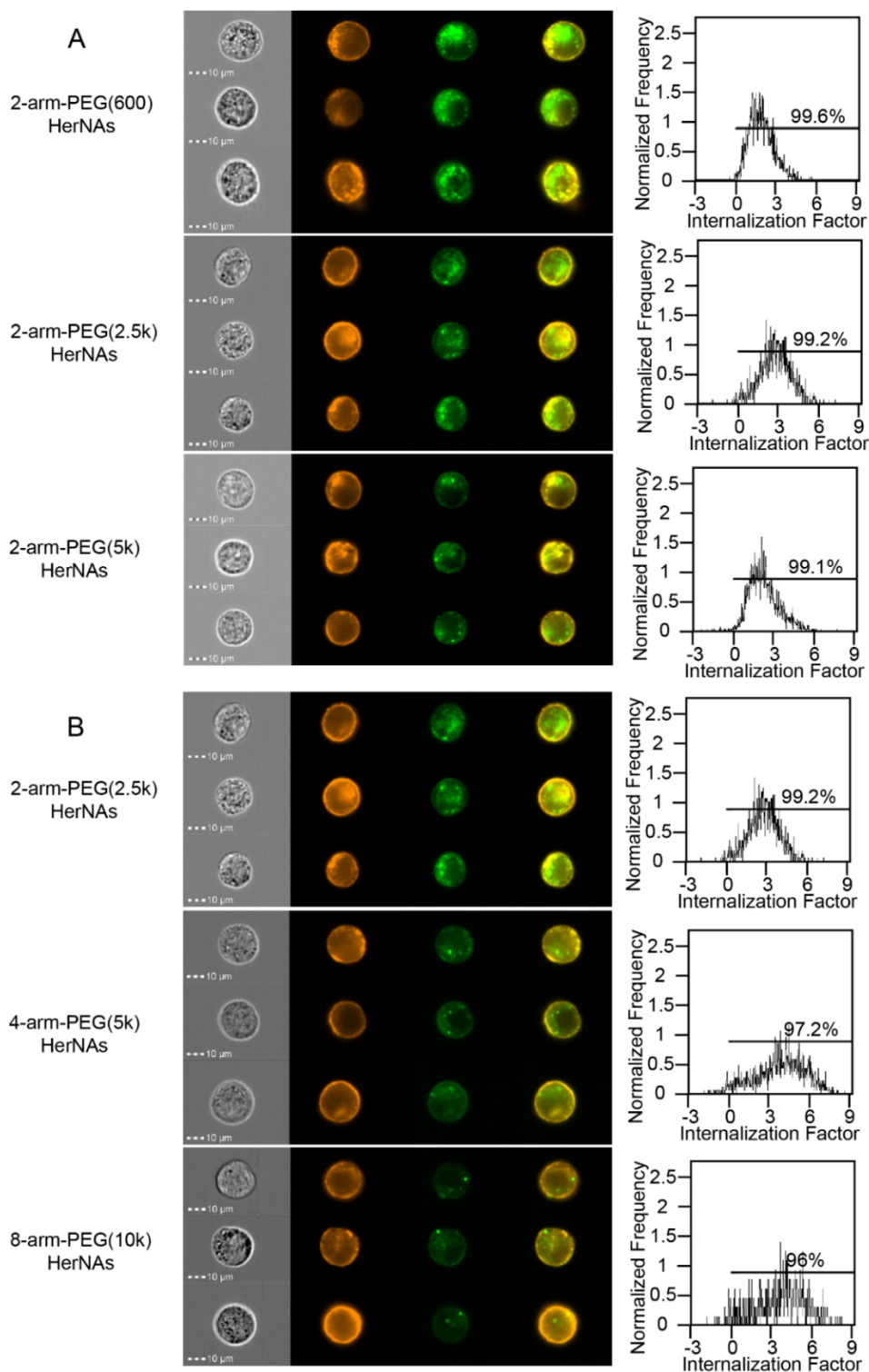


Figure 3.4 BT-474 internalisation profiles of PEGx-HerNAs crosslinked by PEG with different arm lengths (A) and different arm numbers (B), measured by imaging flow cytometry. Cell membranes were stained with AF594-WGA (Orange) and Herceptin antibodies were labelled with AF488 (Green) for imaging.

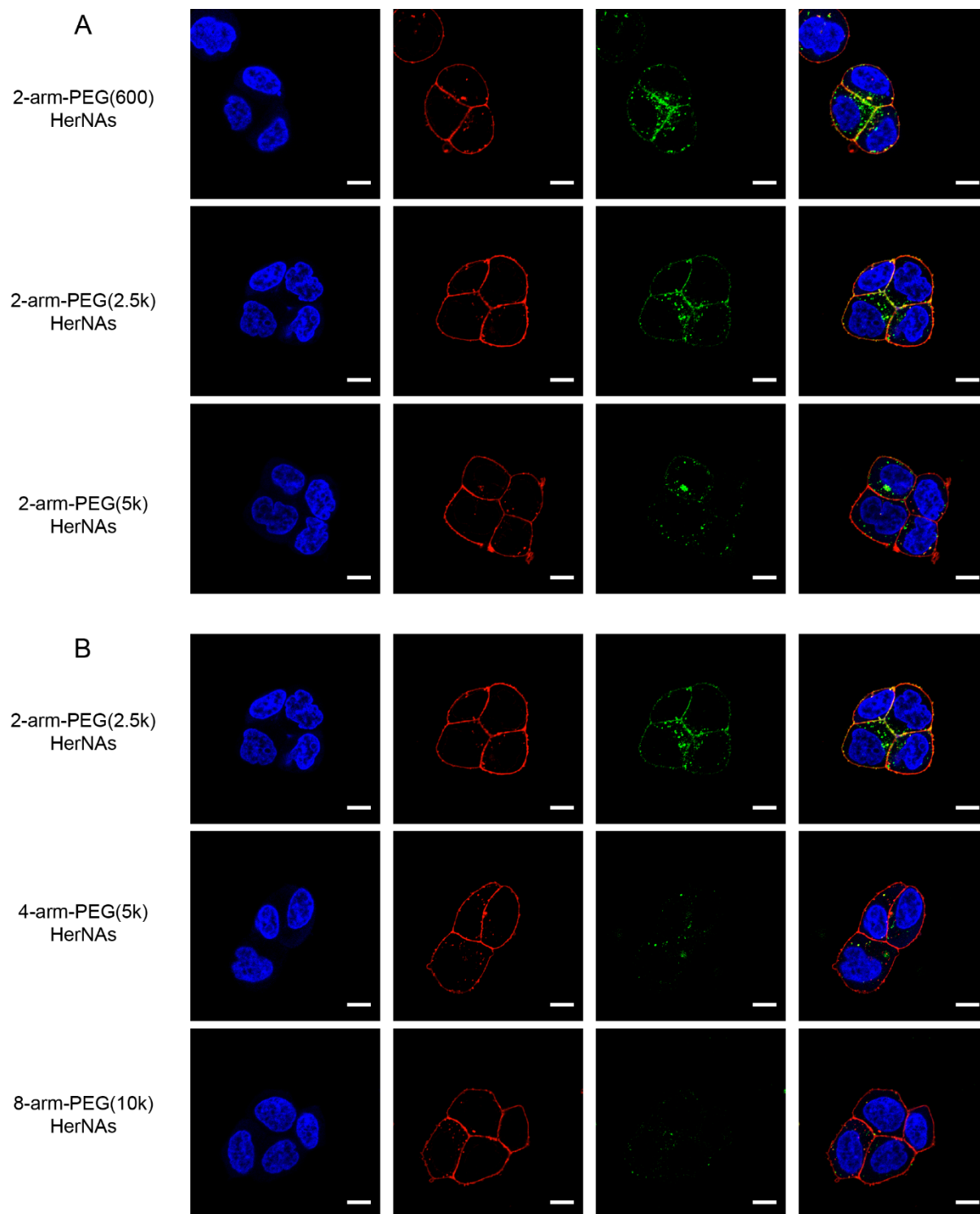


Figure 3.5 Confocal images BT-474 cells treated with PEGx-HerNAs crosslinked by PEG with different arm lengths (A) and different arm numbers (B). The cells were treated for 24 h before fixing. Nuclei were stained with Hoechst 33342 dye (blue); Cell membrane was stained with WGA-594 (red); Herceptin antibodies were labelled with AF488 (green). Scale bars: 10 μ m.

3.4.5 Targeting Cytotoxicity

ErbB2 signalling is known to promote cell proliferation. Herceptin interferes with ErbB2-triggered signalling by preventing ErbB2 receptor dimerization, which eventually inhibits cell growth.^{23, 24} To investigate the cell growth inhibition of PEGx-HerNAs, the cell viability of ErbB2+ BT-474 cells and ErbB2- MDA-MB-231 cells were examined by XTT assay after treatment with free Herceptin, PEGx-HerNAs or cell culture medium (blank control) for 48h. Results showed that the cell viability of BT-474 reduced to varying degrees after treatment with 20 $\mu\text{g mL}^{-1}$ PEGx-HerNAs when compared with the cell viability of blank control, whereas no reduction effect was found in MDA-MB-231 cells after the treatment (**Figure 3.6**). The results confirm that the PEGx-HerNAs retain the selective cytotoxicity of Herceptin antibody molecules.

Importantly, the cytotoxicity of PEGx-HerNAs to BT-474 cells dropped gradually when the PEG arm length increased. Specifically, 43.1% of cells were eradicated by 2-arm-PEG(600)-HerNAs, 27.2% by 2-arm-PEG(2.5k)-HerNAs, and 21.6% by 2-arm-PEG(600)-HerNAs (**Figure 3.6A**). However, when the PEG arm number increased, the corresponding PEGx-HerNAs showed significantly decreased cytotoxicity. Only 7.7% and 9.6% of cells were eliminated by 4-arm-PEG(5k)-HerNAs and 8-arm PEG(10k)-HerNAs, respectively. (**Figure 3.6B**). A similar trend was observed when cells were treated with PEGx-HerNAs at a lower concentration (50 $\mu\text{g mL}^{-1}$, **Figure S3.9**).

The effect of PEGylation on the cytotoxicity of the PEGx-HerNAs is consistent with the cellular association, as cell growth inhibition is the downstream effect initiated by the binding of Herceptin antibodies to ErbB2 receptors on the cell membrane.

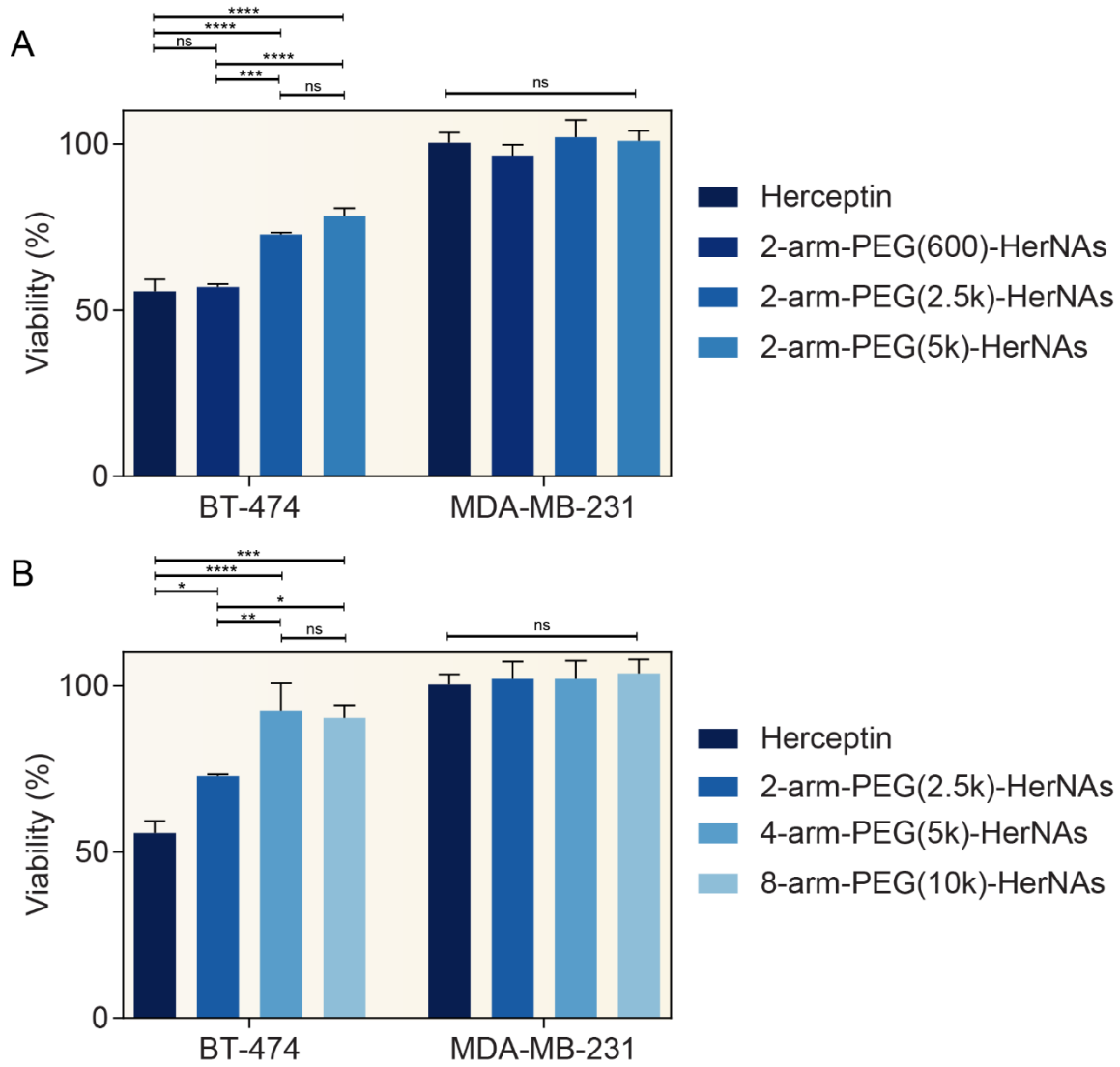


Figure 3.6 Cytotoxicity of PEGx-HerNAs crosslinked by PEG with different arm lengths (A) and different arm numbers (B) in BT-474 and MDA-MB-231 cells, as determined by XTT assay. The cells were treated with free Herceptin or PEGx-HerNAs at an antibody concentration of $20\mu\text{g mL}^{-1}$. Notes: **** Indicates significant difference, $p < 0.0001$, $n = 3$; *** indicates significant difference, $p < 0.001$, $n = 3$; ** indicates significant difference, $p < 0.01$, $n = 3$; * indicates significant difference, $p < 0.05$, $n = 3$; ns indicates no significant difference.

3.4.6 Association with Phagocytes from Human Whole Blood

A major obstacle for a targeted nanoparticle delivery system is the premature capture of nanoparticles by circulating leukocytes and mononuclear phagocytes, which cause their rapid clearance from the blood circulation and ultimately leads to poor targeting.²⁵ To understand if PEGylation can address this issue for the HerNAs system, the association of PEGx-HerNAs with phagocytes in human whole blood was investigated using a previously developed human blood assay.²⁶ AF647 labelled PEGx-HerNAs were incubated in 100 μ L of whole blood at 37 °C for 1 h. After the removal of red blood cells, the remaining cells were labelled with fluorophore-conjugated antibodies to identify distinct blood cell populations. Phagocytes including dendritic cells, monocytes and neutrophils were identified and the association of PEGx-HerNAs with these phagocytes was analysed by multi-colour flow cytometry. Results showed that around 12 – 18% of dendritic cells, 4 – 10% of monocytes and 0.6 – 2% neutrophils associated with PEGx-HerNAs. With the increase in PEG arm length, the association of PEGx-HerNAs with circulating phagocytes decreased (**Figure 3.7A i**) but with the increase in PEG arm numbers, the association showed no statistically significant difference (**Figure 3.7A ii**). 2-arm-PEG(5k)-HerNAs and 4-arm-PEG(5k)-HerNAs showed the lowest association with phagocytes in whole blood (**Figure S3.10**). This finding suggests that a PEG crosslinker with fewer arm numbers but longer arm length is more beneficial for avoiding phagocyte capture in blood circulation.

Besides, the association with lymphocytes (B cells, T cells and NK cells) from human blood was also studied but no consistent trend was discovered with the changing of PEG arm structures (**Figure S3.11**).

The association study was also performed *in vitro* on an immortal macrophage cell line RAW 267.4. Cells were treated with AF488 labelled PEGx-HerNAs for 24 h and then analysed by

flow cytometry. The association of the particles with RAW 267.4 varied from 40 - 90%. With the increase in PEG arm length, the association percentage dropped from 90 % to 70 % (**Figure 3.7A ii**). When the arm numbers increased to 4 and 8, the association decreased to 39% and 46% (**Figure 3.7A ii**). Overall, 4-arm-PEG(5k)-HerNAs showed minimal association with RAW 267.4 cells (**Figure S3.12**). The *in vitro* association of PEGx-HerNAs exhibited slightly different trends compared to whole blood association due to the limitation of *in vitro* immortal models, as no human serum components, such as complement proteins and antibodies, was presented to facilitate the phagocytic function.

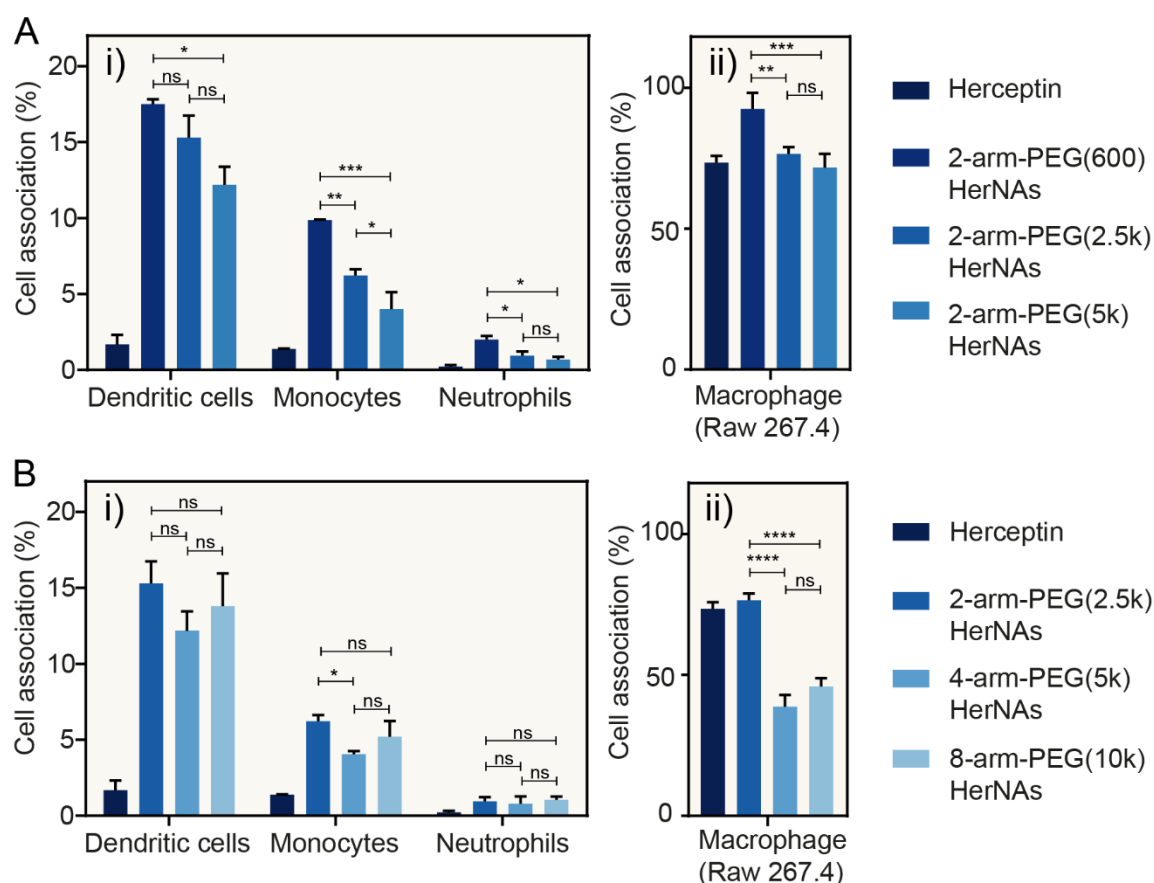


Figure 3.7 Phagocyte association of HerNAs crosslinked with PEG with different arm lengths (A) and different arm numbers (B). i), Association of PEGx-HerNAs with phagocytic cells in human whole blood after 1 h incubation, quantified by flow cytometry. Herceptin antibodies were labelled with AF647. ii), Association of PEGx-HerNAs with a macrophage (Raw 267.4)

cell line *in vitro* after 24 h incubation, quantified by flow cytometry. Herceptin antibodies were labelled with AF488 for detection. Notes: **** Indicates significant difference, $p < 0.0001$, $n = 3$; *** indicates significant difference, $p < 0.001$, $n = 3$; ** indicates significant difference, $p < 0.01$, $n = 3$; * indicates significant difference, $p < 0.05$, $n = 3$; ns indicates no significant difference.

3.5 Conclusions

In this chapter, PEGylated antibody nano-assemblies (PEGx-AbNAs) were prepared using various PEG crosslinkers with different arm lengths and arm numbers to understand the effect of PEGylation on bio-nano interactions of antibody nano-assemblies. The arm length and arm numbers of PEG crosslinker affect the targeting ability and phagocytic association of AbNAs. With increasing PEG arm length, the interaction of PEGx-HerNAs with target cancer cells varied negligibly, but the interaction with phagocytes decreased significantly. In contrast, when the PEG arm numbers increased, the interaction with target cells decreased rapidly but the interaction with phagocytes showed no significant difference. The results suggest that PEG with less arm number but longer arm length is more beneficial for antibody nano-assemblies to achieve both minimal phagocytic capture and optimal targeting. This fundamental study improves our understanding on how PEGylation affects the behaviour of antibody nano-assemblies, especially with respect to specific and phagocytic antibody-cell binding, and provides guidance on the PEGylation strategy of antibody nano-assemblies for *in vivo* studies.

3.6 Supporting Information

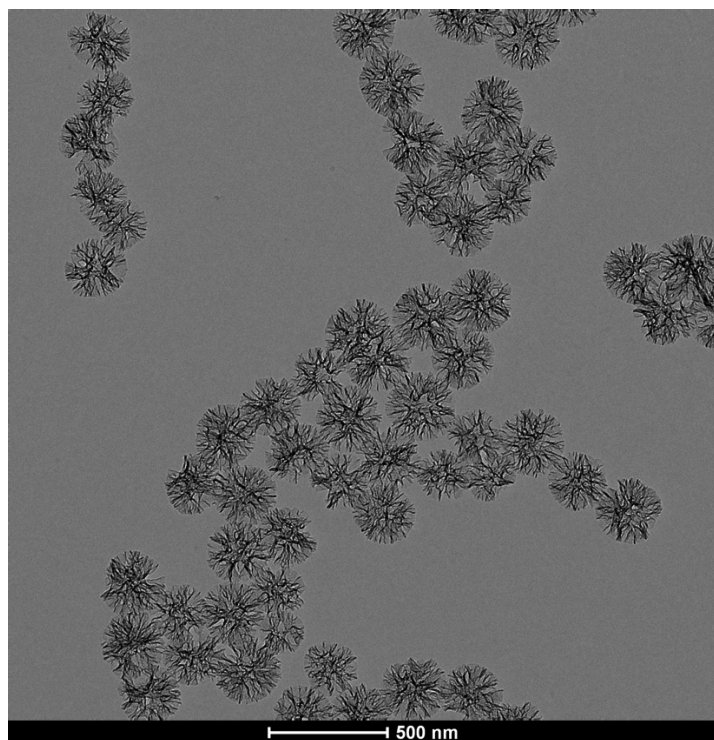


Figure S3.1 TEM image of mesoporous silica templates

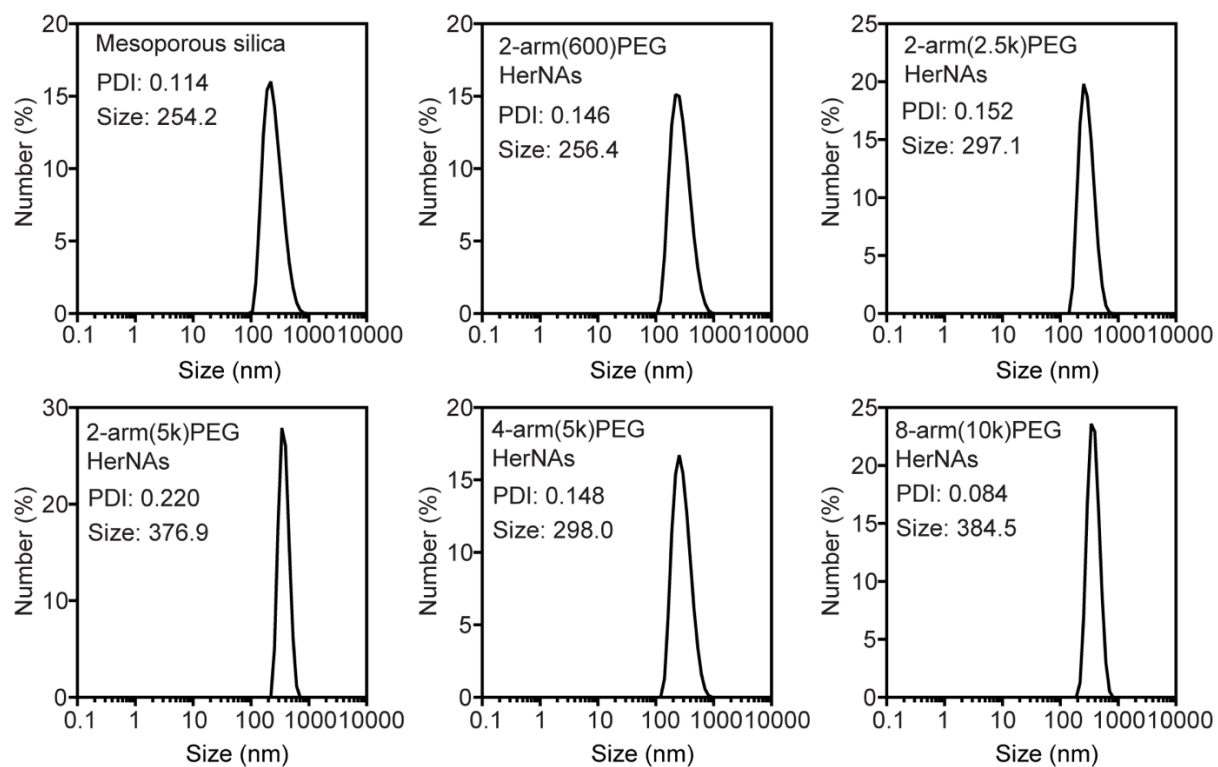


Figure S3.2 Size distribution profiles of PEG_x-HerNAs in DPBS solution.

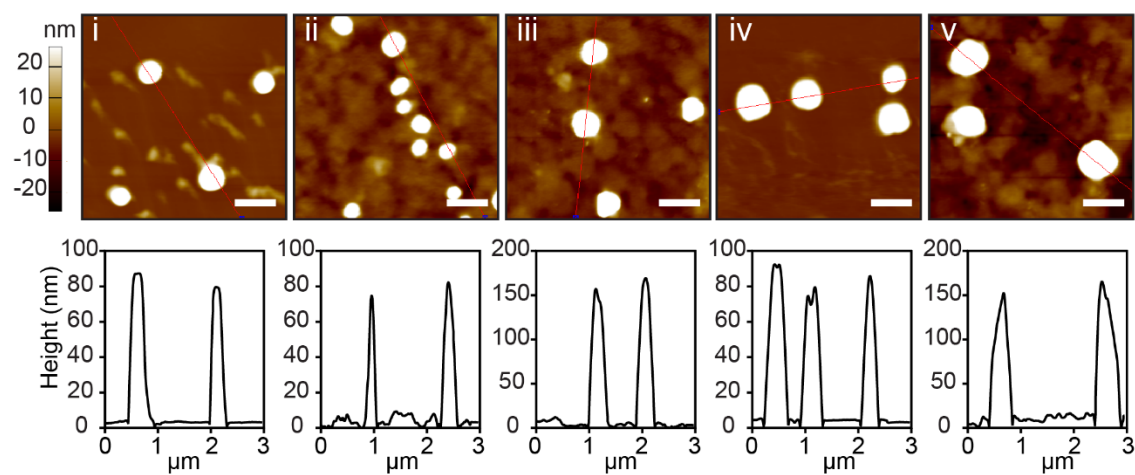


Figure S3.3 Height profiles of PEGx-HerNAs, measured by a Cypher ES AFM (Asylum Research, US). Scale bar:500nm.

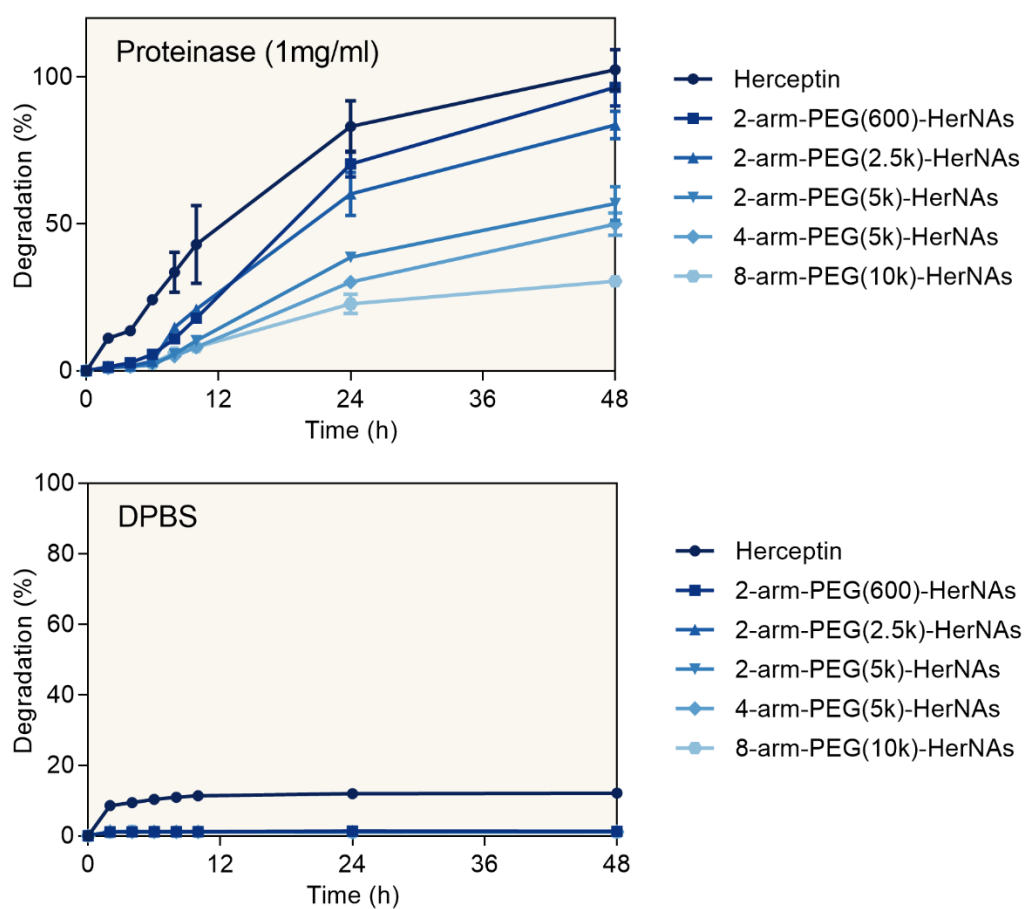


Figure S3.4 Degradation profiles of PEGx-HerNAs after incubation in 1 mg mL^{-1} of proteinase and DPBS.

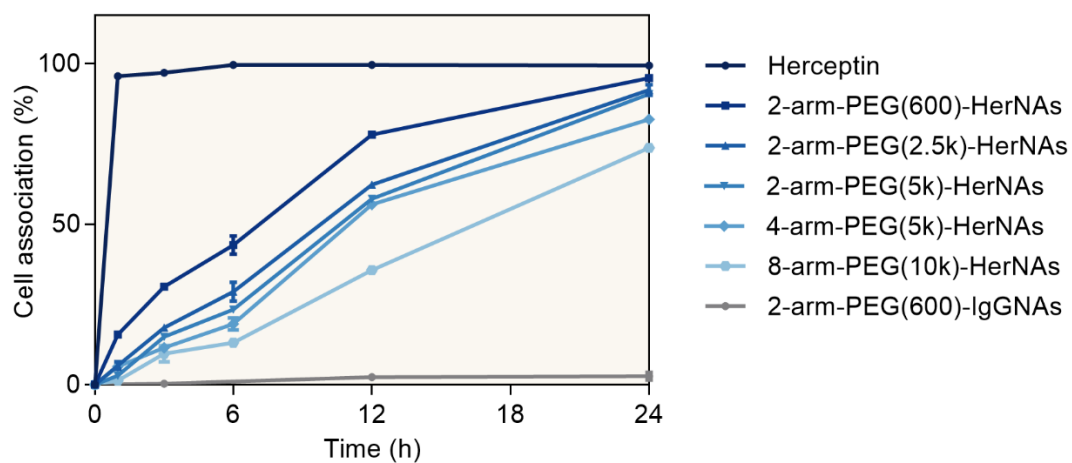


Figure S3.5 Association of PEGx-HerNAs with BT-474 cells at various time points (1, 3, 6, 12, 24 h).

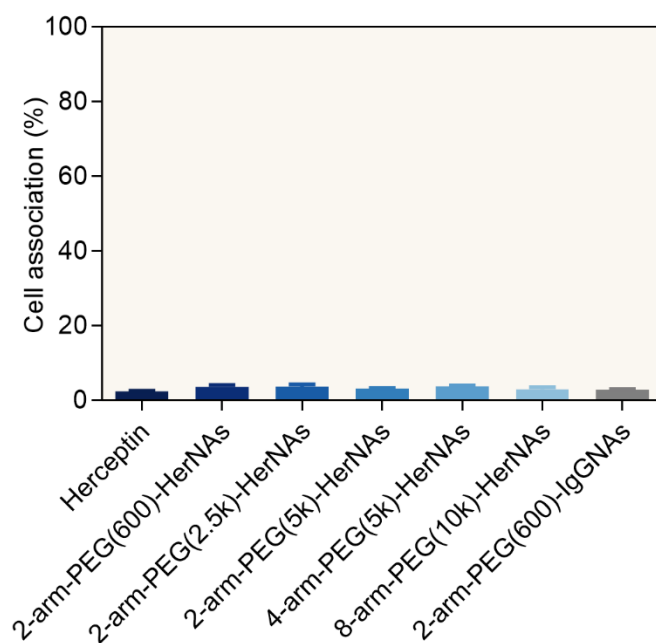


Figure S3.6 Association of PEGx-HerNAs with MDA-MB-231 cells after 24 h incubation.

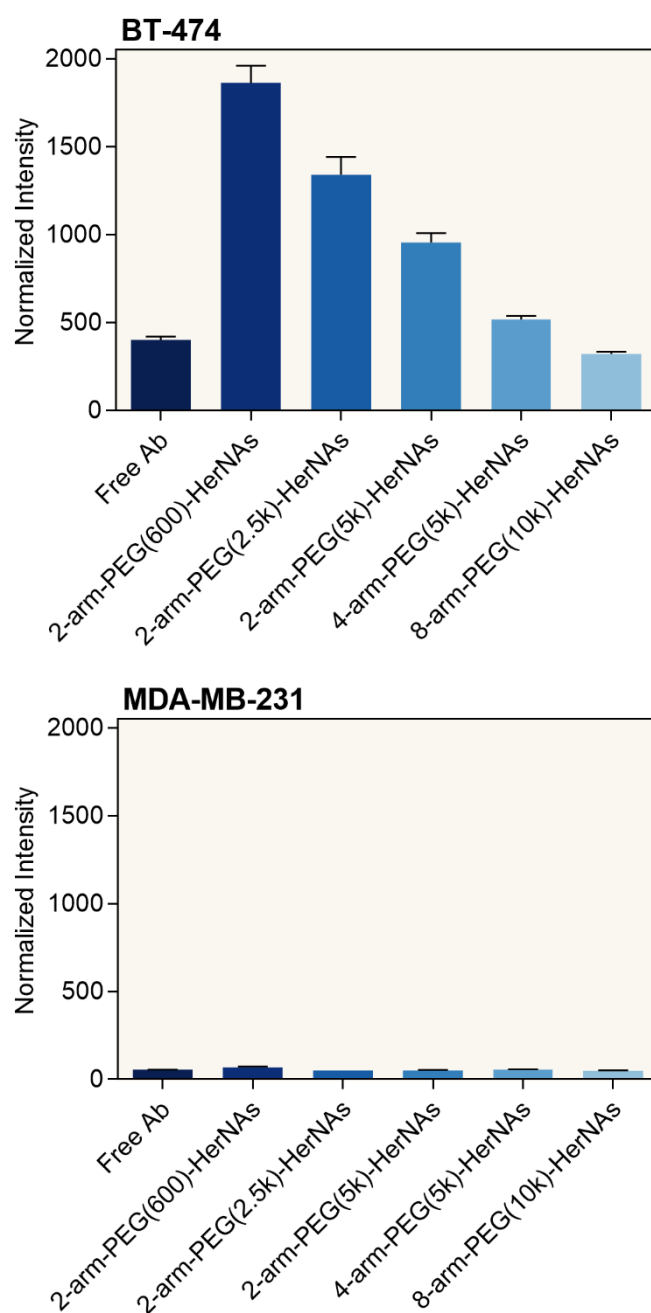


Figure S3.7 Fluorescence intensity of BT-474 and MDA-MB-231 cells after incubation with AF488 labelled PEGx-HerNAs for 24 h.

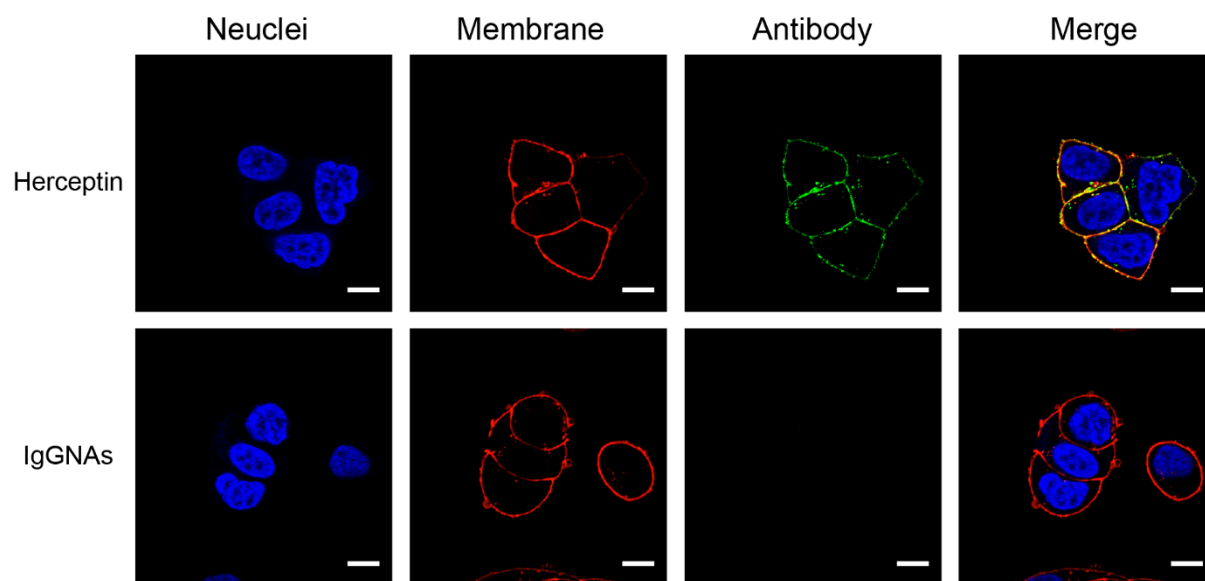


Figure S3.8 Confocal images of BT-474 cells treated with Free Herceptin and IgGNAs.

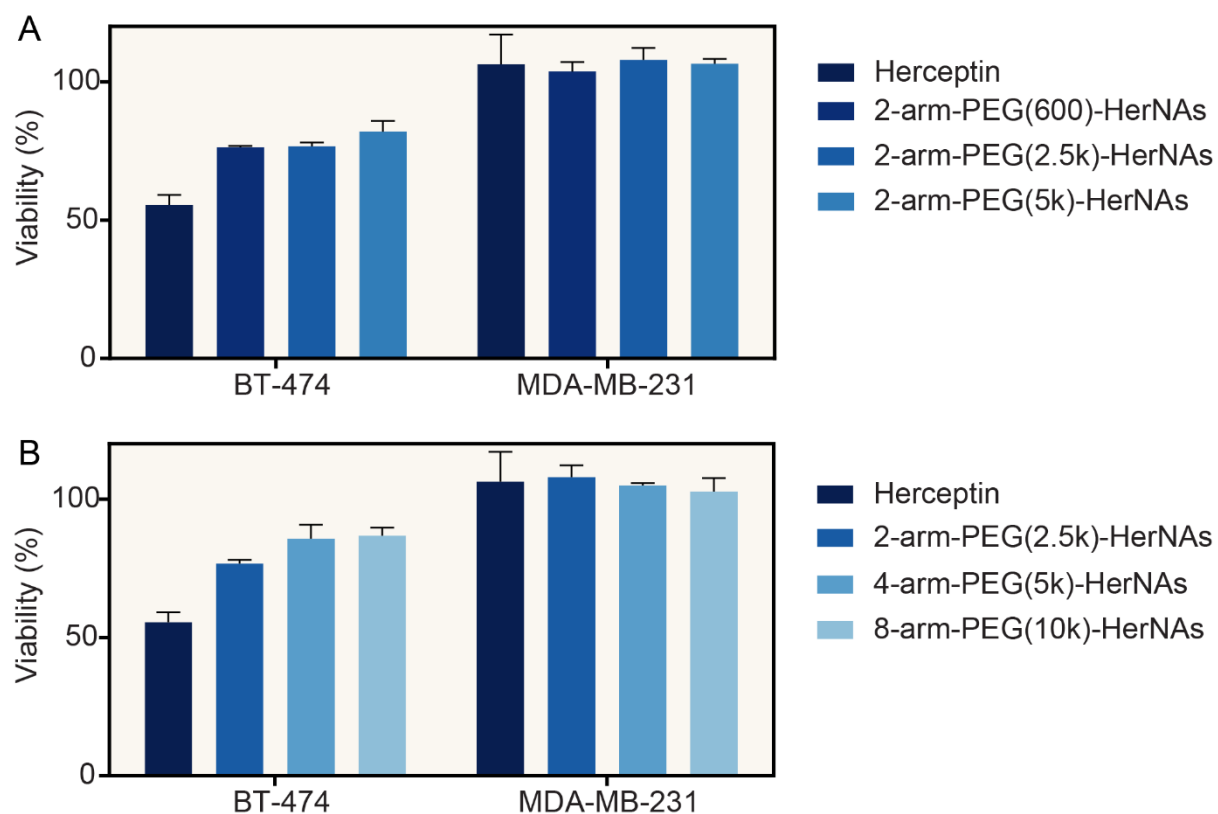


Figure S3.9 Cytotoxicity of PEGx-HerNAs ($5 \mu\text{g mL}^{-1}$) crosslinked by PEG with different arm lengths and different arm numbers in BT-474 and MDA-MB-231 cells.

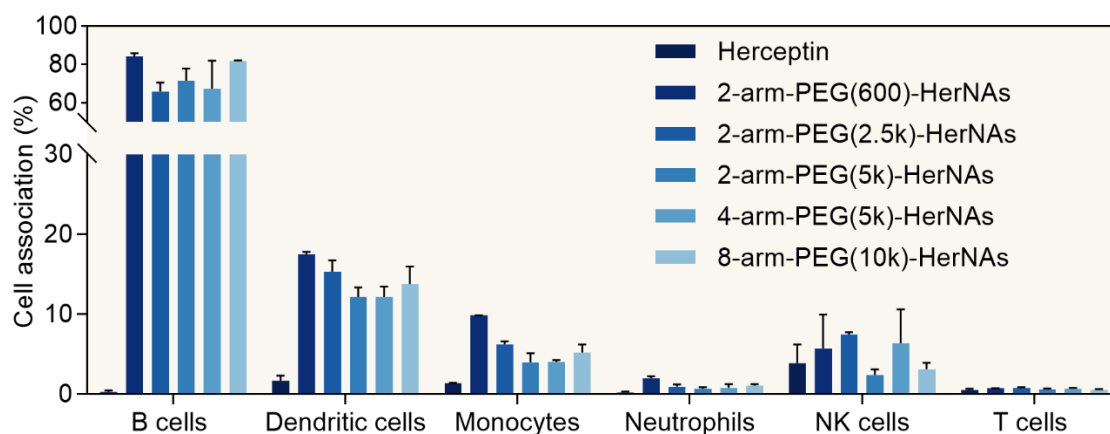


Figure S3.10. Association of PEGx-HerNAs crosslinked by various PEG with immune cells from human blood.

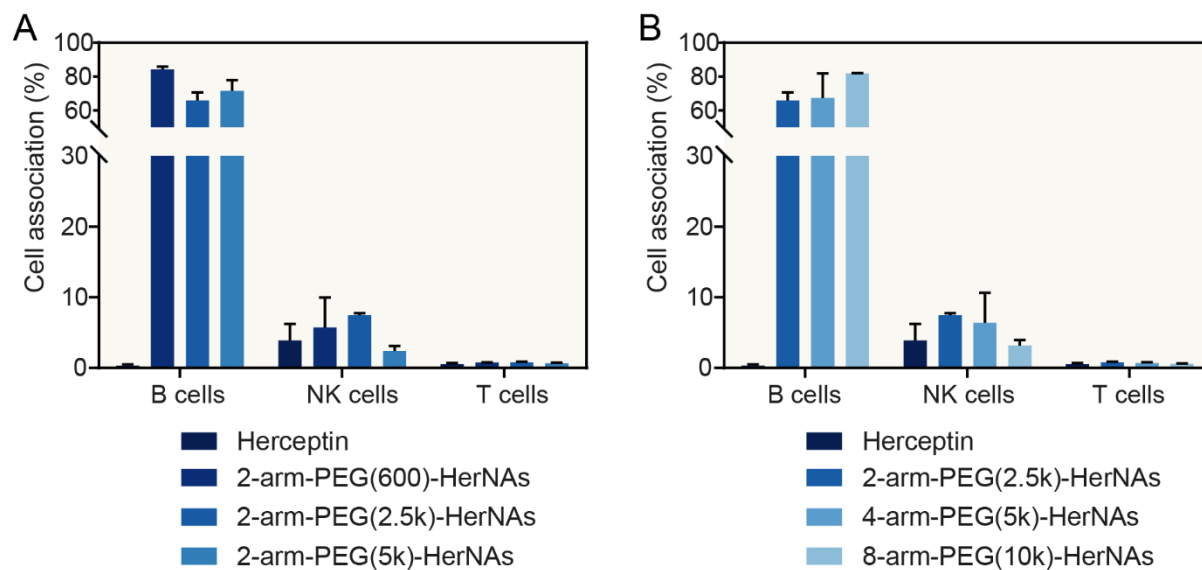


Figure S3.11 Association of PEGx-HerNAs crosslinked by PEG with different arm lengths (A) and different arm numbers (B) with lymphocytes from human blood.

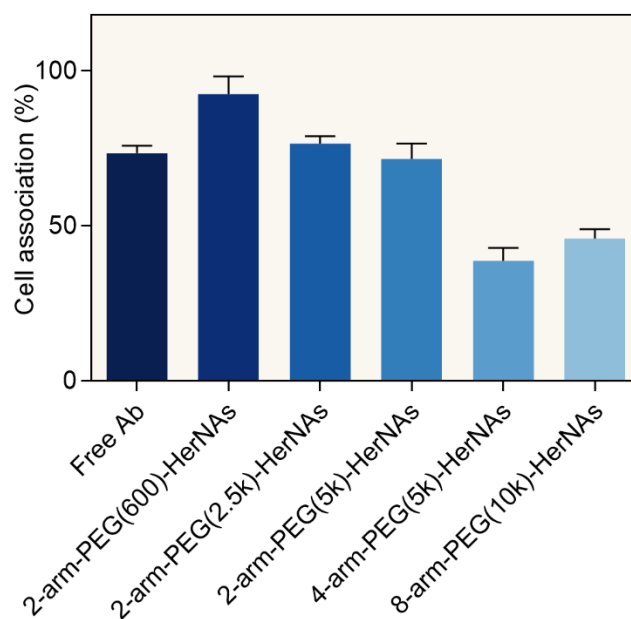


Figure S3.12 Association of PEGx-HerNAs crosslinked by various PEG with macrophage cells (Raw 264.7).

3.7 References

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Chapter 4

Engineering Single-Domain Antibody

Nano-Assemblies for Enhanced Selective

Cell Association

Contents

4.1	Aims.....	124
4.2	Introduction.....	124
4.3	Experimental Section.....	126
4.3.1	Materials	126
4.3.2	Preparation of Nano-Assemblies	126
4.3.3	Antibody Labelling	127
4.3.4	Particle Characterisation	128
4.3.5	Cell Culture.....	128
4.3.6	Cellular Association Assessed by Flow Cytometry.....	128
4.3.7	Imaging by Confocal Laser Scanning Microscopy.....	129
4.3.8	Cell Viability Assessed by XTT Assay	129
4.3.9	Fc Receptor Blocking	130
4.3.10	<i>Ex vivo</i> Human Whole Blood Assay.....	130
4.4	Results and Discussion	132
4.4.1	Synthesis and Characterisation	132
4.4.2	Cellular Targeting	133
4.4.3	Cell Growth Inhibition.....	137
4.4.4	Association with Immortalised Monocytic THP-1 Cells.....	139
4.4.5	Association with Leukocytes in Human Whole Blood.....	141
4.5	Conclusions.....	145
4.6	Supporting Information.....	146
4.7	References.....	154

4.1 Aims

Nanobodies, also known as single-domain antibodies, are considered the smallest antigen-binding units (12-15 kDa) that solely bind to target antigens. Their unique structure offers several desirable features, such as small size, high stability, strong antigen-binding affinity, and low immunogenicity, which make them suitable candidates for antibody nano-assembly engineering. In this chapter, the aims are to synthesise nanobody nano-assemblies and to investigate their targeting ability and stealth ability in comparison with full-sized antibody nano-assemblies. Anti-ErbB2 nanobodies and anti-ErbB2 antibodies were used as model molecules, and the bioactivities of the nano-assemblies were investigated using BT-474, MDA-MB-231, and THP-1 cell lines and human blood cells.

4.2 Introduction

Monoclonal antibodies have been successfully applied in clinical therapy for many types of human malignancies, thereby inspiring the engineering of antibody-modified or antibody-based nanoparticles for improved therapy outcome. However, in the application of cancer therapy and nanomedicine, conventional monoclonal antibodies have several limitations including inadequate pharmacokinetics, poor tissue accessibility, and crystallisable fragment (Fc)-mediated immunogenicity.^{1,2} Such limitations have inspired the development of antigen-binding fragments with improved structures and properties for enhanced targeting efficacy. These therapeutic antibody fragments include antigen-binding fragments (Fab), single-chain variable fragments (scFv), and ‘third generation’ (3G) molecules, nanobodies.³ Among currently developed therapeutic fragments, nanobodies have potential to achieve optimal targeting efficacy.

A nanobody, also known as a single-domain antibody (sdAb), consists of one single monomeric variable domain derived from the naturally occurring heavy-chain-only antibodies in Camelidae serum. It is the smallest antigen-binding unit (12-15 kDa) that solely binds to the target antigen with high affinity and specificity.^{4, 5} Owing to this unique structure, nanobodies offer several desirable features, such as small size, high stability, strong antigen-binding affinity, and low immunogenicity,⁶ thus making them suitable as next-generation antibody derivatives. Numerous nanomedicine systems have already incorporated nanobodies as target motifs for active targeting delivery.⁷⁻⁹

Compared with full-sized antibodies, nanobodies with smaller size offer more active antigen binding sites with stronger affinity when incorporated into nanoparticles. Moreover, nanobodies are expected to be less immunogenic after immobilization on the particle surface, owing to the lack of Fc domain that could be recognized by various immune cells via Fc receptors. These desirable features make nanobodies excellent candidates for antibody nano-assembly engineering to achieve enhanced selective association and reduced immunogenicity.

In this chapter, anti-ErbB2 nanobodies and anti-ErbB2 antibodies were used as model molecules (referred simply as ‘nanobody’ and ‘antibody’ hereinafter) to synthesise nanobody nano-assemblies (NanoNAs) and antibody nano-assemblies (AbNAs) via the mesoporous silica template-assisted assembly platform. Generic human immunoglobulin G (IgG) molecules were used as a non-specific control to synthesise control particle IgG nano-assemblies (IgGNAs). The cellular association of the nano-assemblies with ErbB2⁺ and ErbB2[−] breast cancer cells and downstream cell growth inhibition were studied to investigate the targeting efficacy of NanoNAs in comparison with AbNAs. Furthermore, the *ex vivo* whole blood assay was performed to investigate the interactions of NanoNAs with immune cells in human blood.

4.3 Experimental Section

4.3.1 Materials

Cetyltrimethylammonium bromide (CTAB), Poly (acrylic acid) (PAA), ammonium hydroxide solution, tetraethyl orthosilicate (TEOS), hydrofluoric acid (HF, 48 wt%), ammonium fluoride (NH_4F), sodium carbonate (Na_2CO_3), dimethyl sulfoxide (DMSO), phenazine methosulfate (PMS), and immunoglobulin G (IgG) from sheep serum were purchased from Sigma-Aldrich. 8-arm-polyethylene glycol N-hydroxysuccinimide (8-arm-PEG-NHS, MW 10k) were purchased from Jenkem. Alexa Fluor 647 carboxylic acid succinimidyl ester (AF647), RPMI 1640 medium, Dulbecco's modified Eagle's medium (DMEM), Fetal bovine serum (FBS), Dulbecco's phosphate-buffered saline (DPBS) buffer, trihydrochloride (Hoechst 33342), 2,3-Bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide inner salt (XTT) were purchased from Thermo Fisher Scientific. Human BD FC block was purchased from BD Pharmingen. Anti-ErbB2 nanobodies were generously provided by Professor Christopher Porter's research group at Monash University. Anti-ErbB2 antibodies (Herceptin) were purchased from F. Hoffmann-La Roche Ltd. Ultrapure water with a resistance of greater than 18 M Ω cm (Milli-Q water) was obtained from a three-stage Millipore Milli-Q Plus 185 purification system.

4.3.2 Preparation of Nano-Assemblies

Mesoporous silica nanoparticle (MSN) templates were synthesised via a literature method using CTAB as a cationic surfactant, TEOS as a silica source, and TEA as a catalyst.¹⁰⁻¹² Briefly, 1.1 g of CTAB and 4.3 g of PAA were added in 50 mL of water under vigorous stirring at room temperature to form a clear solution. 3.5 mL of ammonium hydroxide solution was

subsequently added in the solution under continuous stirring, forming a turbid suspension. Following 20 min of stirring, 4.46 mL of TEOS was added to the above suspension to initiate the formation of MSN. The suspension was further stirred for 20 min, then transferred into a Teflon-sealed autoclave and left at 100 °C for 48 h. The resultant particles were collected and washed with water and ethanol for three times, and then dried at 80 °C. The final MSNs were obtained after calcination at 550 °C for 6 h.

Subsequently, 500 μL of anti-ErBb2 nanobody solution (2 mg mL^{-1}) was added to 500 μL of MSN solution (7 mg mL^{-1}) in sodium acetate buffer (0.1 M, pH 5.0). The particle/protein dispersion was allowed to incubate for 2 h at room temperature on an Eppendorf thermomixer to allow the nanobody molecules to infiltrate the pores of the template via electrostatic adsorption. Excess antibodies were removed by centrifugation (~ 5000 g, 5 min, $\times 3$), and the nanobody-loaded nanoparticles were dispersed in DPBS (pH 7.4). 8-arm-PEG(10k)-NHS were added to the Herceptin-loaded MSNs in PBS buffer and allowed to incubate for 4 h at room temperature with constant agitation. Nanobody nano-assemblies (NanoNAs) were finally obtained by dissolving the MSN template with 1 M HF/3 M NH_4F solution (pH ~ 5), followed by washing with DPBS buffer three times. *Caution! HF is highly toxic. Extreme care should be taken when handling HF solution, and only small quantities should be prepared.* anti-ErBb2 antibody nano-assemblies (AbNAs) and non-specific control particles IgG nano-assemblies (IgGNAs) were prepared following the same methods.

4.3.3 Antibody Labelling

For nanobody and antibody labelling, 2.5 μL AF647 (1 mg mL^{-1}) in DMSO was added to 200 μL of nanobody or antibody solution (5 mg mL^{-1}) in DPBS buffer containing 0.1 M Na_2CO_3 (pH 8.3) for 2 h at room temperature with constant agitation. Excess free fluorophores were

removed using Zeba™ spin desalting columns (7 K molecular weight cut-off for nanobodies and 40 K molecular weight cut-off for antibodies). The labelled antibodies were then used to prepare NanoNAs and AbNAs for imaging.

4.3.4 Particle Characterisation

Transmission electron microscopy (TEM) images were taken on an FEI Tecnai Spirit microscope operated at 120 kV. Samples were prepared by placing a drop of a sample suspension (suspended in deionized water) on a carbon-coated copper grid and drying under ambient conditions. Fluorescent microscopy (TEM) images were taken on an Olympus IX71 inverted fluorescence microscope. Hydrodynamic size distribution and Zeta potentials were measured using a Zetasizer Nano ZS (Malvern Instruments, UK).

4.3.5 Cell Culture

ErBb2⁺ human breast cancer BT-474 cells were maintained in RPMI medium 1640 supplemented with 10% (v/v) FBS. ErbB2[−] human breast cancer MDA-MB-468 cells and immortalised monocytic THP-1 cells were incubated in DMEM with 10% (v/v) FBS. All cultures were maintained at 37 °C in a 5% CO₂-humidified atmosphere.

4.3.6 Cellular Association Assessed by Flow Cytometry

Cells were seed at a density of 10⁵ per well in 24-well plates and incubated at 37 °C overnight. Then the culture medium was removed and replaced with fresh medium containing fluorescent labelled NanoNAs, AbNAs, control particle IgGNAs, free nanobodies or free antibodies. The dosage was 10 µg mL^{−1} protein for all samples. After incubation for various time periods, the

cells were gently washed with DPBS, followed by treatment with 0.25% trypsin at 37 °C for 5 min. The cells were collected via centrifugation at 300 g for 5 min and the resulting pellet was then resuspended in DPBS. Finally, the cells were analysed by an Apogee A50-Micro flow cytometer. At least 10^4 cells were analysed for each sample.

4.3.7 Imaging by Confocal Laser Scanning Microscopy

Cells were seeded in an eight-well Lab-Tek II chambered slide at a density of 5×10^4 cells per well for 20 h. After incubation, cells were treated with fluorescent labelled NanoNAs, AbNAs, control particle IgGNAs, free nanobodies or free antibodies for 24 h. Then the cells were washed with DPBS, fixed with 4% paraformaldehyde for 15 min at room temperature, and counterstained with Hoechst 33342 for 5 min. The cells were washed with DPBS after staining and finally immersed in DPBS before imaging, which was performed on a Nikon A1R+ confocal microscope.

4.3.8 Cell Viability Assessed by XTT Assay

Cells were seeded at a density of 1×10^4 cells per well in triplicate in 96-well microplates and allowed to adhere overnight. NanoNAs, AbNAs, control particle IgGNAs, free nanobodies or free antibodies were added and incubated with cells for 24 or 72 h. The concentration of protein was kept at 5, 10 and 20 $\mu\text{g mL}^{-1}$. After incubation, the culture medium was replaced by fresh medium containing 0.2 mg mL^{-1} of activated XTT (10 mL of 0.2 mg mL^{-1} XTT in complete medium were activated by adding 20 μL of 1mM PMS in DPBS), and cells were allowed to incubate further at 37 °C for 3 h. Cell viability was measured under the Infinite M200

microplate reader (Tecan, Switzerland) at 475 nm, with 675 nm as the reference wavelength. Cell viability was expressed as a percentage by normalizing absorbance to untreated cells.

4.3.9 Fc Receptor Blocking

Cells were suspended at a concentration of 1 million per 50 μ L medium. 50 μ L of Human BD Fc block ($50 \mu\text{g mL}^{-1}$) was added into each 50 μ L of cell suspensions, and incubated for 10 minutes at room temperature. The Fc blocked cells were then treated with AF647 labelled NanoNAs or AbNAs at a concentration of $10 \mu\text{g mL}^{-1}$. The respective cellular association was analysed by flow cytometry following the detailed methods described above.

4.3.10 *Ex vivo* Human Whole Blood Assay

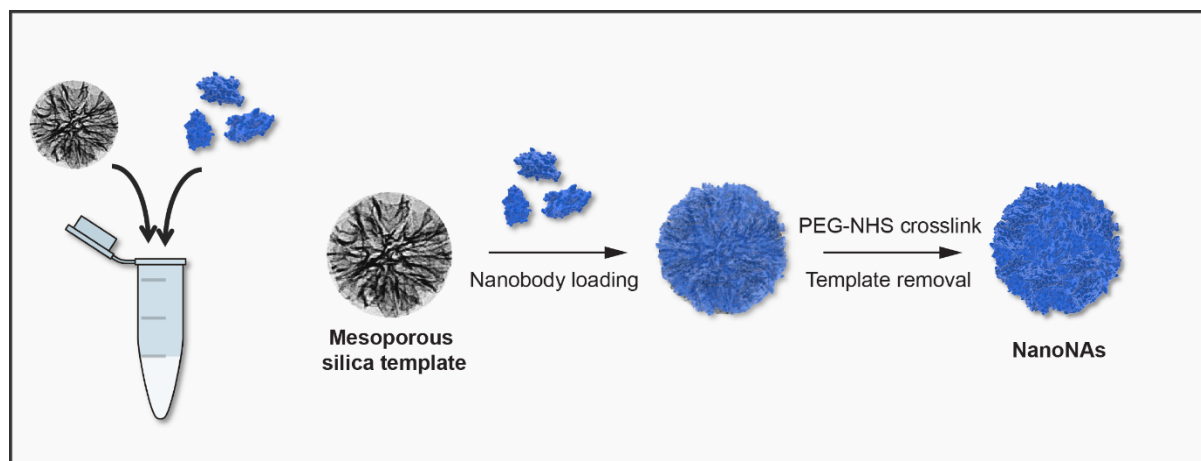
Fresh blood was collected from healthy human volunteers (Donor 1 and 2) into VACUETTE sodium heparin tubes (Greiner Bio-One) after obtaining informed consent in accordance with The University of Melbourne Human Ethics Approval#1443420 and the Australian National Health and Medical Research Council Statement on Ethical Conduct in Human Research. AF647 labelled NanoNAs, AbNAs, control particle IgGNAs, free nanobodies or free antibodies ($1 \mu\text{g}$) were incubated in 100 μ L of whole blood at 37°C for 1 h. Subsequently, the samples were treated with lysing buffer (BD Pharm Lyse) following the manufacturer's protocol to lyse red blood cells. The rest of the blood cells were further washed with flow cytometry staining (FCS) buffer (PBS containing 0.5 % w/v BSA and 2 mM EDTA) at 500 g, 7 min and resuspended in 100 μ L of FCA buffer. Titrated concentration of fluorophore-conjugated antibodies cocktail (CD3 AF700 (SP34-2, BD), CD14 APC-H7 (MΦP9, BD), CD66b BV421 (G10F5, BD), CD45 V500 (HI30, BD), CD56 PE (B159, BD), lineage-1 cocktail APC (BioLegend), HLA-DR PerCP-Cy5.5 (G46-6, BD), and CD19 BV650 (HIB19,

BioLegend)) was added and incubated for 1h on ice to identify Monocytes, granulocytes, B cells, T cells, natural killer (NK) cells and dendritic cells. Unbound antibodies were removed by washing twice with cold (4 °C) FCS buffer (500 g, 7 min). Cells were fixed with 2% w/v formaldehyde in PBS and directly analysed by flow cytometry (CytoFLEX, Beckman Coulter Life Sciences), and data were processed using FlowJoV10.

4.4 Results and Discussion

4.4.1 Synthesis and Characterisation

Nanobody nano-assemblies (NanoNAs) were synthesised via the MSN-assisted method reported in Chapter 1. Briefly, nanobodies were loaded in mesoporous silica template followed by crosslinking with PEG-NHS and removal of MSN template (**Scheme 4.1**). In this study, MSN with a size of 500 nm was used as the template and 8-arm-PEG-NHS (MW 10k) was used as the crosslinker. Antibody nano-assemblies (AbNAs) and non-specific IgG nano-assemblies (IgGNAs) were synthesised via the same method.



Scheme 4.1 The synthesis of NanoNAs via the template-assisted assembly platform. Briefly, Nanobodies were loaded in MSN template followed by crosslinking with 8-arm-PEG(10k)-NHS and removal of MSN template.

The synthesised nano-assemblies possess a uniform spherical morphology under microscopes (**Figure 4.1A and 4.1B**). The transmission electron microscopy (TEM) image shows that NanoNAs and AbNAs deposited on a carbon film had an average diameter around 500 nm (**Figure 4.1A**), which corresponded with the size of the MSN templates and was consistent with the hydrodynamic size distribution measured by dynamic light scattering (**Figure 4.1C**). Both NanoNAs and AbNAs are slightly negatively charged with a zeta potential value around

-10 mV (**Figure 4.1D**). The control IgGNAs had similar morphology, size distribution and zeta potential (**Figure S4.1**)

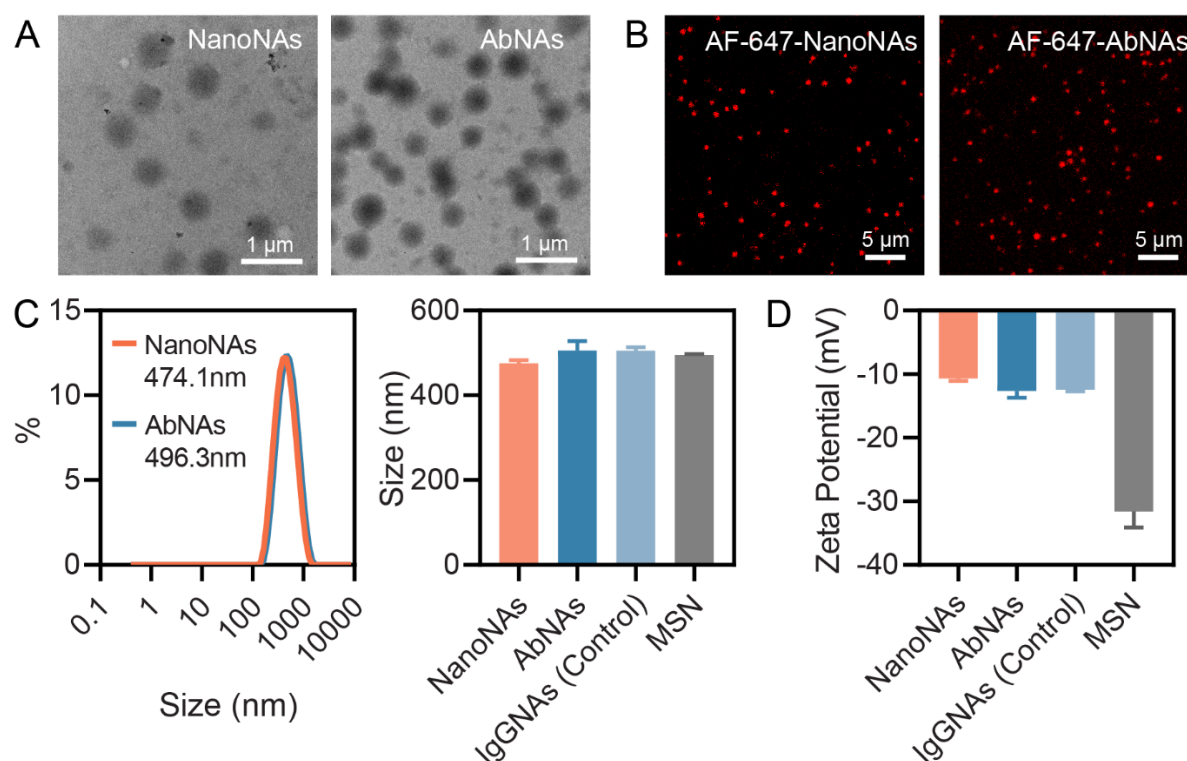


Figure 4.1 Characterisations of NanoNAs and AbNAs. A, TEM images of NanoNAs and AbNAs. B, Fluorescence microscopy images of NanoNAs and AbNAs. Nanobodies and antibodies were labelled with Alexa Fluor 647 before assembled into NanoNAs and AbNAs. C, Size distribution of NanoNAs and AbNAs by dynamic light scattering. D, Zeta potential of NanoNAs and AbNAs measured by Zetasizer.

4.4.2 Cellular Targeting

Nanobodies that specifically target the ErbB2 receptor overexpressed in ~25% of invasive breast carcinomas¹³ was used as model molecules to prepare the NanoNAs in this study. Full-sized antibodies with the same target were used to prepare the corresponding AbNAs. Their ability to associate with ErbB2 overexpressed (ErbB2+) BT-474 breast cancer cells were

investigated by flow cytometry analysis, while ErbB2 negative (ErbB2⁻) MDA-MB-231 breast cancer cells were used as control cells. The NanoNAs and AbNAs were incubated with both types of cells in culture medium at 37° C for 3, 6 and 20 h. Nonspecific IgGNAs was included as control particles. Conjugation AF647 dye to the protein molecules aided the detection.

Results showed NanoNAs specifically associated with BT-474 (ErbB2⁺) cells in a time-dependent manner but no association with MDA-MB-231 (ErbB2⁻) cells was detected (**Figure 4.2, S4.2 and S4.3**). 18.8 % of BT-474 cells were associated with NanoNAs after 3 h incubation. The association percentage increased to 30.5% at 6 h and reached 90% at 20h (**Figure 4.2A**). Comparing with free nanobody, the NanoNAs showed a slightly lower target association percentage at 3 and 6 h, but eventually reached the same level after 20 h of incubation. The above findings suggested NanoNAs retained the selective targeting ability of nanobody molecules to recognise the ErbB2 receptors on the cell membrane. Notably, AbNAs had limited association with BT-474 cells at all time points. The reduction of targeting ability was largely caused by the mask of PEG on the particle surface that spatially blocks the antigen binding sites from the target receptor. Comparing with the full-sized antibody (150 kDa), nanobody with only one single monomeric variable domain (12 – 15 kDa) allowed 10 times more active sites to be incorporated in the particles, which leads to the significantly enhanced targeting ability of NanoNAs.

Furthermore, the AF647 fluorescence intensity of the treated BT-474 cells was quantified as an indicator of the number of nanobodies/antibodies associated with each cell (**Figure 4.2B**). The intensity of cells treated with NanoNAs increased with the incubation time. At 3 and 6 h, the same intensity level was observed in cells treated with NanoNAs and free nanobody. But after 20 h of incubation, cells treated with NanoNAs exhibited a significantly higher intensity compared to cells treated with free nanobody, which suggested that NanoNAs allowed a substantially higher number of nanobody molecules to associate to each target cell.

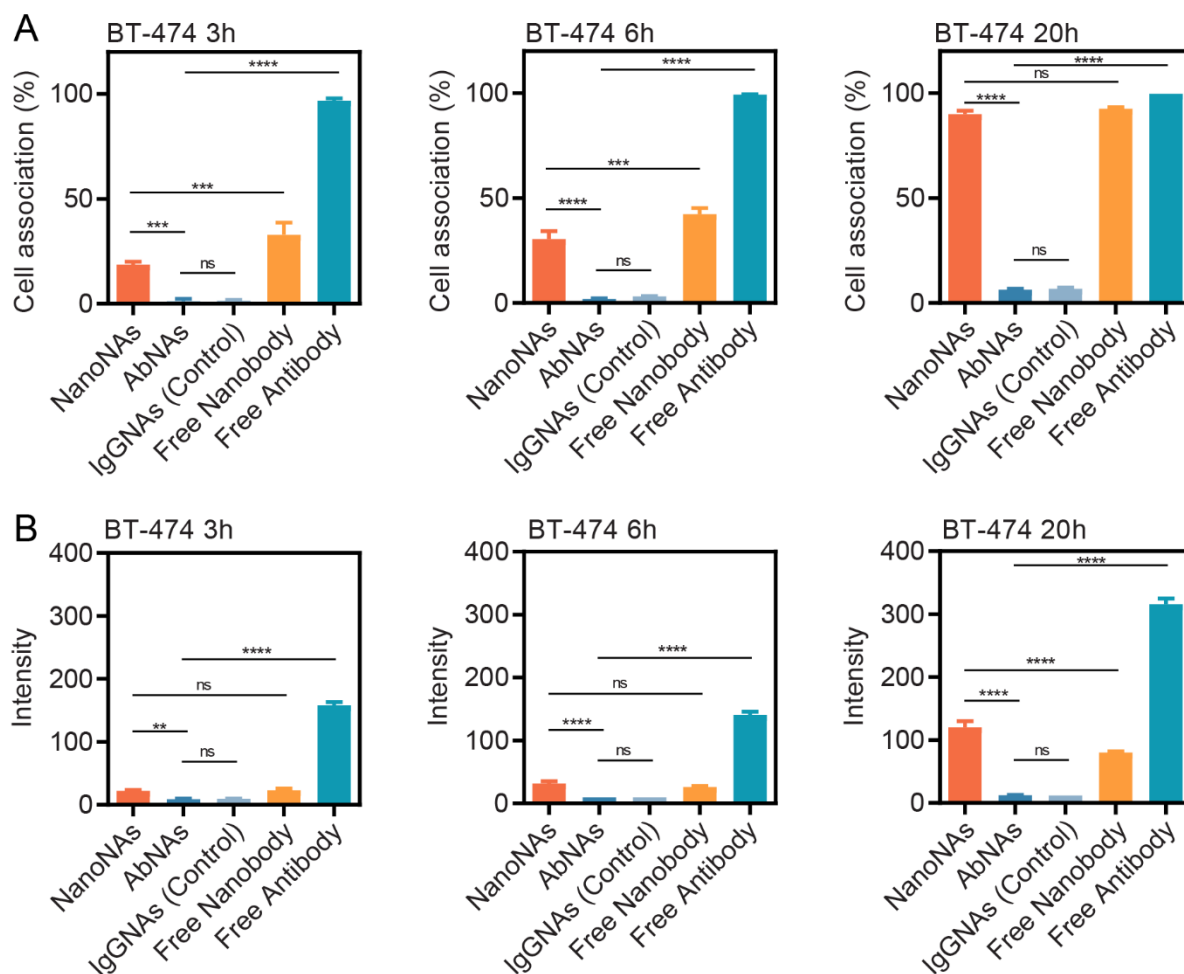


Figure 4.2 Association profiles of NanoNAs and AbNAs with ErBb2+ breast cancer BT-474 cell, quantified by flow cytometry analysis. Nanobodies, antibodies and their nano-assemblies were labelled with AF647. BT-474 cells were incubated with the labelled NanoNAs, AbNAs, free nanobody, free antibody and control particle IgGNAs for 3, 6 or 24 h. The dosage was $10 \mu\text{g mL}^{-1}$ protein for all samples. The percentage of associated cells (A) and their florescent intensity (B) were quantified by flow cytometry analysis. Notes: **** Indicates significant difference, $p < 0.0001$, $n = 3$; *** Indicates significant difference, $p < 0.001$, $n = 3$; ** Indicates significant difference, $p < 0.01$, $n = 3$; * Indicates significant difference ($p < 0.05$, $n = 3$); ns Indicates there is no significant difference ($p > 0.05$, $n = 3$).

The association study was further confirmed by confocal laser scanning microscopy (CLSM). The BT-474 (ErbB2+) cells were incubated AF647 labelled NanoNAs and AbNAs at 37°C for

20 h, then fixed with paraformaldehyde. Before imaging, the nuclei were stained with Hoechst 33342. The CLSM images were captured by a Nikon A1R+ confocal microscope. The imaging results were consistent with the association study conducted by flow cytometry. As shown in **Figure 4.3**, a large amount of NanoNAs were associated with BT-474 cells but only a few AbNAs was found, while both free nanobody and free antibody were associated with all BT-474 cells. In the meantime, the control IgGNAs was not observed in BT-474 (ErbB2+) cells (**Figure S4.4**), and no association were observed in MDA-MB-231 (ErbB2-) cells (**Figure S4.5**).

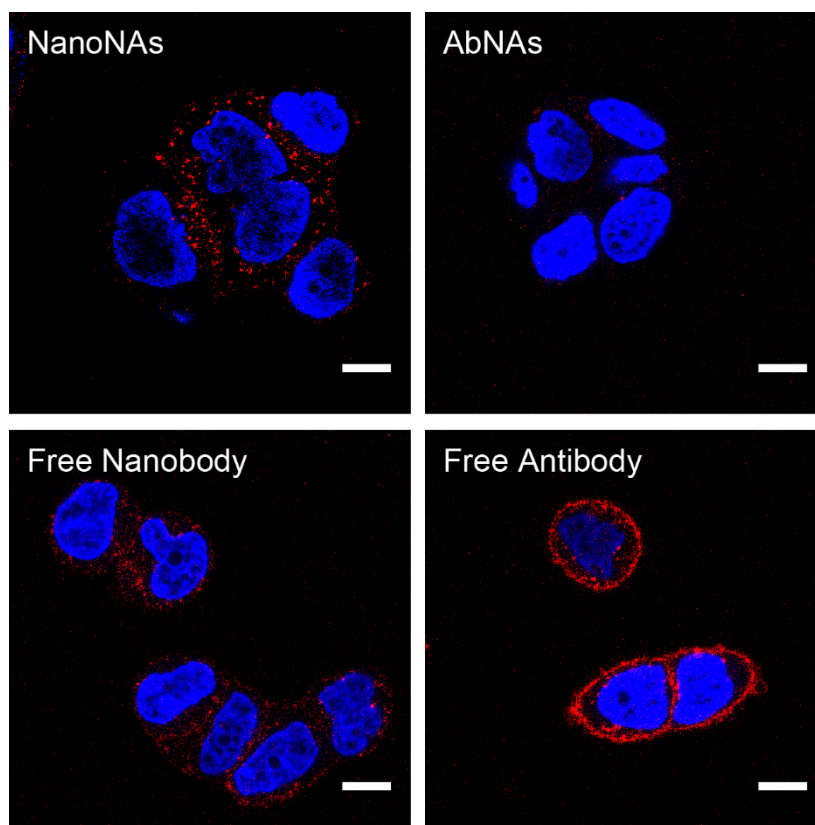


Figure 4.3 Images of BT-474 (ErbB2+) cells treated with NanoNAs, AbNAs, free nanobody and free antibody, captured by CLSM. Nanobodies, Antibodies and their nano-assemblies were labelled with AF647 (Red). The BT-474 cells were treated for 20 h. The dosage was $10 \mu\text{g mL}^{-1}$ protein for all samples. Nuclei were stained with Hoechst 33353 dye (Blue) before imaging. Scale bar: $10 \mu\text{m}$

4.4.3 Cell Growth Inhibition

As well reported previously, ErbB2 signalling promotes cell proliferation. The anti-ErbB2 nanobodies and antibodies interfere with ErbB2-triggered signalling by preventing ErbB2 receptor dimerization, which eventually inhibits cell growth.^{14, 15} To investigate the cell growth inhibition triggered by NanoNAs and AbNAs, the cell viability of ErbB2+ BT-474 cells and ErbB2– MDA-MB-231 cells were examined by XTT assay after treatment with NanoNAs, AbNAs and controls at the concentration of 5 $\mu\text{g mL}^{-1}$, 10 $\mu\text{g mL}^{-1}$, and 20 $\mu\text{g mL}^{-1}$ for 24 h and 72 h. After treated with 5 $\mu\text{g mL}^{-1}$ NanoNAs for 24 h, 85.9% of BT-474 cells were still viable when compared with the cell viability of blank control. With the concentration increased to 10 $\mu\text{g mL}^{-1}$ and 20 $\mu\text{g mL}^{-1}$, the viability of cells treated with NanoNAs dropped to 76.2% and 73.3%. Cells treated with AbNAs at 5 $\mu\text{g mL}^{-1}$, 10 $\mu\text{g mL}^{-1}$ and 20 $\mu\text{g mL}^{-1}$ for 24 h showed 99.5%, 92.4% and 73.8% viability respectively (**Figure 4.4A**). When incubation time prolonged to 72h, the viability of cell treated with NanoNAs at 5 $\mu\text{g mL}^{-1}$, 10 $\mu\text{g mL}^{-1}$ and 20 $\mu\text{g mL}^{-1}$ dropped to 62.5%, 61.3% and 50.9%, and the viability of cells treated with AbNAs at 5 $\mu\text{g mL}^{-1}$, 10 $\mu\text{g mL}^{-1}$ and 20 $\mu\text{g mL}^{-1}$ decreased to 73.5%, 75.8% and 72.7% (**Figure 4.4B**). As indicated from the results above, NanoNAs showed an improved growth inhibition ability on target cancer cells when compared with AbNAs at all treatment conditions. In addition, NanoNAs achieved the same level of growth inhibition when compared with free nanobodies, while AbNAs showed reduced inhibition compared with free antibodies, which also demonstrated the advantage of NanoNAs over AbNAs on cell growth inhibition.

No cell growth inhibition was found in MDA-MB-231 (ErbB2–) cells at all treatment (**Figure S4.6**). It indicated the NanoNAs and AbNAs retained the selective growth inhibition of nanobody and antibody molecules.

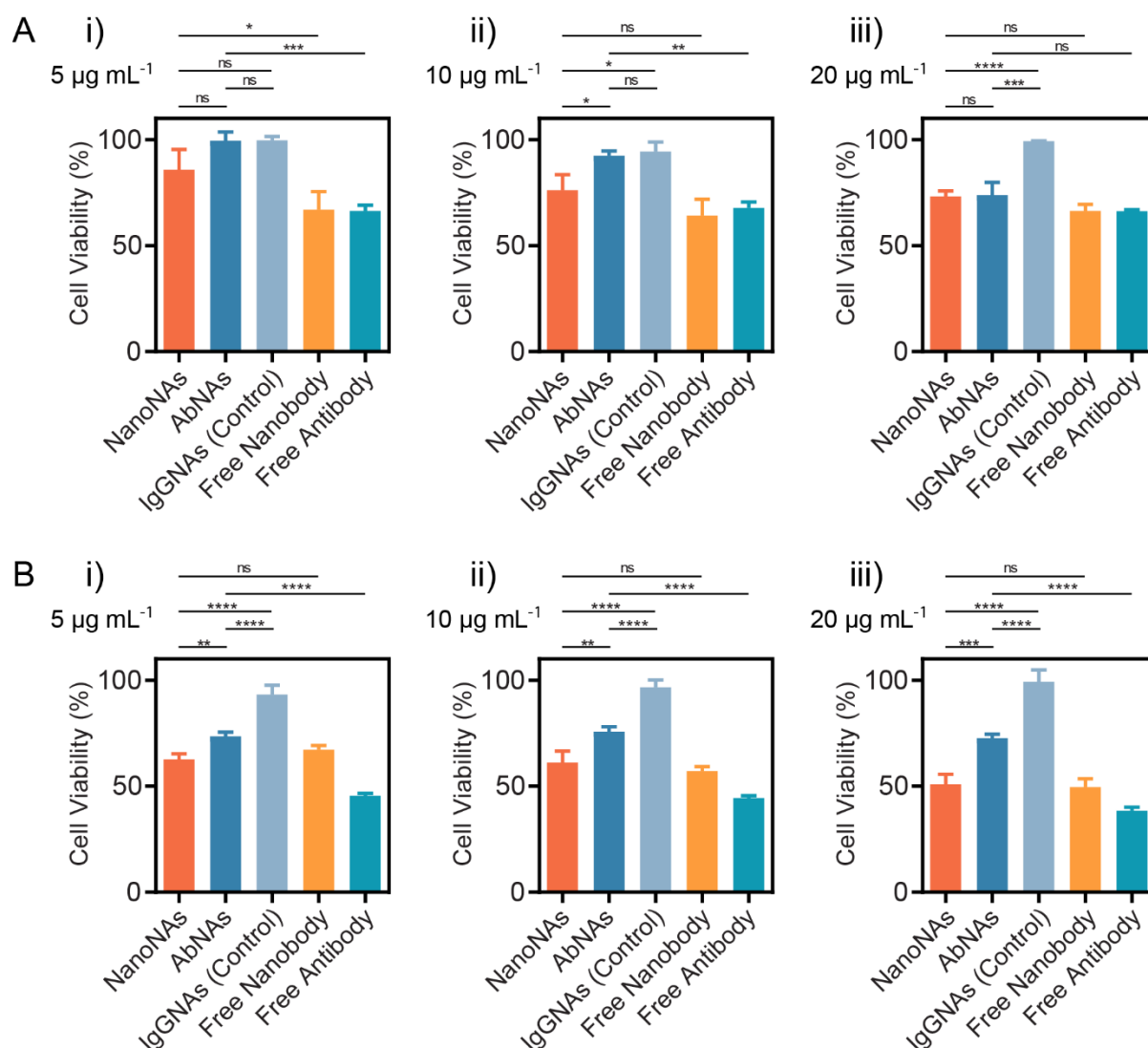


Figure 4.4 Inhibition effect of NanoNAs and AbNAs on BT-474 (ErbB2+) cell growth. The cells were treated with NanoNAs, AbNAs, control particle IgGNAs, free nanobody and free antibody for 24 h (A) and 72 h (B) at the concentration of i) 5 $\mu\text{g mL}^{-1}$, ii) 10 $\mu\text{g mL}^{-1}$, and iii) 20 $\mu\text{g mL}^{-1}$. The cell viability was then examined by 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay. Notes: **** Indicates significant difference, $p < 0.0001$, $n = 3$; *** Indicates significant difference, $p < 0.001$, $n = 3$; ** Indicates significant difference, $p < 0.01$, $n = 3$; * Indicates significant difference ($p < 0.05$, $n = 3$); ns Indicates there is no significant difference ($p > 0.05$, $n = 3$).

4.4.4 Association with Immortalised Monocytic THP-1 Cells

Compared with whole antibodies, nanobodies are expected to be less immunogenic after mobilized on the particle surface, owing to the lack of Fc domain that could be recognized by various immune cells via Fc receptors. To investigate the immunogenicity of NanoNAs, the association study was performed *in vitro* on immortal monocytic cell line THP-1 that express Fc γ receptors.¹⁶ The cells were treated with AF647 labelled NanoNAs, AbNAs and controls at 10 $\mu\text{g mL}^{-1}$ for 20 and 80 h, then the percentages of associated cells and their fluorescent intensity was analysed via flow cytometry. Results showed only 1 % cells were associated with NanoNAs and 5.6 % cells were associated with AbNAs after 20 h of incubation. When incubation time increased to 80 h, still only 1 % of cells were associated with NanoNAs but 48.5 % were associated with AbNAs (**Figure 4.5A**). The intensity data also showed a similar trend (**Figure 4.5B**). The results showed that NanoNAs had a significantly lower association with immortalised monocytic cells compared to AbNAs, which indicated NanoNAs could avoid the recognition and capture by phagocytic cells to achieve prolonged blood circulation.

To further investigate if the improved stealth ability of NanoNAs was owing to the lack of Fc region, THP-1 cells were treated with Human BD Fc block to block Fc γ receptors on the cell membrane. Then the Fc blocked THP-1 cells and untreated normal THP-1 cells were incubated with AF647 labelled NanoNAs and AbNAs for 20 h and 80 h. As shown in **Figure 4.6**, no association difference was observed between Fc blocked cells and untreated cells when treated with NanoNAs. However, Fc blocked cells had a significantly lower association with AbNAs compared with untreated cells. The results suggested the presence of Fc regions contributed to the easy capture of AbNAs by phagocytic cells with Fc receptors, and the NanoNAs exhibited a great advantage in avoiding phagocytic capture owing to the lack of Fc region.

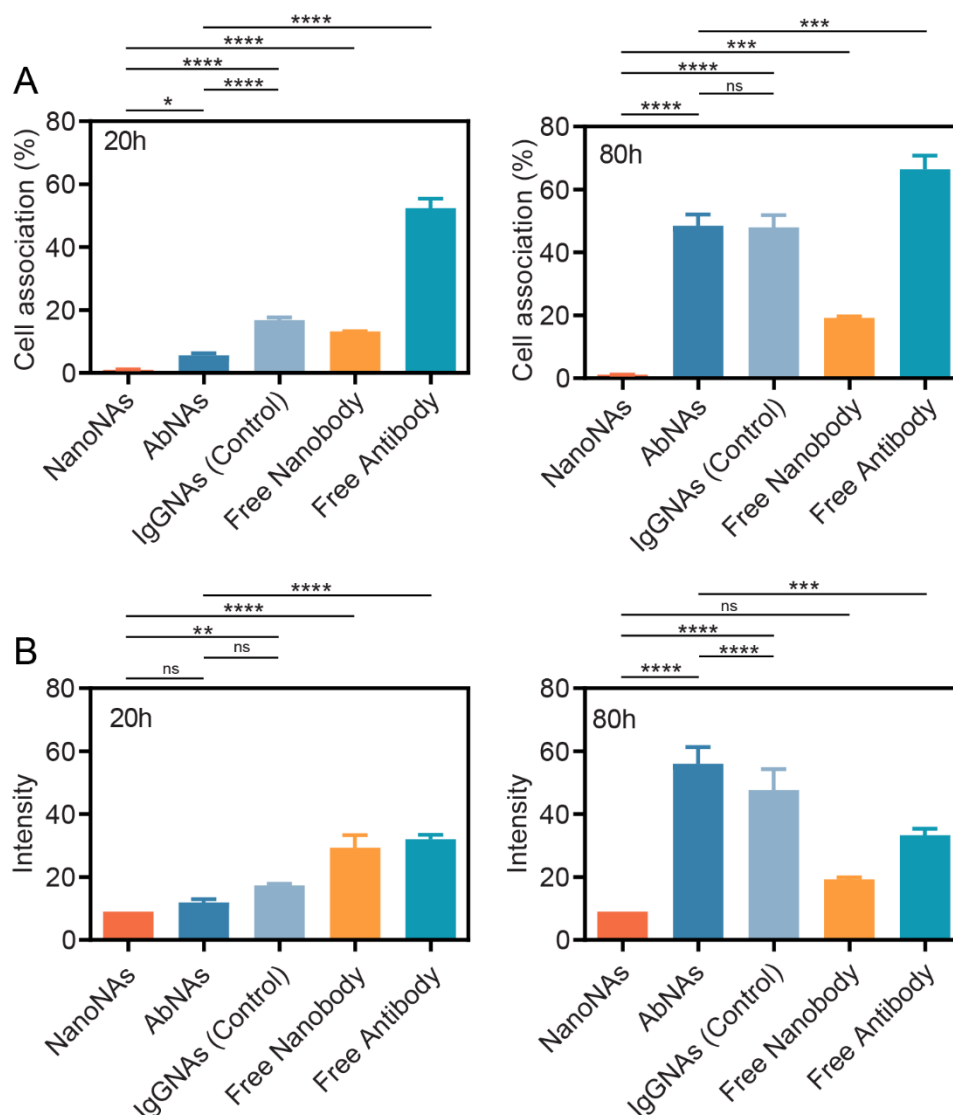


Figure 4.5 Association profiles of NanoNAs and AbNAs with immortalised human monocytic THP-1 cell, quantified by flow cytometry analysis. Nanobodies, antibodies and their nano-assemblies were labelled with AF647. THP-1 cells were incubated with the labelled NanoNAs, AbNAs, free nanobody, free antibody and control particle IgGNAs for 20 h and 80 h. The dosage was $10 \mu\text{g mL}^{-1}$ protein for all samples. The percentage of associated cells (A) and their florescent intensity (B) were quantified by flow cytometry analysis. Notes: **** Indicates significant difference, $p < 0.0001$, $n = 3$; *** Indicates significant difference, $p < 0.001$, $n = 3$; ** Indicates significant difference, $p < 0.01$, $n = 3$; * Indicates significant difference ($p < 0.05$, $n = 3$); ns Indicates there is no significant difference ($p > 0.05$, $n = 3$).

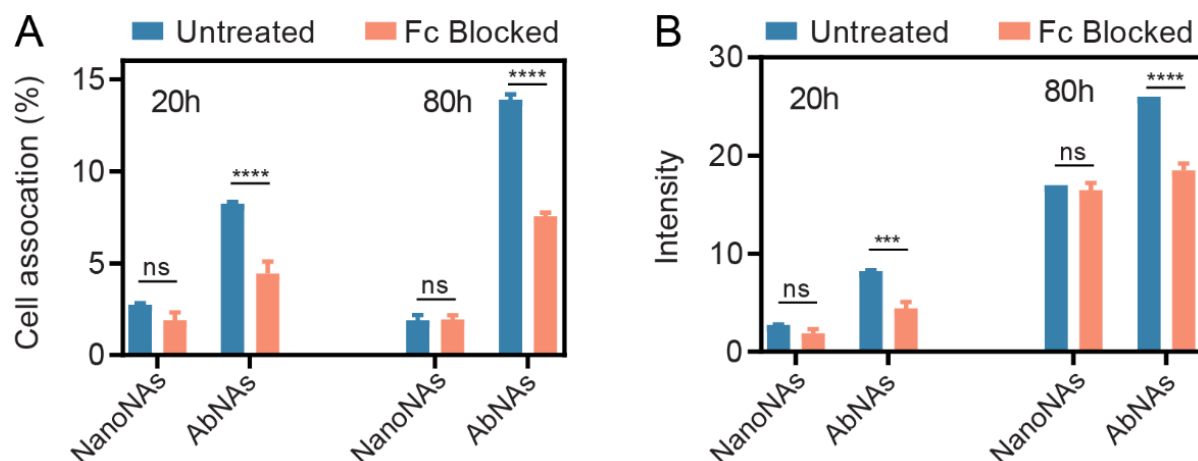


Figure 4.6 Association of NanoNAs and AbNAs with Fc γ receptors blocked THP-1 cells, quantified by flow cytometry analysis. NanoNAs and AbNAs were labelled with AF647. THP-1 cells were treated with 25 $\mu\text{g mL}^{-1}$ Fc block for 10 minutes at room temperature to block the Fc γ receptors. Untreated and Fc blocked THP-1 cells were incubated with Alexa Fluor 647 labelled NanoNAs, AbNAs, free nanobody, free antibody and control particle IgGNAs for 20 h and 80 h. The dosage was 10 $\mu\text{g mL}^{-1}$ protein for all samples. The percentage of associated cells (A) and their florescent intensity (B) were quantified by flow cytometry analysis. Notes: **** Indicates significant difference, $p < 0.0001$, $n = 3$; *** Indicates significant difference, $p < 0.001$, $n = 3$; ** Indicates significant difference, $p < 0.01$, $n = 3$; * Indicates significant difference ($p < 0.05$, $n = 3$); ns Indicates there is no significant difference ($p > 0.05$, $n = 3$).

4.4.5 Association with Leukocytes in Human Whole Blood

The *in vitro* immortal models often have limitations to mimic the *in vivo* environment. To better understand the immunogenicity of NanoNAs, the association with leukocytes in human whole blood was investigated using a previously developed human blood assay.¹⁷ Fresh blood was collected from healthy human volunteers in accordance with The University of Melbourne Human Ethics Approval #1443420 and the Australian National Health and Medical Research

Council Statement on Ethical Conduct in Human Research. AF647 labelled NanoNAs, AbNAs and controls ($10 \mu\text{g mL}^{-1}$) were incubated in $100 \mu\text{L}$ of whole blood at 37°C for 1 h. Subsequently, red blood cells were lysed with lysing buffer. The rest of the blood cells were stained by fluorophore-conjugated antibodies cocktails and further analysed by flow cytometry. Leukocytes including monocytes, granulocytes, B cells, T cells, natural killer (NK) cells and dendritic cells were identified and the association of NanoNAs and AbNAs with the leukocytes was analysed using Flowjo V10. As well studied in previous researches, Fc γ receptors are widely expressed on virtually all the leukocytes except T cells, but the expression level varies among different cell types.^{18, 19} Results showed the majority of associated particles were captured by B cells, granulocytes and monocytes (**Figure S4.7**) owing to the heterogeneity of Fc γ receptor expression.^{20, 21} As shown in **Figure 4.7**, 55.4 % B cells, 13.1 % granulocytes and 71.3 % monocytes were associated with NanoNAs, while 83.6 % of B cells, 50.4 % granulocytes and 50.3 % monocytes were associated with AbNAs. NanoNAs showed a significantly lower association with B cells and granulocytes but a higher association with monocytes compared with AbNAs. The same trend was found when repeated the study with fresh blood from another healthy donor (**Figure S4.8**). Notably, the free nanobody had an extremely high association with monocytes ($\sim 100\%$). Therefore, the unexpected high association of NanoNAs with monocytes might cause by an inherited feature of the nanobody.

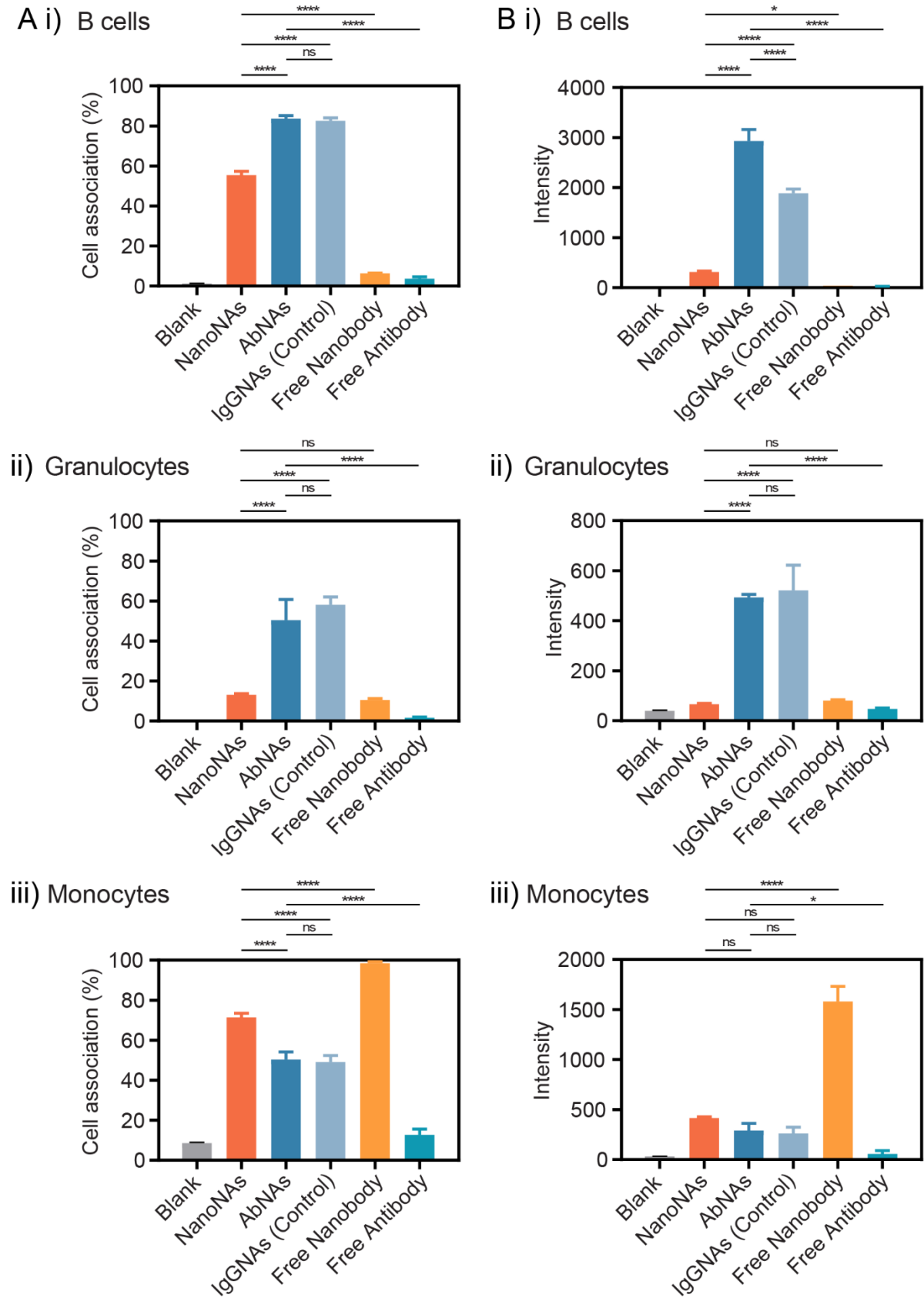


Figure 4.7 Association profiles of NanoNAs and AbNAs with B cells, granulocytes and monocytes from human whole blood (donated by donor 1), quantified by flow cytometry

analysis. Nanobodies, antibodies and their nano-assemblies were labelled with Alexa Fluor 647. The labelled NanoNAs, AbNAs, free nanobody, free antibody and control particle IgGNAs were incubated in fresh human whole blood for 1 h at 37 ° C. B cells, granulocytes and monocytes were identified by specific fluorescent labelled antibodies. The percentage of associated cells (A) and their florescent intensity (B) were quantified by flow cytometry analysis. Notes: **** Indicates significant difference, $p < 0.0001$, $n = 3$; *** Indicates significant difference, $p < 0.001$, $n = 3$; ** Indicates significant difference, $p < 0.01$, $n = 3$; * Indicates significant difference ($p < 0.05$, $n = 3$); ns Indicates there is no significant difference ($p > 0.05$, $n = 3$).

4.5 Conclusions

In this chapter, nanobodies that consist of only one single monomeric variable domain were used to synthesise nanobody nano-assemblies (NanoNAs) via mesoporous silica template-assisted assembly. Compared with AbNAs composed of full-sized antibody, the NanoNAs exhibited significantly enhanced selective association to target cancer cells owing to the small size of nanobodies that afforded a large number of active binding sites on the particle surfaces. Furthermore, NanoNAs showed significantly improved stealth when exposed to monocytic cells (THP-1) *in vitro*, owing to the lack of Fc domain that could be recognized by various immune cells via Fc receptors, potentially prolonging the circulation of NanoNAs in the blood system. However, when NanoNAs were exposed to leukocytes in human whole blood, cell-specific association behaviour was observed. The majority of associated nanoparticles was captured by B cells, granulocytes, and monocytes. In comparison with AbNAs, NanoNAs displayed significantly lower association with B cells and granulocytes but higher association with monocytes. This result could be due to a combined effect of Fc receptor-mediated recognition and the inherent property of the nanobody, which is worth investigating in future studies. Overall, the nanobody-based nano-assemblies exhibited enhanced antigen-targeting efficacy and reduced immunogenicity, owing to the unique structure of the nanobodies, and therefore have potential in the application of cancer targeting therapy.

4.6 Supporting Information

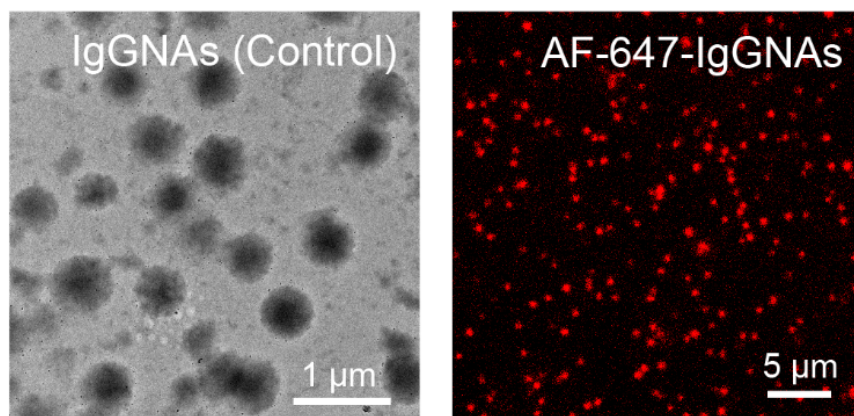


Figure S4.1 TEM image of IgGNAs and fluorescence microscopy image of AF647 labelled IgGNAs.

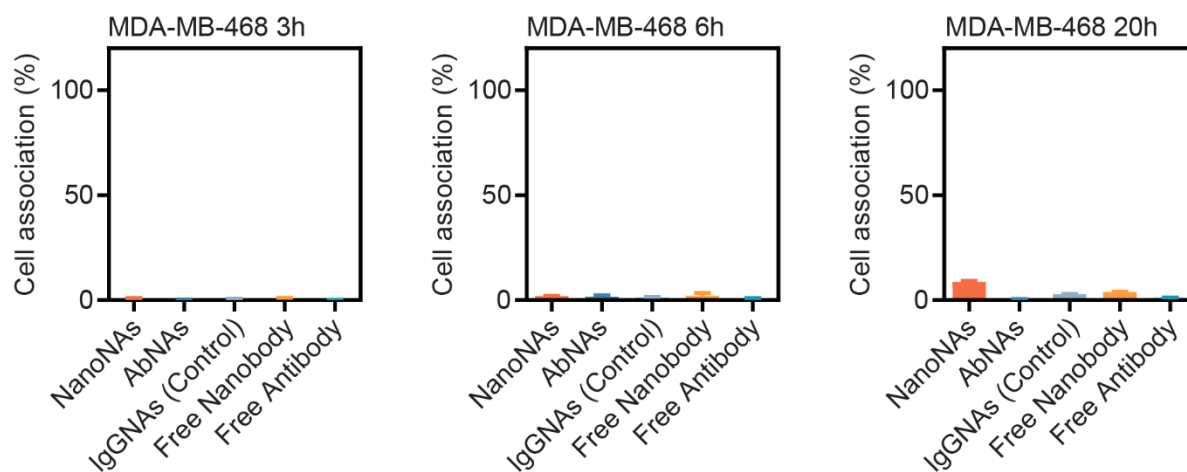


Figure S4.2 Association (%) profiles of NanoNAs and AbNAs with ErbB2⁺ breast cancer MDA-MB-468 cells, quantified by flow cytometry analysis. Nanobodies, antibodies and their nano-assemblies were labelled with AF647. MDA-MB-468 cells were incubated with the labelled NanoNAs, AbNAs, free nanobody, free antibody and control particle IgGNAs for 3, 6 or 24 h. The dosage was $10 \mu\text{g mL}^{-1}$ protein for all samples. The percentage of associated cells were quantified by flow cytometry.

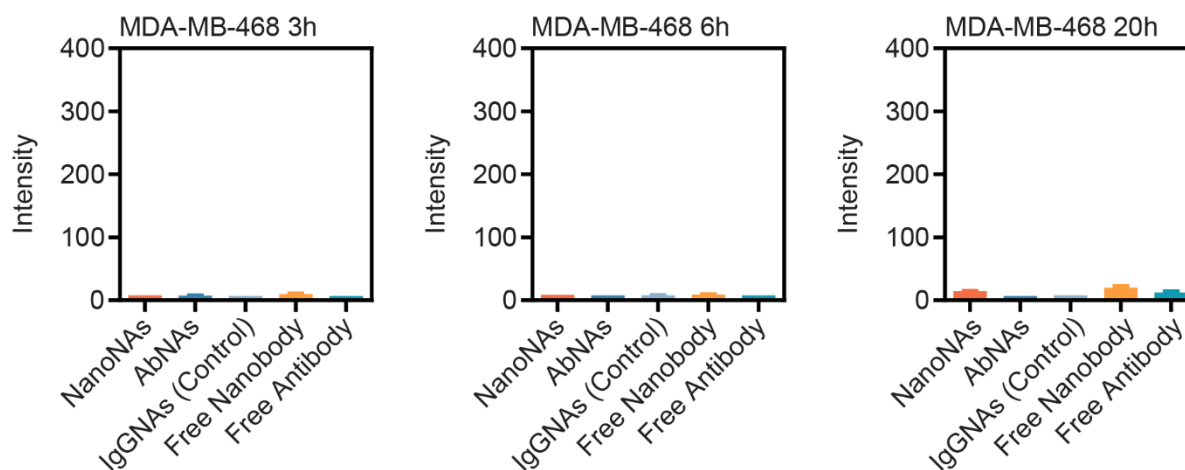


Figure S4.3 Association (intensity) profiles of NanoNAs and AbNAs with ErbB2⁺ breast cancer MDA-MB-468 cells, quantified by flow cytometry analysis. Nanobodies, antibodies and their nano-assemblies were labelled with AF647. MDA-MB-468 cells were incubated with the labelled NanoNAs, AbNAs, free nanobody, free antibody and control particle IgGNAs for 3, 6 or 24 h. The dosage was $10 \mu\text{g mL}^{-1}$ protein for all samples. The fluorescent intensity of the associated cells was quantified by flow cytometry.

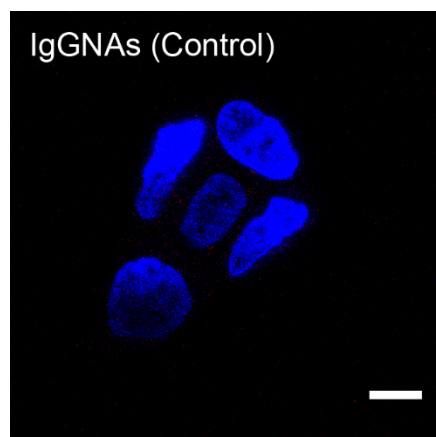


Figure S4.4 Images of BT-474 (ErbB2+) cells treated with IgGNAs, captured by a confocal laser scanning microscope. The BT-474 cells were treated with AF647 labelled IgGNAs (Red) at a dosage of $10 \mu\text{g mL}^{-1}$ for 24 h. Nuclei were stained with Hoechst 33353 dye (Blue) before imaging. Scale bar: $10 \mu\text{m}$

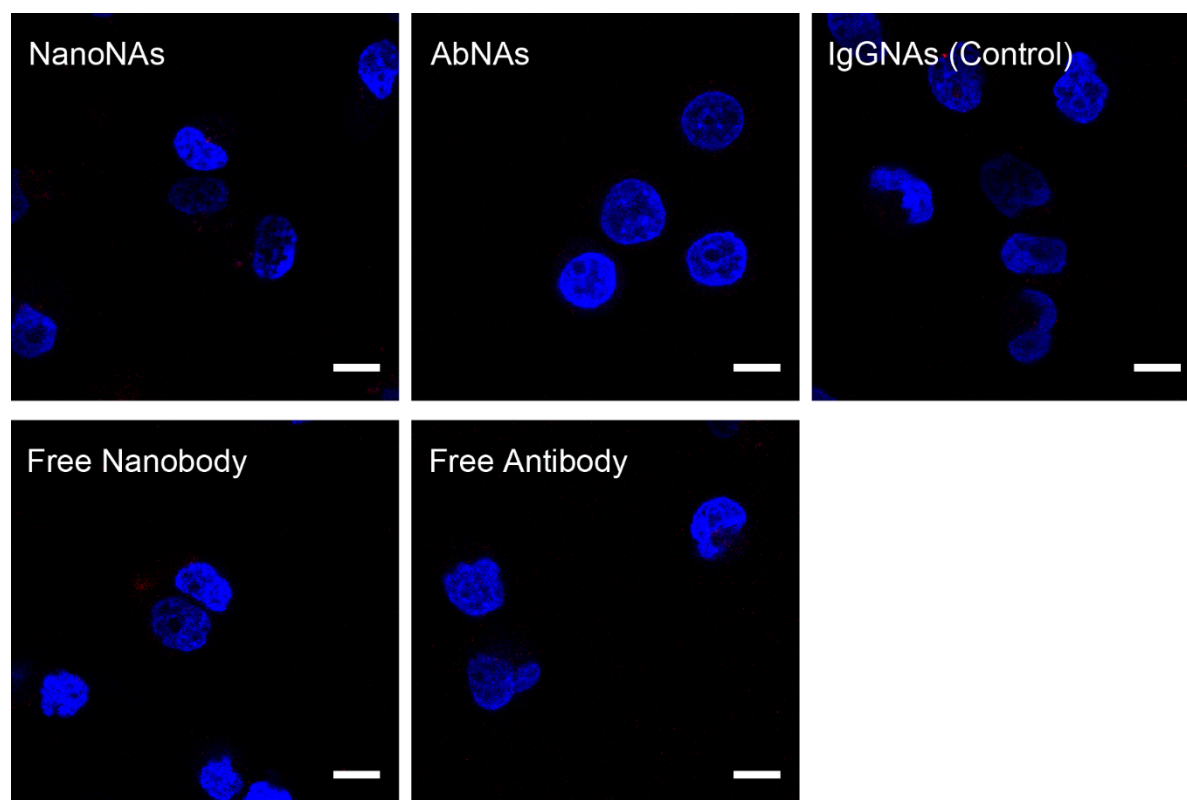


Figure S4.5 Images of MDA-MB-468 (ErbB2⁻) cells treated with NanoNAs, AbNAs, control particle IgGNAs, free nanobody and free antibody, captured by CLSM. Nanobodies, Antibodies and their nano-assemblies were labelled with AF647 (Red). The BT-474 cells were treated for 24 h. The dosage was $10 \mu\text{g mL}^{-1}$ protein for all samples. Nuclei were stained with Hoechst 33353 dye (Blue) before imaging. Scale bar: $10 \mu\text{m}$

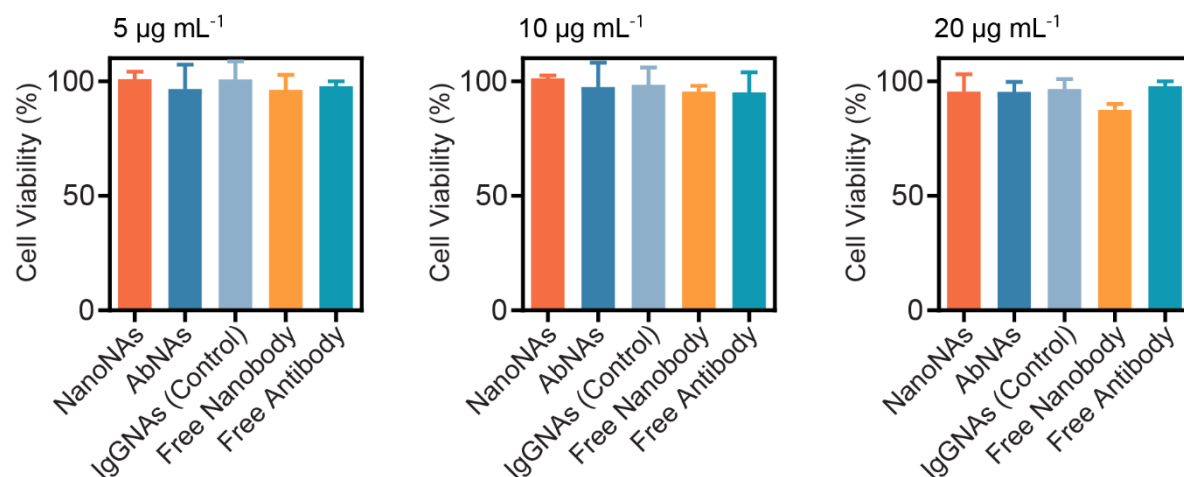


Figure S4.6 Inhibition effect of NanoNAs and AbNAs on MDA-MB-468 (ErbB2-) cell growth. The cells were treated with NanoNAs, AbNAs, control particle IgGNAs, free nanobody and free antibody for 72 h at the concentration of 5 µg mL⁻¹, 10 µg mL⁻¹, and 20 µg mL⁻¹. The cell viability was then examined by 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay.

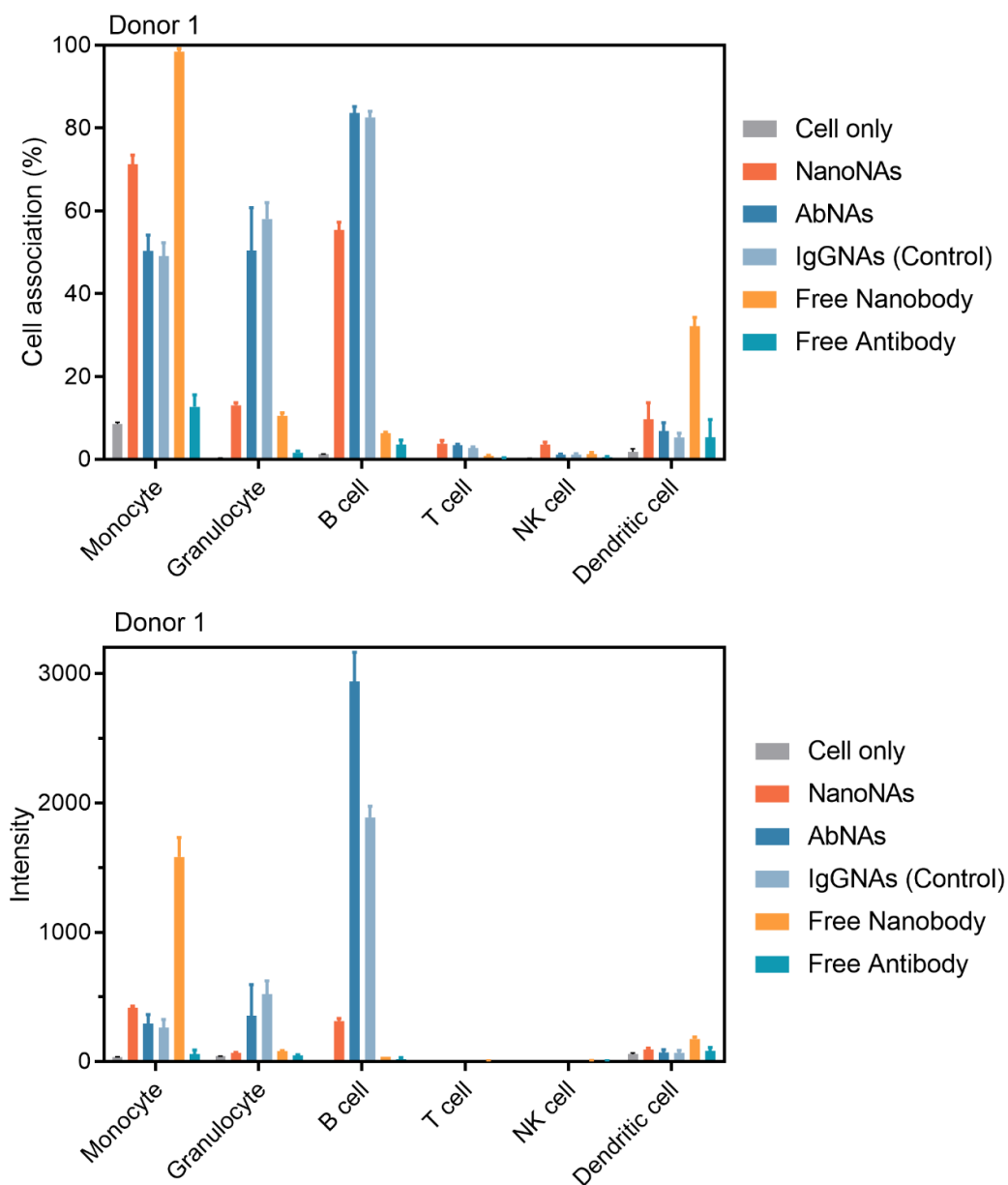


Figure S4.7 Association profiles of NanoNAs and AbNAs with leucocytes from human whole blood (collected from donor 1), quantified by flow cytometry analysis. Nanobodies, antibodies and their nano-assemblies were labelled with AF647. The labelled NanoNAs, AbNAs, free nanobody, free antibody and control particle IgGNAs were incubated in fresh human whole blood for 1 h at 37 ° C. Monocytes, granulocytes, B cells, T cells, natural killer (NK) cells and dendritic cells were identified by specific fluorescent labelled antibodies. The percentage of associated cells and their florescent intensity were quantified by flow cytometry analysis.

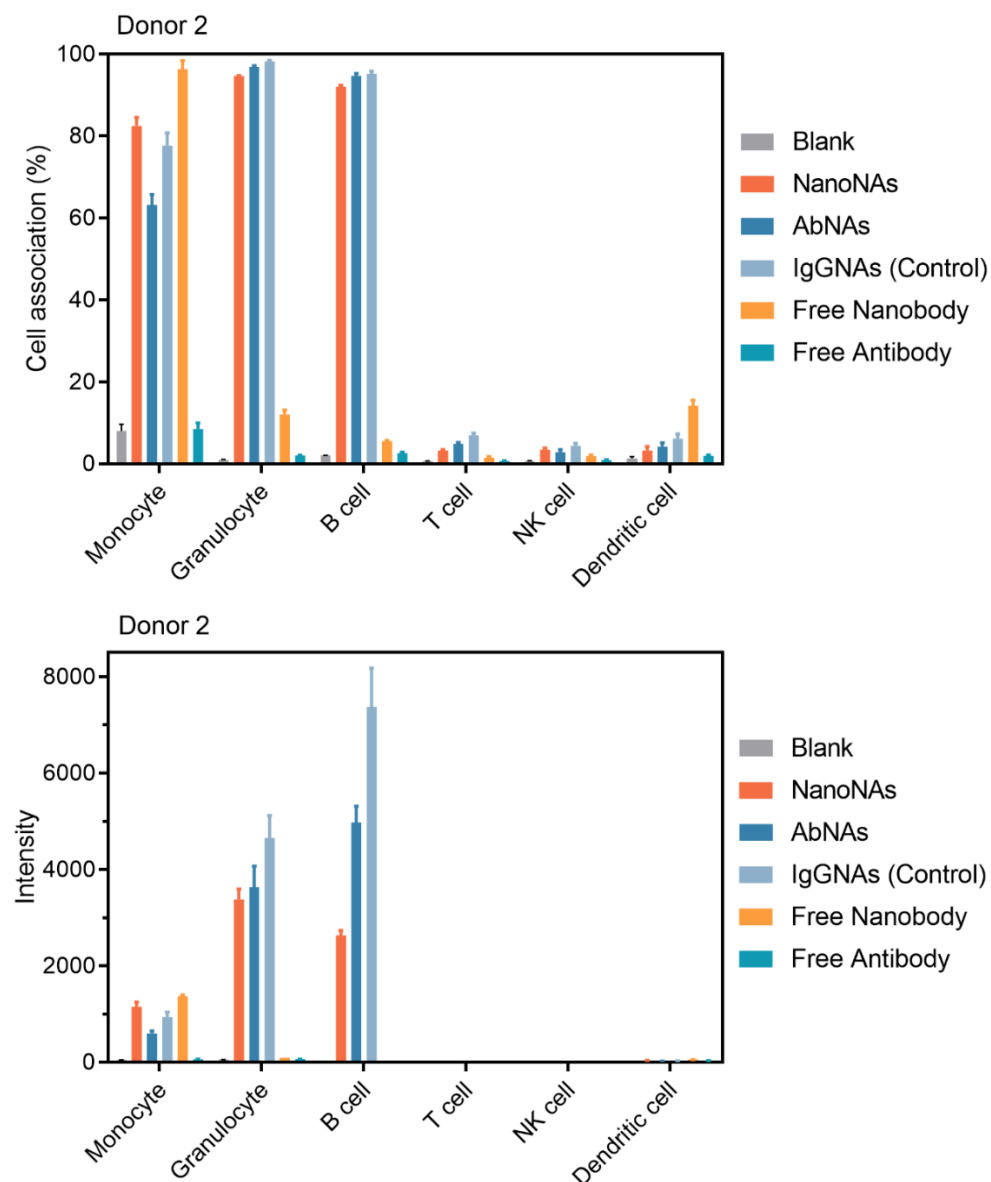


Figure S4.8 Association profiles of NanoNAs and AbNAs with leucocytes from human whole blood (collected from donor 2), quantified by flow cytometry analysis. Nanobodies, antibodies and their nano-assemblies were labelled with AF647. The labelled NanoNAs, AbNAs, free nanobody, free antibody and control particle IgGNAs were incubated in fresh human whole blood for 1 h at 37 ° C. Monocytes, granulocytes, B cells, T cells, natural killer (NK) cells and dendritic cells were identified by specific fluorescent labelled antibodies. The percentage of associated cells and their florescent intensity were quantified by flow cytometry analysis.

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Chapter 5

Conclusions and Perspectives

Over the past few decades, monoclonal antibodies have successfully transitioned from being subjects of benchtop medical research to clinically efficient drugs. Approximately 100 antibody-based drugs have been approved for clinical treatment of several diseases, including cancer, auto-immune diseases, chronic inflammatory diseases, and viral infections. The clinical success of monoclonal antibodies has inspired studies in the understanding the fundamental molecular basis of antibody–antigen interactions and engineering of antibodies with enhanced or novel properties. With the emergence of nanomedicine, the application of antibodies as targeting ligands on the surface of nanotechnology-enabled therapeutic modalities has attracted attention owing to the specificity that antibodies impart on nanoparticles. However, studies on assembled antibodies or pure antibody nanoparticles and how they interact with their antigens in biological systems have been limited, largely because of the challenges in preparing well-defined antibody nanoparticles.

In Chapter 2, mesoporous silica template-assisted assembly was employed to fabricate nano-assemblies of therapeutic antibodies, including Herceptin and the antibody-drug conjugate Kadcyla. Briefly, the antibodies were loaded in mesoporous silica templates, followed by crosslinking with poly(ethylene glycol)-*N*-hydroxysuccinimide (PEG-NHS) and removal of the templates. The resultant antibody nano-assemblies (AbNAs) exhibited a well-defined morphology and retained the antigen specificity of the free antibody. Importantly, the particulate nature of the AbNAs significantly induced antibody internalisation via a receptor-mediated mechanism and enhanced subsequent intracellular responses such as cell growth inhibition. The antibody assembly strategy presents opportunities for understanding bio-nano interaction behaviours of well-defined antibody nano-assemblies and the engineering of nano-assemblies with novel or enhanced properties.

In Chapter 3, the selective targeting and stealth properties of AbNAs were engineered by leveraging the intrinsic properties of the PEG crosslinker, such as chain architecture (PEG arm

number and arm length). PEGylated antibody nano-assemblies (PEGx-AbNAs) were prepared using various PEG crosslinkers with different arm lengths and arm numbers and the established mesoporous silica template-assisted method. As observed, AbNAs crosslinked by PEG with a longer arm length had negligible effect on targeting interactions but displayed significant reduction in phagocytic interactions. In contrast, AbNAs crosslinked by PEG with higher arm numbers (up to 8) adversely affected targeting but insignificant changes in phagocytic interactions were observed. This fundamental study suggests that AbNAs optimized with high selectivity and stealth ability can be obtained using PEG crosslinkers with fewer arm numbers but longer arm length, providing guidelines for future implementation of the PEGylation strategy on AbNAs to achieve desirable *in vivo* performance.

In Chapter 4, nano-assemblies with improved selective cellular association were engineered by substituting conventional full-sized monoclonal antibodies with nanobodies—smallest therapeutic antibody fragments. In comparison with AbNAs composed of full-sized antibodies, NanoNAs exhibited significantly enhanced selective association to target cells owing to the large amount of active binding sites present on the particle surfaces. Moreover, NanoNAs showed significantly improved stealth properties when exposed to monocytes *in vitro*, owing to the lack of the Fc domain, hence avoiding Fc receptor-mediated phagocytic capture. The improved stealth behaviour was also observed when NanoNAs were exposed to B cells and granulocytes in human blood. However, NanoNAs could still be captured by most monocytes in human blood, which might be due to the intrinsic properties of the nanobodies and is worth investigating in future studies.

In summary, this thesis has demonstrated for the first time the versatility of the template-assisted platform for the preparation of antibody assemblies that can be widely applied to therapeutic antibodies, antibody-drug conjugates, and antibody fragments. Facile engineering of AbNAs can be achieved by leveraging the intrinsic properties of crosslinkers and

substituting conventional antibodies with advanced new antibody formats. This thesis provides a new perspective to understand and explore the antigen-antibody interactions and bio-nano behaviour of well-defined antibody nanostructures and facilitates the engineering of antibody-based diagnostics and therapeutics with enhanced or novel functions.

Most of the clinically approved antibody therapeutics target extracellular components such as receptors on the cell membrane. There is significant potential for antibodies with intracellular targets to achieve diagnostic and therapeutic applications. However, the successful intracellular delivery of antibodies remains a challenge. The unique internalization properties of the AbNAs present opportunities for the delivery of monoclonal antibodies at high concentrations and/or against intracellular targets. Moreover, the controlled release of antibodies, such as glutathione-induced or pH-induced release, could be achieved using stimuli-responsive crosslinkers, which could allow the disassembly of AbNAs following internalisation in cells to achieve intracellular targeting. Furthermore, hybrid AbNAs composed of a combination of antibodies can lead to multifunctional assemblies with synergistic properties. For example, combining cell membrane-targeting antibodies and intracellular targeting antibodies could achieve site selectivity and intracellular targeting of specific organelles or intracellular components in cells.

In comparison with AbNAs composed of full-sized antibodies, NanoNAs exhibited superior performance in cancer targeting studies owing to their unique structure, which has inspired the exploration of the potential application of NanoNAs for the treatment of other diseases. Recently, an anti-COVID-19 nanobody was developed by our collaborators. The corresponding full-sized IgG molecules that target the same antigen were synthesised by grafting the anti-COVID-19 nanobodies on human IgG molecules. Future work aims to synthesise anti-COVID-19 NanoNAs and the corresponding anti-COVID-19 IgGNAs using the aforementioned molecules and investigate their interactions with the SARS-CoV-2 virus/SARS-CoV-2-infected cells, as well as their association with leucocytes in the absence

or presence of the virus. The potential application of AbNAs and NanoNAs can be extended to a wide range of human therapies beyond cancer targeting and anti-viral treatment.

The studies in this thesis that focused on the application of AbNAs in tumour targeting show promising results *in vitro* and *ex vivo*. Owing to the complexity of the *in vivo* biological environment, *in vitro* models have limitations in simulating the complex biological system and predicting therapeutic efficacy in the human body. It is worth investigating how AbNAs and NanoNAs behave *in vivo* to understand the effect of engineering on their stealth properties, targeting ability, and therapeutic efficacy. Specifically, future work will explore the influence of the PEG crosslinker with different chain architectures on the biodistribution and blood circulation of AbNAs in mice models and further investigate tumour tissue accumulation and tumour growth reduction in tumour-bearing mice models. The *in vivo* studies of AbNAs could provide valuable predictions and information on biodistribution, efficacy, and safety to assess the clinical potential of antibody nano-assemblies.