

Engineering Particle Systems for Pulmonary Delivery

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Abstract

Pulmonary delivery has proven to be a promising delivery route for either local lung targeting or systemic delivery. A variety of particle systems such as polymeric particles and lipid-based particle systems have been developed as therapeutic delivery carriers for pulmonary delivery. However, the majority of current inhaled particles have limited retention time and low bioavailability in the target lung region, leading to suboptimal efficacy of therapeutic delivery and needing increased drug dosage or dose frequency, which could cause severe side effects. This is mainly due to the clearance and metabolic degradation mechanisms in the lungs. The mucociliary clearance and the alveolar macrophage clearance defend the airway and deep lung region, respectively, and are responsible for the elimination of inhaled particles. Therefore, it is important to understand the interactions of particles with the complex lung physiological environment in order to design more effective drug delivery carriers that can overcome various biological barriers or exploit the defence mechanisms to achieve improved biological outcomes.

This PhD thesis focuses on engineering particle systems for pulmonary drug delivery, with specific aims of studying the interactions between inhalable particles and complex biological systems in the lungs including particle–mucus interactions and the role of a pulmonary corona in the uptake or clearance of particles by alveolar macrophages. Poly (ethylene glycol) (PEG) as a low-fouling material commonly used for ‘stealth’ modification of particles to reduce immune clearance was first investigated. The use of PEG building blocks with various architectures resulted in PEG-based particles with different structures and mechanical properties, which further affected the interactions of particles with proteins and immune cells in a complex biological environment (e.g., human blood). The particle–mucus interactions were then studied in the second part of this PhD research by comparing different polymer particles with potentially mucoadhesive and mucus-penetrating properties, obtaining a basic understanding of mucociliary clearance of particles in the lungs. The role of the pulmonary protein corona in alveolar macrophage clearance of polymer particles was then studied. The presence of a protein corona on particles resulted in increased or reduced macrophage uptake depending on the particle properties. When particles were transferred from one biological environment into another (e.g., blood to lungs), the interplay of protein coronas formed in each environment determined the composition of the eventual mixed protein corona and the subsequent particle–cell interactions. Finally, drug (structurally nanoengineered antimicrobial peptide polymers) loading and intracellular delivery using promising polyphenol-based carriers

were investigated as potential antimicrobial therapies against lung infections (e.g., tuberculosis).

Declaration

This is to certify that:

(i) The thesis comprises only my original work towards the degree of Doctor of Philosophy (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.

Jiaying Song

Preface

In Chapter 3, Thakshila Amarasena assisted with the human blood assays and data analysis. Dr. Yi Ju assisted with the synthesis of mesoporous silica (MS) templates and provided helpful discussions with experimental planning. Zhixing Lin provided assistance with the TEM measurements and the design of project scheme. Dr. Srinivas Mettu and Dr. Md. Arifur Rahim performed the mechanical property measurements and analysis. Dr. Jiajing Zhou conducted the template removal.

In Chapter 4, Dr. Yi Ju assisted with the synthesis of mesoporous silica (MS) templates. Dr. Jiajing Zhou conducted the template removal. Dr. Yuxiu Xia provided assistance with the setup of air-liquid interface (ALI) cell culture. Zhixing Lin provided assistance with the TEM measurements and design of the project scheme. Dr. Christina Cortez-Jugo assisted with experimental planning.

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In Chapter 6, Dr. Steven Shirbin conducted the synthesis of structurally nanoengineered antimicrobial peptide polymers (SNAPPs). Zhixing Lin assisted with the TEM and SEM measurements, and design of the project scheme. Yiyuan Han provided assistance with the preparation of SNAPP-TA capsules. Dr. Shuaijun Pan performed the synthesis of calcium carbonate (CaCO_3) particles. Dr. Christina Cortez-Jugo provided helpful discussions with experimental planning.

Publications

The following publications and manuscripts have been produced during the course of this PhD candidature:

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Oral Presentations

The following presentations were delivered during the course of this PhD candidature:

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2. **Song, J.** “Multi-arm Poly(ethylene glycol) Particles with Tunable Mechanical Properties for Bio-nano Interactions” *Nanostructured Interfaces and Materials Science (NIMS) Group Meeting*, **November 2019**, The University of Melbourne, Australia.
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Table of Content

Abstract.....	i
Declaration.....	iii
Preface.....	iv
Publications.....	v
Oral Presentations.....	vi
Acknowledgements.....	vii
Table of Content.....	ix
List of Figures, Schemes and Tables	xv

Chapter 1. Introduction.....	1
1.1 Introduction	3
1.2 Lung Physiology and Lung Diseases	4
1.2.1 Lung Physiology.....	4
1.2.2 Lung Diseases.....	6
1.3 Pulmonary Delivery	10
1.3.1 Particle-Based Pulmonary Delivery	12
1.3.2 Aerosol Delivery Approaches	19
1.4 Biological Barriers in the Lungs	21
1.4.1 Mucociliary Clearance.....	22
1.4.2 Macrophage Clearance	23
1.5 Interactions between Particle Systems and Biological Environments	24
1.5.1 Interactions between Particles and Mucus.....	25
1.5.2 Pulmonary Surfactant and Pulmonary Corona	31
1.6 Scope of Thesis	32
1.7 References	34
 Chapter 2. Instrumentation and Techniques	 51
2.1 Aim.....	53

2.2 Instrumentation and Techniques	53
2.2.1 Differential Interference Contrast (DIC) Microscopy	53
2.2.2 Fluorescence Microscopy	54
2.2.3 Confocal Laser Scanning Microscopy (CLSM)	57
2.2.4 Transmission Electron Microscopy (TEM)	59
2.2.5 Scanning Electron Microscopy (SEM)	60
2.2.6 Atomic Force Microscopy (AFM)	61
2.2.7 Ultraviolet–Visible (UV–vis) Spectroscopy	63
2.2.8 Fluorescence Spectroscopy	64
2.2.9 Dynamic Light Scattering (DLS)	65
2.2.10 Microelectrophoresis	66
2.2.11 Quartz Crystal Microbalance with Dissipation Monitoring (QCM-D)	68
2.2.12 Flow Cytometry	70
2.2.13 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)	71
2.2.14 Mass Spectrometry	72
2.2.15 Nebulisation	74
2.3 References	75

Chapter 3. Engineering Stiffness of Poly(ethylene glycol)-Based Particles

for Regulating Particle–Immune Cell Interactions	78
3.1 Aim	80
3.2 Introduction	80
3.3 Experimental section	82
3.3.1 Materials	82
3.3.2 Synthesis of Mesoporous Silica (MS) Particles	83
3.3.3 Synthesis of MS@PEG and PEG Particles	84
3.3.4 Particle Characterisation	84
3.3.5 Mechanical Property Measurements and Analysis	85
3.3.6 Raw 264.7 Cell Association	86
3.3.7 RAW 264.7 Cell Association Imaging by Confocal Microscopy	87
3.3.8 Human Whole Blood Cell Association	87
3.3.9 Human Washed Blood Cell Association	88
3.3.10 Formation of Biomolecular Corona-Coated Particles	88

3.3.11 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).....	89
3.4 Results and Discussions	89
3.4.1 Synthesis and Characterisation of MS@PEG and PEG Particles	89
3.4.2 Mechanical Property Analysis.....	92
3.4.3 Cell Association.....	93
3.4.4 Protein Corona.....	99
3.5 Conclusions	101
3.6 Supporting Information	102
3.7 References	114

Chapter 4. Mucoadhesive vs. Mucus-Penetrating Particles: Nebulisation

and Interaction with Mucus.....118

4.1 Aim.....	120
4.2 Introduction	120
4.3 Experimental Section	123
4.3.1 Materials	123
4.3.2 Synthesis of Mesoporous Silica (MS) Particles	124
4.3.3 Synthesis of MS@PEG and PEG Particles	124
4.3.4 Poly(methacrylic acid) (PMA) Functionalisation.....	125
4.3.5 Synthesis of MS@PMA _{SH} and PMA _{SH} Particles	125
4.3.6 Particle Characterisation.....	126
4.3.7 Nebulisation of Particles.....	127
4.3.8 Preparation of Artificial Mucus.....	127
4.3.9 Quartz Crystal Microbalance with Dissipation (QCM-D) Analysis	127
4.3.10 Air-Liquid Interface (ALI) Cell Culture.....	128
4.4 Results and Discussions	128
4.4.1 Particle Synthesis and Characterisation.....	128
4.4.2 Nebulisation of Particles.....	130
4.4.3 Particle–Mucus Interactions by QCM-D Analysis.....	132
4.4.4 Particles in ALI Cell Culture	133
4.5 Conclusions	135
4.6 Supporting Information	135
4.7 References	138

Chapter 5. The Interplay of Pulmonary Corona and Plasma Protein

Corona on Macrophage Uptake of Polymer Particles.....142

5.1 Aim.....	144
5.2 Introduction	144
5.3 Experimental Section	147
5.3.1 Materials	147
5.3.2 Synthesis of Mesoporous Silica (MS) Particles	148
5.3.3 Synthesis of MS@PEG and PEG Particles	148
5.3.4 Poly(methacrylic acid) (PMA) Functionalisation.....	149
5.3.5 Synthesis of MS@PMA _{SH} and PMA _{SH} Particles	149
5.3.6 Particle Characterisation.....	150
5.3.7 Collection of Mouse Bronchoalveolar Lavage Fluid (BALF).....	150
5.3.8 Formation of Protein Corona-Coated Particles	151
5.3.9 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).....	151
5.3.10 Proteomics Analysis by Mass Spectrometry	151
5.3.11 Cell Culture.....	153
5.3.12 Cell Association by Flow Cytometry	153
5.3.13 Cell Association by Confocal Microscopy	153
5.4 Results and Discussions	154
5.4.1 Particle Preparation and Characterisation.....	154
5.4.2 Protein Corona Effects on Particle Surface Charge.....	155
5.4.3 Protein Corona Effects on Cell Association	156
5.4.4 Protein Corona Analysis by SDS-PAGE and Mass Spectrometry	161
5.5 Conclusions	166
5.6 Supporting Information	167
5.7 References	172

Chapter 6. Immobilisation and Intracellular Delivery of Structurally

Nanoengineered Antimicrobial Peptide Polymers Using Polyphenol-Based

Capsules 175

6.1 Aim.....	178
6.2 Introduction	178

6.3 Experimental Section	180
6.3.1 Materials	180
6.3.2 Synthesis of Structurally Nanoengineered Antimicrobial Peptide Polymers (SNAPPs)	181
6.3.2.1 Synthesis of D,L-Valine N-Carboxyanhydrides (Val NCA) and (Z)-L-Lysine N- Carboxyanhydrides (Lys NCA)	181
6.3.2.2 Synthesis of Poly(Z-L-Lysine- <i>r</i> -D,L-Valine) _{arm} PAMAM-(NH ₂) _{m,core} Star Peptide Polymers	182
6.3.2.3 Deprotection of Star Peptide Polymers	182
6.3.2.4 FITC Labelling of SNAPP	183
6.3.3 Synthesis of Calcium Carbonate Particles	183
6.3.4 Preparation of SNAPP-Tannic Acid (SNAPP-TA) Capsules	183
6.3.5 Preparation of SNAPP-loaded MPN (SNAPP-Fe ^{III} -TA) Capsules	184
6.3.6 Capsule Characterisation	184
6.3.7 Nebulisation of Capsules	185
6.3.8 SNAPP Loading Capacity of SNAPP-TA and SNAPP-Fe ^{III} -TA Capsules	185
6.3.9 <i>In Vitro</i> SNAPP Release	186
6.3.10 Capsule Permeability Measurements	186
6.3.11 Cell Culture	186
6.3.12 Cell Association Analysis by Flow Cytometry	187
6.3.13 Cell Association Imaging by Confocal Microscopy	187
6.3.14 Cell Internalisation Analysis by Imaging Flow Cytometry	187
6.3.15 Cell Viability Analysis by XTT Assay	188
6.3.16 Co-localisation by Confocal Laser Scanning Microscopy	188
6.3.17 <i>In Vitro</i> Antimicrobial Activity	189
6.4 Results and Discussions	189
6.4.1 Preparation and Characterisation of SNAPP-TA and SNAPP-Fe ^{III} -TA Capsules	189
6.4.2 SNAPP Loading and <i>In Vitro</i> Release	192
6.4.3 <i>In Vitro</i> Cell Interactions Study	193
6.4.4. Intracellular Delivery	195
6.4.5. <i>In Vitro</i> Antimicrobial Activity	196
6.5 Conclusions	198
6.6 Supporting Information	199
6.7 References	208

Chapter 7. Conclusions and Outlook	211
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List of Figures, Schemes and Tables

Figures

Figure 1.1. Schematic illustration of the human respiratory system.....	5
Figure 1.2. Schematic illustration of the human respiratory system in detail.....	5
Figure 1.3. Organ distribution of liposomes (A) and nanostructured lipid carriers (NLC, B) after intravenous and inhalation delivery.....	11
Figure 1.4. A timeline of the development of nanoengineered particle systems for biomedical applications.....	13
Figure 1.5. Schematic illustration of key physicochemical properties of nanoengineered particles that affect the drug delivery efficacy.	14
Figure 1.6. Factors affecting drug metabolism, adsorption and clearance in the lungs.....	21
Figure 1.7. (A) general schematic representation of mucus secretion from goblet cells and submucosal glands; (B) schematic representation of the generally accepted structure of airway surface liquid.....	22
Figure 1.8. Schematic illustration of the immune responses in the alveoli.....	24
Figure 1.9. Timeline illustration of some examples of bio-nano interactions studied using particles assembled through templating methods in the Caruso group.....	25
Figure 1.10. Schematic illustration of the fate of conventional mucoadhesive particles (CP) and mucus-penetrating particles (MPP) after administration to a mucosal surface. CP are mostly immobilised in the luminal mucus layer, while MPP tend to penetrate through the luminal mucus layer and enter the underlying adherent mucus layer.....	26
Figure 1.11. (a) Mucus disruption induced by particles. A mucus fibre network is depicted on the left with the introduction of particles leading to their entanglement with mucus resulting in a change of the fibre network. (b) The potential effects of mucoadhesive particles on the mucus structure. Mucoadhesive particles can increase mucus pore sizes by bundling mucin fibres with adhesive interactions.....	27

Figure 1.12. A schematic illustration of a Transwell diffusion system containing A: acceptor compartment; B: semipermeable membrane; C: mucus layer; D: donor compartment.....	29
Figure 1.13. Scheme of the experiment setup to study particle–mucus interactions by QCM-D.....	29
Figure 1.14. The movement of each particle (P) was captured by video as individual particle trajectories (A) over a minimum of 30 consecutive frames with the criterion that any individual particle tracked must display a continuous presence in the X – Y plane throughout the respective 30 sequential frames. For example, P1 in (A) above tracked from (Δt_1) to (Δt_{30}) . The distance moved in the X – Y trajectory by each particle over a time interval (Δt) , i.e. camera frame rate, is expressed as a squared displacement (SD) (B), $SD = (X_{\Delta t})^2 + (Y_{\Delta t})^2$ ($\Delta t_{n = 1-30}$).....	30
Figure 1.15. Schematic illustration of (A) submerged and (B) air-liquid interface culture of primary human bronchial epithelial cells grown on porous culture inserts.....	30
Figure 1.16. Formation of protein corona on particle surface and its effect on immunological identity and targeting ability of nanoparticles.....	31
Figure 1.17. Schematic illustration of plasma corona and pulmonary surfactant corona.....	32
Figure 3.1. Fluorescence microscopy, TEM and AFM images of (a1–a3) 3-arm PEG particles, (b1–b3) 4-arm PEG particles, (c1–c3) 6-arm PEG particles and (d1–d3) 8-arm PEG particles. Scale bars are 10 μ m in (a1–d1) and 2 μ m in (a2–d2) and (a3–d3).....	90
Figure 3.2. Zeta potential of MS particles, MS@PEG particles and PEG particles in phosphate buffer (pH 7.4, 5 mM). Data are shown as the mean $\pm$ standard error of at least three independent experiments (Student t -test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ versus the column of ‘MS’).....	92
Figure 3.3. (a) Schematic illustration of the elastic modulus measurements between a glass bead probe and PEG particles by CP-AFM. (b) Representative force–deformation (F – δ) curves of 3-arm PEG, 4-arm PEG, 6-arm PEG and 8-arm PEG particles. The F – δ curve of a glass substrate is also shown for calibration. (c) Young’s modulus derived from the F – δ analysis for the PEG particles.....	93
Figure 3.4. RAW 264.7 cell association of (a) MS@PEG and PEG particles after 12 h of incubation at a particle-to-cell ratio of 100:1 and (b) PEG particles after 24 h of incubation at	

a particle-to-cell ratio of 100:1 and 1000:1. Data are shown as the mean $\pm$ standard error of at least three independent experiments. Statistical data are presented in Figure S3.6.....94

Figure 3.5. Confocal microscopy images of RAW 264.7 cells after incubation with AF488-labelled (a1) MS@3-arm PEG, (b1) MS@4-arm PEG, (c1) MS@6-arm PEG, (d1) MS@8-arm PEG, (a2) 3-arm PEG, (b2) 4-arm PEG, (c2) 6-arm PEG, or (d2) 8-arm PEG particles (green) for 12 h at a particle-to-cell ratio of 100:1. The cell membrane was stained with wheat germ agglutinin Alexa Fluor 594 conjugate (WGA-594, red) and the nuclei were stained with Hoechst 33342 (blue). Scale bars are 20 μ m.....95

Figure 3.6. Cell association of (a) MS@PEG and (b) PEG particles with granulocytes, monocytes and B cells after incubation in human whole blood for 1 h at varying particle-to-cell ratios. Data are shown as the mean $\pm$ standard error of three independent experiments, with at least 100 000 leukocytes analysed in each individual experiment. Cell associations with the other blood cell populations, i.e. NK cells, T cells and dendritic cells, are shown in Figure S3.8.....97

Figure 3.7. Cell association of (a) MS@PEG and (b) PEG particles with granulocytes, monocytes, and B cells after incubation in (1) human whole blood, (2) blood plasma followed by human washed blood or (3) human washed blood. Based on the different incubation conditions, either a ‘hard’ and ‘soft’ corona (1 and 2) or ‘no’ corona (3) was formed on the particles. The particle-to-cell ratio was set at 10:1 for MS@PEG particles and 100:1 for PEG particles. Data are shown as the mean $\pm$ standard error of three independent experiments, with at least 100 000 leukocytes analysed in each individual experiment. Control refers to the cell populations without particle treatment in the incubation medium.....99

Figure 3.8. (a) Fluorescence intensity and (b) zeta potential of MS@PEG and PEG particles without or with protein corona derived from human blood plasma. Data are shown as the mean $\pm$ standard error of at least three independent experiments (Student *t*-test, ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).....101

Figure S3.1. Molecular structure of (a) building blocks: 3-arm-PEG-NH₂, 4-arm-PEG-NH₂, 6-arm-PEG-NH₂ and 8-arm-PEG-NH₂, and (b) crosslinker: 8-arm-PEG-NHS.....102

Figure S3.2. (a) SEM and (b) TEM images and (c) size distribution (determined by dynamic light scattering) of MS particles.....103

Figure S3.3. Representative AFM images and corresponding height profiles of (a) 3-arm PEG, (b) 4-arm PEG, (c) 6-arm PEG and (d) 8-arm PEG particles. Scale bars are 2 μm	104
Figure S3.4. Fluorescence intensity of AF488-labelled PEG particles.....	105
Figure S3.5. (a) Optical microscopy image of the silica colloidal probe used for the CP-AFM force measurements. Fluorescence microscopy images of AF488-labelled 8-arm PEG particles at the base of a FluoroDish (b) before and (c) after the force measurements. Scale bars are 50 μm in (a) and 20 μm in (b) and (c).....	105
Figure S3.6. RAW 264.7 cell association of (a) MS@PEG and PEG particles after 12 h of incubation at a particle-to-cell ratio of 100:1 and (b) PEG particles after 24 h of incubation at a particle-to-cell ratio of 100:1 or 1000:1. Data are shown as the mean $\pm$ standard error of at least three independent experiments (Student <i>t</i> -test, ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).).....	106
Figure S3.7. (a) Gating method used to identify white blood cell populations. Side and forward scatter were first applied to gate white blood cells before doublets were excluded. The specific cell types were identified according to expression of surface markers: CD66b ⁺ granulocytes; CD14 ⁺ monocytes; CD19 ⁺ B cells; CD56 ⁺ NK cells; CD3 ⁺ T cells; and HLA-DR PE-CF594 dendritic cells. (b) Percentage of each cell type positive for the AF647-labelled particles was then measured and an example of the gating and particle association values for one sample is shown.....	107
Figure S3.8. Cellular association of (a) MS@PEG and (b) PEG particles with NK cells, T cells and dendritic cells after incubation in human whole blood for 1 h at varying particle-to-cell ratios. Data are shown as the mean $\pm$ standard error of three independent experiments, with at least 100 000 leukocytes analysed in each individual experiment.....	108
Figure S3.9. Association of MS@PEG particles with granulocytes, monocytes and B cells after incubation in human washed blood at a particle-to-cell ratio of 10:1. Data are shown as the mean $\pm$ standard error of three independent experiments, with at least 100 000 leukocytes analysed in each individual experiment (Student <i>t</i> -test, ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).....	109
Figure S3.10. Fluorescence intensity distribution of MS@PEG and PEG particles without or with protein corona, measured by flow cytometry (Apogee flow).....	110

Figure S3.11. Fluorescence intensity distribution of (a) MS@PEG and (b) PEG particles without protein corona, (c) MS@PEG and (d) PEG particles with protein corona, measured by flow cytometry (Apogee flow).....	111
Figure S3.12. SDS-PAGE image of proteins separated from ‘hard’ corona of MS@PEG and PEG particles after 1 h of incubation in human blood plasma at 37 °C. The reference bands related to particular molecular weights are shown on the left in the image as indicated by an arrow. The results of blood plasma are shown on the right.....	112
Figure 4.1. Fluorescence microscopy images of AF488-labelled MS@PMA _{SH} , PMA _{SH} , MS@PEG and PEG particles before (a1–d1) and after (a2–d2) nebulisation. The scale bars are 10 µm.....	131
Figure 4.2. TEM images of MS@PMA _{SH} , PMA _{SH} , MS@PEG and PEG particles before (a1–d1) and after (a2–d2) nebulisation. The scale bars are 2 µm.....	131
Figure 4.3. Size distribution of MS@PMA _{SH} , PMA _{SH} , MS@PEG and PEG particles before and after nebulisation determined by DLS.....	132
Figure 4.4. Changes in frequency (Δf) with time recorded by QCM-D for interactions between artificial mucus with PMA _{SH} particles and PEG particles. The frequency values quoted are for the third overtone only; the fifth, seventh, and ninth overtones all followed similar trends...	133
Figure 4.5. Confocal microscopy images of ALI cell culture treated with (a) MS@PMA _{SH} , (b) PMA _{SH} , (c) MS@PEG and (d) PEG particles (green), captured as a volume view. The nuclei and mucus (blue) were stained with Hoechst 33342. The scale bars are 50 µm.....	134
Figure S4.1. (a) SEM and (b) TEM image of 800 nm MS particles. (c) TEM image of 400 nm MS particles.	135
Figure S4.2. (a) Size distribution and (b) zeta potential of 400 nm and 800 nm mesoporous silica particles with or without amine group modification (denoted as MS-NH ₂ and MS, respectively).....	136
Figure S4.3. Aerosol size distribution of nebulised water, and MS@PMA _{SH} , PMA _{SH} , MS@PEG and PEG particle suspensions as evaluated by laser diffraction.....	136
Figure 5.1. Fluorescence microscopy images of AF488 fluorescently labelled (a1) MS@PMA _{SH} , (b1) PMA _{SH} , (c1) MS@PEG and (d1) PEG particles. TEM images of (a2)	

MS@PMA_{SH}, (b2) PMA_{SH}, (c2) MS@PEG and (d2) PEG particles. Scale bars are 10 μ m in (a1–d1), and 1 μ m in (a2–d2). Modified from Figure 4.1 and 4.2.....155

Figure 5.2. (a) Zeta potential and (b) corresponding statistical analysis of MS@PMA_{SH}, PMA_{SH}, MS@PEG or PEG particles without a protein corona (PC) (A: bare) or with different protein coronas (B: PC-Plasma; C: PC-Lung; D: PC-Plasma+lung; and E: PC-Lung+plasma). Data are shown as the mean $\pm$ standard error of at least three independent experiments. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (one-way ANOVA with Tukey's multiple comparisons test).....156

Figure 5.3. (a) Cell association and (b, c) corresponding statistical analysis of MS@PMA_{SH}, PMA_{SH}, MS@PEG or PEG particles without a protein corona (PC) (bare, A) or with different protein coronas (B: PC-Plasma; C: PC-Lung; D: PC-Plasma+lung; and E: PC-Lung+plasma) with MH-S cells and RAW 264.7 cells after 2 h incubation at a particle-to-cell ratio of 100:1. Data are shown as the mean $\pm$ standard error of at least three independent experiments. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (one-way ANOVA with Tukey's multiple comparisons test).....159

Figure 5.4. Confocal microscopy images of MH-S cells after incubation with AF488-labelled MS@PMA_{SH}, PMA_{SH}, MS@PEG or PEG particles without a protein corona (PC) (bare) or with different protein coronas (PCs) (PC-Plasma, PC-Lung, PC-Plasma+lung, and PC-Lung+plasma) (green) for 2 h at a particle-to-cell ratio of 100:1. The cell membranes were stained with Alexa Fluor 594-conjugated wheat germ agglutinin (WGA-594, red), and the nuclei were stained with Hoechst 33342 (blue). Scale bars are 20 μ m.....160

Figure 5.5. Confocal microscopy images of RAW 264.7 cells after incubation with AF488 fluorescently labeled MS@PMA_{SH}, PMA_{SH}, MS@PEG or PEG particles without a protein corona (PC) (bare) or with different protein coronas (PCs) (PC-Plasma, PC-Lung, PC-Plasma+lung, and PC-Lung+plasma) (green) for 2 h at a particle-to-cell ratio of 100:1. The cell membranes were stained with Alexa Fluor 594-conjugated wheat germ agglutinin (WGA-594, red), and the nuclei were stained with Hoechst 33342 (blue). Scale bars are 20 μ m.....161

Figure 5.6. SDS-PAGE images of separated corona proteins derived from (a) MS@PMA_{SH}, (b) PMA_{SH}, (c) MS@PEG and (d) PEG particles. A = PC-Plasma, B = PC-Lung, C = PC-Lung+plasma, and D = PC-Plasma+lung.....163

Figure 5.7. The top 20 most abundant proteins found in plasma+lung protein corona and lung+plasma protein corona on (a) MS@PMA _{SH} particles and (b) PMA _{SH} particles. Data represent the mean value $\pm$ standard deviation ($n = 3$). ** $p < 0.01$, **** $p < 0.0001$ (two-way ANOVA with Tukey's multiple comparisons test).....	164
Figure 5.8. The top 20 most abundant proteins found in plasma+lung protein corona and lung+plasma protein corona on (a) MS@PEG particles and (b) PEG particles. Data represent the mean value $\pm$ standard deviation ($n = 3$). * $p < 0.05$, **** $p < 0.0001$ (two-way ANOVA with Tukey's multiple comparisons test).....	165
Figure S5.1. (a) SEM and (b) TEM images of MS particles.....	167
Figure S5.2. Size distribution of MS particles with or without amine group modification (denoted as MS-NH ₂ and MS, respectively) measured by dynamic light scattering (DLS)....	167
Figure S5.3. Size distribution of MS@PMA _{SH} , PMA _{SH} , MS@PEG and PEG particles measured by dynamic light scattering (DLS).....	167
Figure S5.4. Bovine serum albumin (BSA) standard curve produced using a Micro BCA protein assay. Data represent the mean value $\pm$ standard deviation ($n = 3$).....	168
Figure S5.5. Heatmap of identified proteins from plasma+lung protein corona or lung+plasma protein corona on MS@PMA _{SH} particles. Data represent the mean value from at least two replicates. Blank cells refer to absence of proteins.....	169
Figure S5.6. Heatmap of identified proteins from plasma+lung protein corona or lung+plasma protein corona on MS@PEG particles. Data represent the mean value from at least two replicates. Blank cells refer to absence of proteins.....	170
Figure S5.7. Heatmap of identified proteins from plasma+lung protein corona or lung+plasma protein corona on MS@PMA _{SH} , PMA _{SH} , MS@PEG or PEG particles. Data represent the mean value from at least two replicates.	171
Figure 6.1. DIC microscopy, fluorescence microscopy, TEM, SEM, and AFM images of (a1–a5) ^L SNAPP-TA capsules and (b1–b5) ^L SNAPP-Fe ^{III} -TA capsules. Scale bars are 10 μ m in (a1, a2, b1, b2) and 2 μ m in (a3–a5 and b3–b5).....	191
Figure 6.2. (a) Loading amount and (b) loading efficiency of FITC-labelled ^L SNAPP in ^L SNAPP-TA or ^L SNAPP-Fe ^{III} -TA capsules as a function of ^L SNAPP loading concentration. <i>In vitro</i> time-dependent release of FITC-labelled ^L SNAPP from (c) ^L SNAPP-Fe ^{III} -TA capsules or	

(d) ^LSNAPP-TA capsules at pH 3.0, 4.5, 7.4 and 9.0. Data presented are averages of three replicates, and error bars represent standard deviations.....193

Figure 6.3. *In vitro* cell interactions study. (a) Association of ^LSNAPP-TA or ^LSNAPP-Fe^{III}-TA capsules with MH-S cells following incubation for 8 or 24 h at 37 °C and a capsule-to-cell ratio of 50:1. Imaging flow cytometry of the quantification of the internalisation of (b) ^LSNAPP-TA and (c) ^LSNAPP-Fe^{III}-TA capsules in MH-S cells after incubation for 24 h at 37 °C at a capsule-to-cell ratio of 50:1. The capsules were fluorescently labelled by loading with FITC-labelled ^LSNAPP (green) and the cell membranes were stained with WGA-594 (orange). The degree of cell internalisation was expressed as an internalisation factor. The insets show the representative images of cells with internalised capsules (positive IF). Confocal microscopy images of the MH-S cells treated in the (d) absence of capsules (control) and (e, g) presence of ^LSNAPP-TA or (f, h) ^LSNAPP-Fe^{III}-TA capsules for 8 h (e, f) or 24 h (g, h) at 37 °C at a capsule-to-cell ratio of 50:1. The capsules were fluorescently labelled by loading with FITC-labelled ^LSNAPP (green). The cell membranes were stained with WGA-594 (red) and the nuclei were stained with Hoechst 33342 (blue). Scale bars are 10 µm in (d–h). 194

Figure 6.4. (a) Intracellular distribution of ^LSNAPP-TA and ^LSNAPP-Fe^{III}-TA capsules in MH-S cells after 24 h incubation at 37 °C at a capsule-to-cell ratio of 50:1, as evaluated by confocal microscopy. The capsules were fluorescently labelled by loading with FITC-labelled ^LSNAPP (green). The endo/lysosomes were stained with LysoTracker Red (red). The nuclei were stained with Hoechst 33342 (blue). The cellular boundaries were represented by dashed lines according to the corresponding bright-field images. Scale bars are 10 µm. (b) Representative colour scatter plots of ^LSNAPP-TA and ^LSNAPP-Fe^{III}-TA capsules vs endo/lysosomes. (c) Corresponding Pearson's correlation coefficient (PCC) values of ^LSNAPP-TA and ^LSNAPP-Fe^{III}-TA capsules vs endo/lysosomes (mean ± standard deviation, *n* > 50). **** *p* < 0.0001 from the unpaired *t*-test.....196

Figure 6.5. Relative growth (%) of *E. coli* as a function of the concentration of ^LSNAPP released from (a) ^LSNAPP-TA or (b) ^LSNAPP-Fe^{III}-TA capsules. The MIC, defined as the lowest concentration of SNAPP that inhibited 99% of bacterial growth (i.e., the *X* value at *Y* = 1%), was determined from the fitted exponential regression. Data represent the mean value ± standard deviation (*n* = 3). 198

Figure S6.1. Zeta potential of SNAPP (^L SNAPP and ^S SNAPP), SNAPP-TA and SNAPP-Fe ^{III} -TA capsules prepared from ^L SNAPP and ^S SNAPP at different pH (mean ± standard deviation, <i>n</i> = 3).	200
Figure S6.2. (a) SEM and (b) TEM images of CaCO ₃ particles showing their porous surface structure.	200
Figure S6.3. (a) DIC microscopy, (b) fluorescence microscopy, (c) TEM, (d) SEM and (e) AFM images of thick ^L SNAPP-Fe ^{III} -TA capsules (thickness of 269 ± 26 nm). Scale bars are 10 μm in (a, b) and 2 μm in (c–e).	200
Figure S6.4. (a, b) Fluorescence microscopy and (c, d) TEM images of ^L SNAPP-TA (a, c) and ^L SNAPP-Fe ^{III} -TA (b, d) before nebulisation. (e, f) Fluorescence microscopy and (g, h) TEM images of ^L SNAPP-TA (e, g) and ^L SNAPP-Fe ^{III} -TA (f, h) after nebulisation. Scale bars are 10 μm in (a, b, e, f), and 2 μm in (c, d, g, h).	201
Figure S6.5. <i>In vitro</i> time-dependent release of ^L SNAPP from thick ^L SNAPP-Fe ^{III} -TA capsules (thickness of 269 ± 26 nm) at pH 3.0, 4.5, 7.4 and 9.0. Data presented are averages of three replicates and error bars represent standard deviations.....	201
Figure S6.6. Loading amount of FITC-labelled ^L SNAPP and ^S SNAPP in SNAPP-TA and SNAPP-Fe ^{III} -TA capsules as a function of SNAPP loading concentration. Data presented are averages of three replicates and error bars represent standard deviations.....	202
Figure S6.7. <i>In vitro</i> time-dependent release of FITC-labelled ^S SNAPP from (a) ^S SNAPP-Fe ^{III} -TA capsules, (b) thick ^S SNAPP-Fe ^{III} -TA capsules and (c) ^S SNAPP-TA capsules at pH 3.0, 4.5, 7.4 and 9.0. Data presented are averages of three replicates and error bars represent standard deviations.....	202
Figure S6.8. Molecular weight-dependent permeability of ^L SNAPP-Fe ^{III} -TA capsules and ^L SNAPP-TA capsules at different pH. Representative confocal microscopy images of (a) ^L SNAPP-Fe ^{III} -TA capsules and (b) ^L SNAPP-TA capsules incubated with FITC-dextran (3–5, 59–77 and 2000 kDa) at pH 4.5. Scale bars are 5 μm. The percentage of permeable (c) ^L SNAPP-Fe ^{III} -TA capsules and (d) ^L SNAPP-TA capsules plotted as a function of the molecular weight of FITC-dextran.....	203
Figure S6.9. UV–vis absorbance standard curve of (a) FITC-labelled ^L SNAPP and (b) ^S SNAPP in Milli-Q water.	203

Figure S6.10. Fluorescence intensity standard curves of FITC-labelled ^L SNAPP at different pH (the measurement parameter was adjusted to achieve a reasonably high fluorescence intensity with the detectable range).	204
Figure S6.11. Fluorescence intensity standard curves of FITC-labelled ^S SNAPP at different pH (the measurement parameter was adjusted to achieve a reasonably high fluorescence intensity with the detectable range).	205
Figure S6.12. Association of SNAPP-TA and SNAPP-Fe ^{III} -TA capsules prepared from ^L SNAPP or ^S SNAPP with MH-S cells after incubation for 24 h at 37 °C at a capsule-to-cell ratio of 50:1 (mean ± standard deviation, <i>n</i> = 3).....	206
Figure S6.13. Cell (MH-S) cytotoxicity of SNAPP-TA and SNAPP-Fe ^{III} -TA capsules prepared from ^L SNAPP or ^S SNAPP after incubation at 24 h at 37 °C at a capsule-to-cell ratio of 10:1 or 50:1. Cell viability was evaluated by XTT assay (mean ± standard deviation, <i>n</i> = 3). The viability of cells treated in the absence of capsules was normalised to 100%.....	206
Figure S6.14. Growth curves of <i>E. coli</i> in the absence and presence of (a) ^L SNAPP and (c) ^S SNAPP at different concentrations. Relative OD ₆₀₀ at 4 h of these samples compared to the control (0 µg mL ⁻¹ , 100%) was fitted to an exponential function, of which the <i>X</i> value at <i>Y</i> = 1% was established as the MIC of (b) ^L SNAPP and (d) ^S SNAPP (mean ± standard deviation, <i>n</i> = 3).....	207

Schemes

Scheme 2.1. Simplified illustration of the basic components and principle of a DIC microscope.....	54
Scheme 2.2. Simplified Jablonski diagram of fluorescence photoluminescence. An electron is excited by an excitation light, followed by internal conversion and vibrational relaxation to the lowest excited state (S1) owing to instability at the high-energy state. Further relaxation to the ground state (S0) results in the emission of fluorescence light.	55
Scheme 2.3. Simplified illustration of the basic components and principle of a fluorescence microscope.....	56

Scheme 2.4. Simplified illustration of the basic components and principle of a DIC/fluorescence microscope configuration.....	57
Scheme 2.5. Simplified illustration of the setup of a confocal laser scanning microscope.....	58
Scheme 2.6. Simplified illustration of the setup of a transmission electron microscope.....	60
Scheme 2.7. Simplified illustration of the setup of a scanning electron microscope.....	61
Scheme 2.8. Typical configuration of an atomic force microscope.....	62
Scheme 2.9. Simplified illustration of a typical UV–vis spectrophotometer.....	64
Scheme 2.10. Simplified illustration of a typical fluorometer.....	65
Scheme 2.11. Simplified illustration of DLS measurement of two particle systems comprising particles of different sizes.....	66
Scheme 2.12. Illustration of the distribution of ions within an electrical double layer around a charged particle in solution. The zeta potential is measured at the slipping plane.....	68
Scheme 2.13. Illustration of the working principle of QCM-D.	69
Scheme 2.14. Simplified illustration of a typical flow cytometry system.....	70
Scheme 2.15. Illustration of the setup of gel electrophoresis to separate biomolecules.....	71
Scheme 2.16. Simplified illustration of basic SDS-PAGE procedure.....	72
Scheme 2.17. Simplified illustration of the setup of a mass spectrometer.....	74
Scheme 2.18. A picture of a PARI air-jet nebuliser, reproduced from Trek® S Portable Aerosol System, PARI, USA.....	75
Scheme 3.1. (a) Schematic illustration of the assembly of MS@PEG (template present) and PEG particles (template removed) using different PEG building blocks (3-arm PEG, M_w 15 kDa; 4-arm PEG, M_w 20 kDa; 6-arm PEG, M_w 30 kDa; and 8-arm PEG, M_w 40 kDa) via MS particle templating. (b) Human blood assays: MS@PEG particles and PEG particles were incubated in (1) human whole blood (with plasma proteins), (2) human blood plasma followed by washed blood (without plasma proteins) or (3) human washed blood (without plasma proteins) and their cellular association with human immune cells was subsequently examined.	82

Scheme 4.1. (a) Schematic illustration of the assembly of MS@PMA_{SH} particles (template present), PMA_{SH} particles (template removed), MS@PEG particles (template present) and PEG particles (template removed) via MS particle templating. (b) Schematic illustration of the nebulisation process of particle suspensions. The PARI air-jet nebuliser figure was reproduced from Trek® S Portable Aerosol System, PARI, USA. (c) Schematic illustration of the particle–mucus interactions in ALI cell culture.....123

Scheme 5.1 (a) Schematic illustration of the assembly of MS@PMA_{SH} particles (template present), PMA_{SH} particles (template removed), MS@PEG particles (template present) and PEG particles (template removed) via mesoporous silica (MS) particle templating. Adapted from Scheme 4.1. (b) Schematic illustration of the experimental setup: MS@PMA_{SH}, PMA_{SH}, MS@PEG, and PEG particles were incubated in (i) plasma, (ii) BALF, (iii) plasma followed by BALF, or (iv) BALF followed by plasma, to form (i) plasma protein corona-coated particles (denoted as PC-Plasma), (ii) lung protein corona-coated particles (denoted as PC-Lung), (iii) plasma plus lung protein corona-coated particles (denoted as PC-Plasma+lung), or (iv) lung plus plasma protein corona-coated particles (denoted as PC-Lung+plasma), respectively, followed by incubating with mouse alveolar macrophage MH-S cells or murine monocyte/macrophage-like RAW 264.7 cells.....146

Scheme 6.1. (a) Schematic of the synthesis of a SNAPP via ring-opening polymerisation (ROP), resulting in the formation of star-shaped polypeptide nanoparticles containing cationic lysine and hydrophobic valine amino acid residues. (b) Schematic depicting the immobilisation and encapsulation of SNAPPs by the assembly of SNAPP-TA and SNAPP-loaded MPN (SNAPP-Fe^{III}-TA) capsules on sacrificial calcium carbonate particle templates.....180

Scheme S6.1. Schematic illustrations of (a) the structure of a generation 2 PAMAM dendrimer (G0 = generation 0; G1 = generation 1; G2 = generation 2) and (b) the structure of ^LSNAPP (M_w 54.0 kDa) and ^SSNAPP (M_w 3.7 kDa).....199

Tables

Table 1.1. Antibiotics used in treatment of TB.....9

Table 1.2. Examples of inhaled medicines.....12

Table 1.3. List of FDA-approved nanomedicines stratified by material category by 2016.....15

Table 1.4. Different active pharmaceutical ingredient (API) molecules incorporated into different particle systems for pulmonary applications.....	18
Table 1.5. Comparison of nebuliser, pMDI and DPI.....	20
Table 1.6. Examples of pulmonary delivery of nanoparticles using dry powder carriers.....	20
Table 1.7. Examples of pulmonary delivery of nanoparticle suspensions using nebulisation...	20
Table 3.1. Average size and thickness of PEG particles in aqueous solution or in dry state.....	91
Table S3.1. Cell count data of human whole blood (before wash) and human washed blood (after wash) for the cell association experiments displayed in Figure 3.6, Figure 3.7 and Figure S3.8.....	113
Table S3.2. Properties of the antibodies used in the human blood assays.....	113
Table S4.1. Droplet size of different particle suspensions.....	137
Table S4.2. Components in complete media vs minimal media for the ALI cell culture.....	137

Chapter 1

Introduction

Chapter 1. Introduction.....	1
1.1 Introduction	3
1.2 Lung Physiology and Lung Diseases	4
1.2.1 Lung Physiology.....	4
1.2.2 Lung Diseases.....	6
1.3 Pulmonary Delivery	10
1.3.1 Particle-Based Pulmonary Delivery	12
1.3.2 Aerosol Delivery Approaches	19
1.4 Biological Barriers in the Lungs	21
1.4.1 Mucociliary Clearance.....	22
1.4.2 Macrophage Clearance	23
1.5 Interactions between Particle Systems and Biological Environments	24
1.5.1 Interactions between Particles and Mucus.....	25
1.5.2 Pulmonary Surfactant and Pulmonary Corona	31
1.6 Scope of Thesis	32
1.7 References	34

1.1 Introduction

The lungs, as a portal entry into the body with the special structures and natures including large surface area suitable for effective absorption, low thickness of the epithelial barrier allowing for rapid penetration, extensive vascularisation and relatively low proteolytic activity in the alveolar region, represent an promising delivery route of pharmaceuticals either for local lung targeting or systemic circulation.¹⁻² Compared to other administration routes such as oral delivery and intravenous administration route, pulmonary delivery can achieve a more rapid onset of drug action, potentially enhanced delivery efficacy and thereby reduced overall drug dosage, decreased systemic toxicity and less severe side effects.³ However, as a major defence organ, the lungs have developed highly efficient clearance mechanisms, including mucociliary clearance, macrophage clearance, and metabolic degradation to protect the body by eliminating foreign substances from entering the systemic circulation, resulting in limited retention time and reduced treatment outcomes of inhaled therapeutics.⁴

Over the past decades, due to the rapid development of material science and nanotechnology, various nanoengineered materials with unique properties and functions have been developed for medical diagnosis, medical imaging, and as drug or gene delivery carriers for disease prevention and treatment.⁵⁻⁷ Particle-based pulmonary delivery, compared to conventional therapeutic formulations, can protect the encapsulated pharmaceuticals against enzymatic and hydrolytic degradation or inactivation. In addition, controlled drug release over prolonged time can be achieved, biological barriers can be overcome, and targeting to specific structures or cells may be possible by manipulating the physicochemical properties of the particles including size, shape, surface chemistry and elasticity.^{5, 8-9}

This thesis will focus on engineering particle systems for pulmonary delivery, with specific aims of investigating the bio-nano interactions between distinct particle systems and complex biological environments, including immune defence, particle-mucus interactions and the effects of protein corona on particle identity and downstream biological responses, to explore the potential biomedical applications of promising particle candidates in pulmonary delivery.

In this chapter, relevant literature is reviewed aiming to provide an overview of : 1) lung physiology and lung diseases (section 1.2); 2) pulmonary delivery, including particle carriers and aerosolisation approaches (section 1.3); 3) biological barriers in the lungs, including mucociliary clearance and macrophage clearance (section 1.4); and 4) interactions between particle systems and biological environments, including particles-mucus interactions and the

effect of pulmonary corona derived from pulmonary surfactant environment in deep lungs (section 1.5).

1.2 Lung Physiology and Lung Diseases

The lungs are a major organ of the respiratory system, responsible for performing gas exchange between the external environment and the internal bloodstream.¹⁰ Due to constant exposure to chemicals and particulates such as pollutants, allergens, and pathogens in ambient air, the lungs are vulnerable to infection and injury, leading to unhealthy lung conditions.¹¹ According to the World Health Organisation (WHO), respiratory diseases make up five of the top 30 common causes of death globally: chronic obstructive pulmonary disease (COPD) is the third, leading to about 3 million deaths each year; lower respiratory tract infection is the fourth, causing nearly 4 million deaths annually; tracheal, bronchial and lung cancer is the sixth, which is the most common cancer worldwide killing 1.6 million people each year; tuberculosis (TB) is the twelfth, causing 1.4 million people died from it in 2015; and asthma is the twenty-eighth with about 334 million people suffering from it.¹²⁻¹⁵ This section provides an overview of lung physiology and lung diseases, which is fundamental background for the development of effective pulmonary therapies and drug delivery carriers.

1.2.1 Lung Physiology

The lower respiratory system can be functionally separated into a conducting zone and a respiratory zone, as shown in Figure 1.1. The conducting zone (also known as upper airways) consisting of oropharynx, larynx, trachea, bronchi, bronchioles and terminal bronchioles is a continuous passageway for air to be inhaled into and exhaled out of the lungs. The respiratory zone (also known as lower airways) is where gas exchange takes place, consisting of the respiratory bronchioles, alveolar ducts and alveolar sacs (alveoli). More detailed structures are shown in Figure 1.2. The unique structural design of the respiratory system maximises delivery of air to each part of the lungs and provides the maximal surface area for gas exchange, to ensure that the breathing process meets the oxygen demands of the body.¹⁰

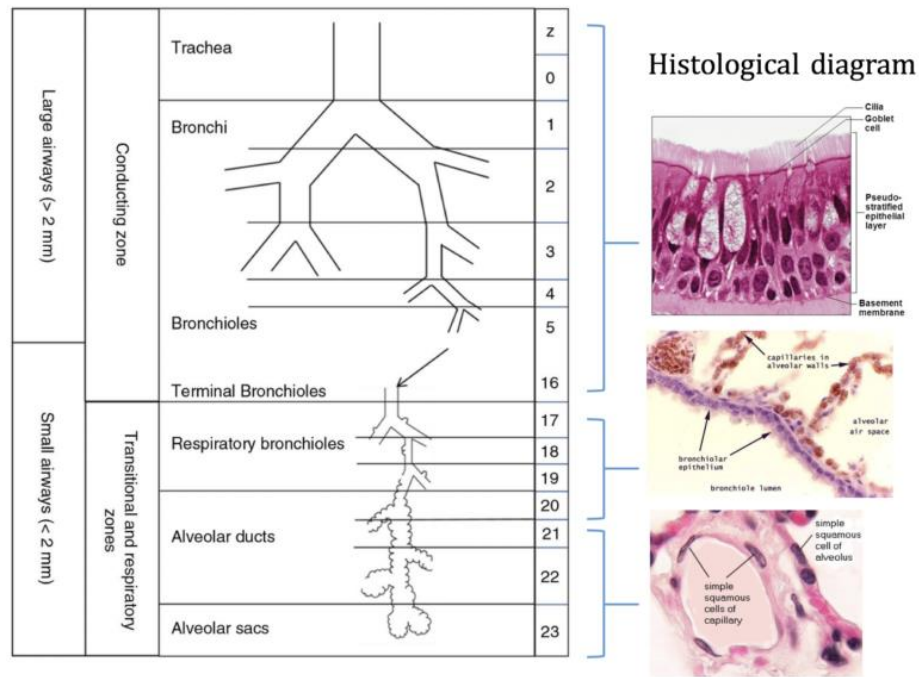


Figure 1.1. Schematic illustration of the human respiratory system. The figure is adapted from reference 16.

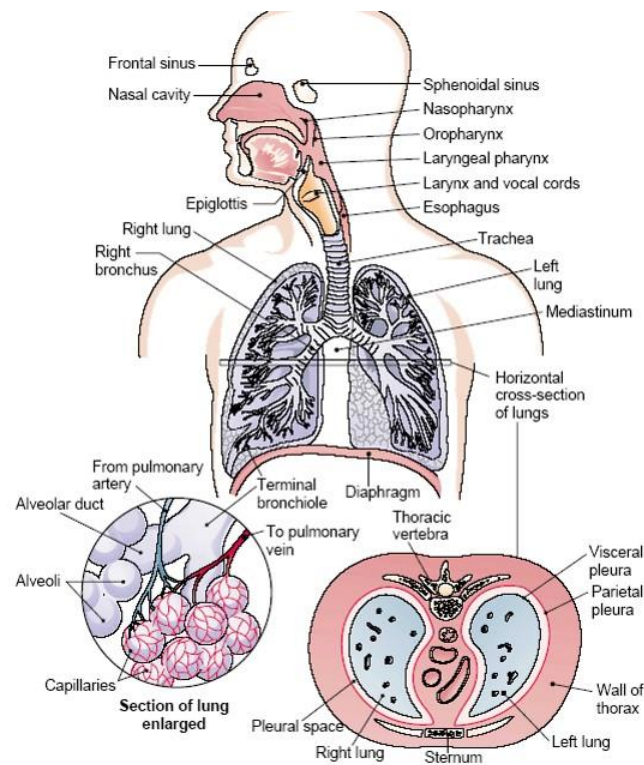


Figure 1.2. Schematic illustration of the human respiratory system in detail. The figure is adapted from reference 17.

1.2.2 Lung Diseases

Lung diseases are some of the most common diseases around the world, killing millions of people at various ages each year, and millions more people are suffering from severe lung conditions.¹⁸⁻²³ Due to our constant exposure to the external environment where various infectious and toxic agents can exist, the lungs are vulnerable to infection and injury.²⁴ There are many factors that may result in unhealthy lungs, of which infections, smoking and genetics are common causes.²⁵⁻²⁷ Lung diseases can result from any problem in any part of the respiratory system, including the above-mentioned airways, tissues and blood vessels located in the lungs.²⁸

Airway diseases such as chronic obstructive pulmonary disease (COPD), asthma, cystic fibrosis (CF), and emphysema, usually cause a narrowing or blockage of airways, making it difficult for the lungs to take in oxygen and release carbon dioxide.²⁸ COPD is the third common cause of death globally, affecting more than 200 million people around the world.²⁹ Tobacco smoke is the primary factor causing the development of COPD, by damaging lung tissue (emphysema) and obstructing the small airways with inflammation compounds and mucus (chronic bronchitis), resulting in shortness of breath and cough.³⁰ In addition to inhaled tobacco smoke, air pollution and genetic syndromes such as α -antitrypsin deficiency are also responsible for the development of COPD.³¹ Avoiding or reducing exposure to the risk factors (e.g., air pollute, and tobacco smoke) are essential for prevention and treatment of COPD.³²⁻³³

Asthma is a chronic inflammatory disease leading to narrowing and swelling of airways, and extra production of mucus, which makes breathing difficult and can trigger coughing and wheezing.³⁴ Asthma afflicts about 334 million people of various ages in the world, and the incidence has experienced rapid increase in global prevalence in the past three decades.³⁵ The causes of most asthma are not well known and there are no effective strategies to prevent it. The development of asthma is sometimes associated with genetic predisposition, exposure to air pollution and environmental allergens and, airway microbiome makeup, dietary factors and abnormal immunological responses.³⁶⁻³⁷

Cystic fibrosis (CF) is a genetic disorder that affects the mucus-producing cells in the lungs, causing dysfunctional mucus secretion.³⁸ The thicker and stickier mucus can block the tubes, ducts and passageways, providing an environment for bacterial growth, which leads to persistent lung infections, inflammation and limited ability to breathe over time.³⁹ Physical airway clearance, inhaled medicines including antibiotics for the treatment of lung infections,

and pancreatic enzyme supplement capsules for enhanced absorption of vital nutrients, are the most common therapies for CF.⁴⁰ Gene therapy has been investigated using animal models but has not been translated to human therapy yet.⁴¹⁻⁴³

Lower respiratory tract infection (LRTI) and pneumonia also affect the lungs, killing around 4 million each year.⁴⁴⁻⁴⁶ Viral infection and bacterial infection are the two factors leading to LRTI, of which 90% is caused by viruses such as respiratory syncytial virus (RSV), influenza, coronavirus, and rhinovirus.⁴⁷⁻⁴⁸ For most bacterial infections which are caused by *Streptococcus pneumoniae*, *Mycoplasma pneumoniae* or *Bordetella pertussis*, antibiotics administration is the primary treatment, even though there is growing bacteria resistance against antibiotics with the increasing administration of antibiotics.⁴⁹⁻⁵¹ For the infections caused by parasites or viruses, antibiotics do not help at all. Actually, most viral infections are self-limited and there is no specific treatment for them.⁵² Immunisations are normally recommended to build up the immune system for prevention against infections. Much attention should be paid to new respiratory pathogens such as the severe acute respiratory syndrome (SARS) in 2003 and the corona virus (SARS-CoV-2) in 2019. These viruses can rapidly spread throughout the world due to the ease of transmission, leading to widespread pandemics and global disaster. Vaccines are necessary and important for the control and prevention of these diseases, which require active international efforts.⁵³

Lung cancer which is the most common and fatal cancer in the world. It kills 1.6 million people each year and the number is rapid growing annually.⁵⁴⁻⁵⁵ Non-small cell lung cancer (NSCLC) (making up 80–85% of all cases) and small cell lung cancer (SCLC) are two major types of lung cancer. Smoking is the primary factor contributing to most cases of lung cancer via damaging DNA and mutating protective genes.⁵⁶⁻⁵⁷ Lung cancer can also happen to people who have never smoked probably due to second-hand smoke, exposure to certain toxins (e.g., biomass fuel, diesel exhaust, radon and asbestos), and family history.⁵⁸⁻⁶⁰ In addition to surgery, chemotherapy and radiation therapy, targeted drug therapy and immunotherapy are considered to be useful treatments for lung cancer.⁶⁰ Inhalation delivery has been an attractive route for lung cancer targeting due to the ease of access to the lungs and the accumulation of drug in local lung region.⁶¹⁻⁶² However, early stage of lung cancer is difficult to diagnose until the cancer has spread to regional lymph nodes or has metastasised to other organs, making lung cancer highly fatal to the patients.⁶³

Tuberculosis (TB) is a serious bacterial infectious disease caused by *Mycobacterium tuberculosis* bacterium (MTB). It killed 1.4 million people in 2015, making it the greatest single infectious agent cause of death among overall deaths in the world.¹⁴ MTB is a small, aerobic, nonmotile bacillus capable of withstanding weak disinfectants and surviving in a dry state for a couple of weeks. It divides every 16–20 hours, which is much slower than other bacteria.⁶⁴ TB is a systemic disease. Besides the lungs, the liver, kidneys, spleen, uterus and bones are also prominent sites of infection. Because the bacteria is present in different organs and cell types in different metabolic states, treatment of TB is a complicated task.⁶⁵ Table 1.1 lists some antibiotics used in the treatment of TB, which can be divided into first-line antibiotics such as isoniazid, rifampicin, ethambutol and pyrazinamide, and second-line drugs, including capreomycin, cycloserine, clofazimine, ciprofloxacin, ofloxacin and streptomycin.⁶⁵ Anti-TB medicines have been used for several decades, however, due to the inappropriate use of drugs, drug resistance has emerged resulting in limited effective antimicrobial options. Therefore, there is a significant and urgent need to develop new alternative antimicrobial agents for the treatments of bacterial infections.^{64, 66} In the past decades, synthetic mimics of antimicrobial peptides (AMPs) have been increasingly developed as a new class of antimicrobial agents, which exhibit high activity, broad-spectra, and relatively low propensity for resistance that is common for natural AMPs.⁶⁷ However, the high toxicity towards mammalian cells has limited their successful translation to the clinic.⁶⁸ Recently, a new class of synthetic AMP mimics, termed ‘structurally nanoengineered antimicrobial peptide polymers’ (SNAPPs) with the formation of star-shaped polypeptide nanoparticles containing cationic lysine and hydrophobic valine amino acid residues has been reported.⁶⁸⁻⁶⁹ The SNAPPs displayed excellent antimicrobial properties with negligible toxicity to mammalian cells and no bacterial resistance, and are considered to be a promising therapeutic for lung diseases caused by bacterial infection (e.g., tuberculosis).⁶⁸⁻⁶⁹

Table 1.1. Antibiotics used in treatment of TB⁶⁵

	Description	MIC ^a	Half-life (hr)#	Water solubility*	Mechanism of action	Route of administration	Biodistribution	Side effect
First-line								
Isoniazid	Antibacterial activity limited to <i>Mycobacteria</i> .	0.02–0.25 (Rastogi et al., 1996, Wanger and Mills, 1996)	Fast acetylators: 0.5–1.6h. Slow acetylators: 2–5h.	140	Halts growth of resting organism, and kill dividing bacteria. Mechanism of action is unknown	Orally	Readily absorbed from the GI. Is widely distributed in the tissues and body fluids. Passes freely into mammalian cells	About 5% of patients – allergic skin eruptions. Also, fever, hepatotoxicity, hematological changes, arthritic symptoms and vasculitis.
Rifampicin	Broad spectra antibiotic and the most active anti-TB drugs.	0.016–0.4 (Rastogi et al., 1996, Wanger and Mills, 1996, Hirata et al., 1995)	3.35 (+/-0.66)	1.4	Inhibits DNA-dependent RNA polymerase in prokaryotic but not in eukaryotic cells.	Orally	Widely distributed in the tissues and body fluids. Enter phagocytic cells.	Relative infrequent, but includes skin eruption, fever and GI disturbance.
Ethambutol	Only affects <i>Mycobacteria</i> .	0.06–0.5 (Rastogi et al., 1996, Wanger and Mills, 1996)	Normal renal function, 3–4h, impaired function, up to 8 h	7.58	Is taken up by bacteria, but mechanism of action is unknown	Orally	Is well absorbed, and can reach therapeutic concentration in CSF	Can cause liver damage. Side effects are uncommon, but include optic neuritis.
Pyrazinamide	Is inactive at neutral pH, but anti-TB at acid pH.	0.25–1.0 (Wanger and Mills, 1996)	9–10	15	Damage bacterial cell membrane and inhibits transcriptions	Orally	Well absorbed and is widely distributed, penetrating well in the meninges	Includes, gout, GI upset, malaise, fever and can cause hepatic damage
Second-line								
Capreomycin	Antibiotic against enteric bacteria and other eubacteria	2.0 (Rastogi et al., 1996)	Not available	Soluble in water as disulfate salt.	Is believed to inhibit protein synthesis, but mechanisms of action is unknown	Intramuscular injection		Included kidney damage, injury to the auditory nerve.
Cycloserine	Broad-spectrum antibiotic	25.0 (Rastogi et al., 1996)	Half-life in patients with normal renal function is 10 hr	Soluble	Competitively inhibits bacterial cell wall synthesis	Orally	Is distributed throughout the tissues and body fluids	Mainly on the central nerve system, ranging from headache and irritability to depression, convulsions and psychotic states.
Clofazimine	Antibacterial activity limited to <i>Mycobacteria</i>	0.1 (Rastogi et al., 1996)	10 days following a single dose, 70 days after long-term, high-dose therapy.	2.25×10^{-4}	Binds to bacterial DNA leading to disruption of cell cycle	Orally		Includes abdominal symptoms, less frequently splenic infarction, bowel obstruction, and GI bleeding.
Ciprofloxacin	Broad spectra antibiotic	0.5 (Rastogi et al., 1996, Shandil et al., 2007)	4	30	Course double stranded DNA breakages	Orally		
Ofloxacin	Broad spectra antibiotic	0.5 (Shandil et al., 2007)	9	28.3	Course double stranded DNA breakages	Orally	65–80% of an administered oral dose is excreted unchanged via the kidneys within 48 hr	LD ₅₀ = 5450 mg/kg (orally in mice)
Streptomycin		1.0 (Rastogi et al., 1996)	5–6h in adults with normal renal function		Binds bacterial 30S ribosomal subunit, causing misreading of rRNA.	Intramuscular injection		Includes nephrotoxicity and ototoxicity.

^aMinimal inhibitory concentration (MIC), µg/mL for H37Rv in broth.
[#](Drugbank.ca).
^{*}(mg/mL at 25°C) (Drugbank.ca).

1.3 Pulmonary Delivery

The lungs, as a portal entry into the body with special structures, including large surface area suitable for absorption (70–140 m² in an adult human), good epithelial permeability owing to low thickness of the epithelial barrier (0.2 µm in alveoli), extensive vascularisation and relatively low proteolytic activity in the alveolar region, represent an promising delivery route for therapeutics either for local lung targeting or systemic circulation.^{3, 70} In contrast to other administration routes such as intravenous administration, pulmonary delivery is needle-free and non-invasive, which is friendly to patients especially those who need frequent dosages, and can achieve a rapid onset of drug action, potentially enhanced delivery efficacy and hence, reduced overall drug dosage and less severe side effects.²

For local lung diseases such as asthma, lung infectious diseases, lung hypertension and lung cancer, pharmaceuticals delivered via inhalation delivery do not need to pass through the digestive system and/or the circulatory system but go to the lung region directly, where a more rapid onset of drug action can be achieved compared to intravenous administration and oral delivery.^{2, 70} Additionally, enhanced accumulation and retention of drugs in the lungs lead to reduced overall drug dosage and consequent less severe systemic side effects, which mainly results from high levels of systemic drug exposure.⁷¹ For example, some poorly-soluble drugs for the treatment of lung diseases such as corticosteroids (ICS) for asthma (e.g., beclomethasone dipropionate (BDP) and fluticasone propionate (FP)) were administered via inhalation and found to be completely absorbed in the lungs even though deposition and absorption occurred in both the conducting airways and alveoli region.⁷²⁻⁷³ Another study compared the organ distribution of liposomes and nanostructured lipid carriers after intravenous and inhalation delivery.⁷⁴ As shown in Figure 1.3, the majority of the injected particles accumulated in the liver, kidney and spleen, and only about one quarter of the injected particles accumulated in the lungs, which indicated that most of the injected particles did not reach the lungs leading to not only reduced treatment efficacy, but also potential severe side effects on other organs. In contrast, the particles delivered via inhalation preferentially accumulated in the lungs.

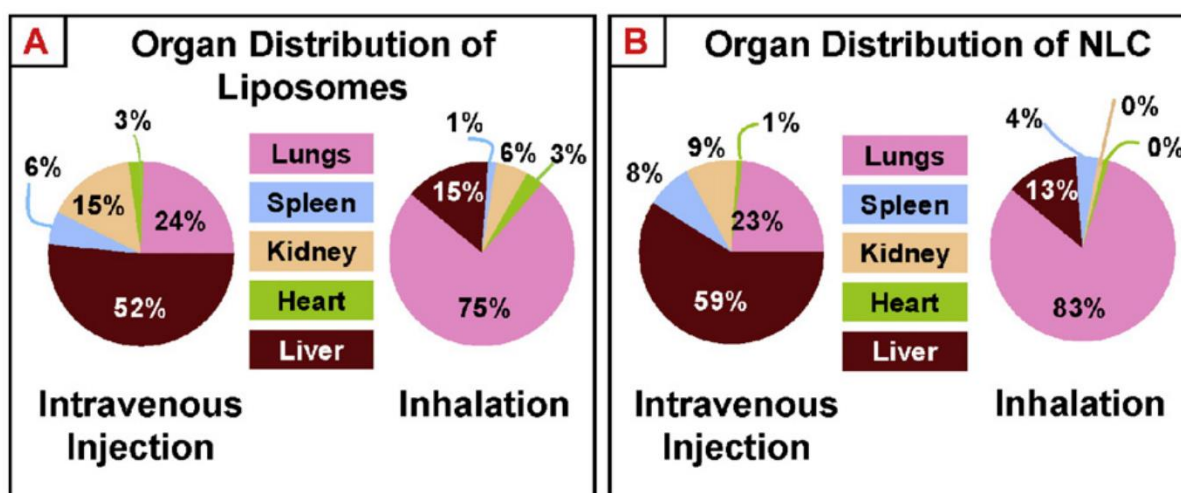


Figure 1.3. Organ distribution of liposomes (A) and nanostructured lipid carriers (NLC, B) after intravenous and inhalation delivery. The figure is modified from reference 74.

For systemic circulation, the lungs are used as a portal of entry to the body. Drugs are delivered into the lungs where they can be rapidly absorbed through the thin layer of epithelial cells and enter the blood circulation system.⁷⁵ Compared to oral delivery, pulmonary delivery can overcome the problem of poor gastrointestinal tract (GI tract), avoid the first-pass metabolism which is coupled with slow absorption, and thereby significantly improve the bioavailability and the delivery efficacy of therapeutics.⁷⁶⁻⁷⁷ Furthermore, pulmonary delivery is a promising administration route for biomacromolecules and biotherapeutics such as peptides and proteins which can't be delivered orally due to enzymatic degradation and poor intestinal membrane permeability.^{76, 78} For example, inhalation of insulin for systemic circulation has been investigated recently. Inhaled insulin is considered to be as reliable as, or more reliable than, subcutaneous injected insulin. Other biotherapeutics such as human growth hormones, glucagons and oxytocin, which are normally delivery via conventional intravenous injection can also be potentially delivered via inhalation.⁷⁹⁻⁸¹ Though exactly which part in the lungs is best for drug deposition and optimum absorption varies with the drug, and is mostly dependent on the properties of drugs especially their molecular weight. Some general principles are emerging. For drugs with high molecular weight such as immunoglobulins of the IgG class (150 kDa), the ideal absorption location might be the middle and upper airways, where IgG is under receptor-mediated transcytosis, while it is better to deposit small molecules and peptides in deep lungs for enhanced absorption rather than in the larger airways.⁸²

Table 1.2 lists some examples of inhaled medicines that have been investigated, including hydrophobic small molecules, peptides and other macromolecules, some of which have been approved for clinical use.

Table 1.2. Examples of inhaled medicines³

Year	Formulation/device	Molecule(s)	Disease
1500 BC	Egyptians used ‘vapors’	?	?
1662 AD	Bennet’s inhalation treatment	?	Tuberculosis
1802	Potter’s cigarettes	?	Asthma
1860	Sales-Giron’s portable nebulizer	?	?
1925	Aqueous/nebulizer	Insulin	Diabetes
1945	Aqueous/nebulizer	Penicillin	Lung infections
1951	Aqueous/nebulizer	Isoprenaline	Asthma
1955	Aqueous/nebulizer	Hydrocortisone	Asthma
1956	First metered-dose inhaler (MDI) (freon)	Albuterol	Asthma
1960	First dry powder inhaler (DPI)	Norsadrenaline	Asthma
1988	First multidose DPI	Terbutaline	Asthma
1996	First protein aqueous/nebulizer	DNAse	Cystic fibrosis
1998	First antibiotic aqueous/nebulizer	Tobramycin	Cystic fibrosis
1997	First hydrofluoralkane MDI	Albuterol	Asthma
2006	First protein DPI	Insulin	Diabetes

1.3.1 Particle-Based Pulmonary Delivery

With the development of material science and nanotechnology, a variety of nanoengineered particle systems with unique properties and functions, including liposomes,⁸³⁻⁸⁴ polymersomes,⁸⁵⁻⁸⁶ protein-drug conjugates,⁸⁷⁻⁸⁸ micelles,⁸⁹⁻⁹⁰ dendrimers,⁹¹⁻⁹² polymer particles,^{8, 93} and polymer capsules,⁹⁴⁻⁹⁵ have been developed as drug carriers for biomedical and therapeutical applications with the aim of improving therapeutic outcomes (Figure 1.4). Compared to conventional delivery of therapeutic formulations, particle-based delivery can protect the loaded-drug against enzymatic and hydrolytic degradation or inactivation. In addition, controlled drug release can be achieved, a variety of biological barriers can be

overcome and targeting to specific structures or cells may be possible by manipulating the physicochemical properties of the particles including composition, size, shape, surface chemistry, elasticity, and surface modification with targeting ligands (Figure 1.5).⁹⁶⁻¹⁰¹

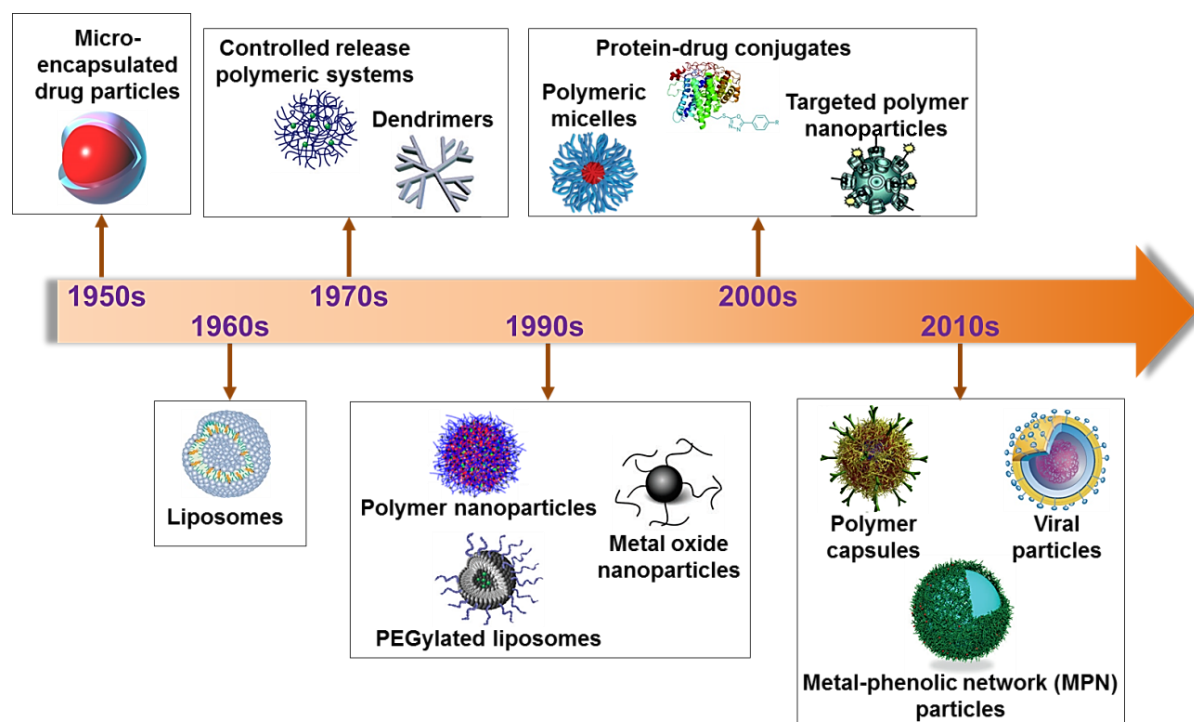


Figure 1.4. A timeline of the development of nanoengineered particle systems for biomedical applications. The figure is adapted from reference 8.

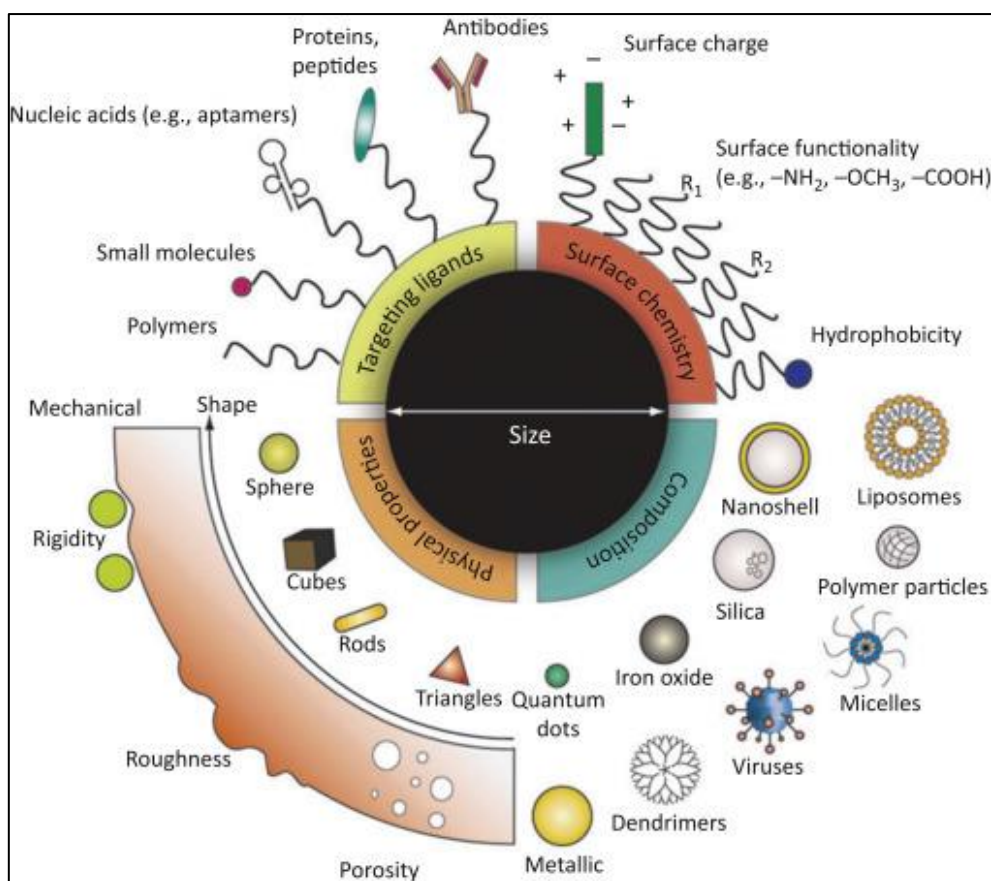


Figure 1.5. Schematic illustration of key physicochemical properties of nanoengineered particles that affect drug delivery efficacy. The figure is adapted from reference 102.

Building on extensive fundamental and translational research, advances in the nanotechnology and biomedical fields are currently converging to revolutionise the treatment of a variety of diseases.¹⁰³ Particles for therapeutic use should be composed of biocompatible and biodegradable materials which will degrade into safe products in the body and then be resorbed through natural pathways or excreted out of the body.¹⁰⁴⁻¹⁰⁵ Table 1.3 lists some examples of particle-based therapeutics which have been approved for clinical use by the FDA or are undergoing clinical investigation, including liposomes, protein nanoparticles, and polymeric nanoparticles.¹⁰⁶ For pulmonary delivery, in addition to polymeric particles which have numerous advantages such as modifiable surface properties and high drug encapsulation, liposomes and lipid-based systems namely, solid lipid nanoparticles (SLN) and solid lipid microparticles (SLM), have received great attention and are a popular delivery system due to their physiological components in the formulation and low toxicity.¹⁰⁷⁻¹¹³ Examples of different drug-incorporated particle systems for pulmonary applications are listed in Table 1.4.

Table 1.3. List of FDA-approved nanomedicines stratified by material category by 2016¹¹⁴

Name	Material Description	Nanoparticle Advantage	Indication(s)	Year(s) approved
Polymer Nanoparticles – synthetic polymer particles combined with drugs or biologics				
Adagen®/pegademase bovine (Sigma-Tau Pharmaceuticals)	PEGylated adenosine deaminase enzyme	Improve circulation time and decreased immunogenicity	Severe combined immunodeficiency disease (SCID)	1990
Cimzia®/certolizumab pegol (UCB)	PEGylated antibody fragment (Certolizumab)	Improved circulation time and greater stability <i>in vivo</i> .	Crohn's disease Rheumatoid arthritis Psoriatic Arthritis Ankylosing Spondylitis	2008 2009 2013 2013
Copaxone®/Glatopa (Teva)	Random copolymer of L-glutamate, L-alanine, L-lysine and L-tyrosine	Large amino-acid based polymer with controlled molecular weight and clearance characteristics	Multiple Sclerosis (MS)	1996
Eligard® (Tolmar)	Leuprolide acetate and polymer (PLGH (poly (DL-Lactide-co-glycolide)))	Controlled delivery of payload with longer circulation time	Prostate Cancer	2002
Macugen®/Pegaptanib (Bausch & Lomb)	PEGylated anti-VEGF aptamer (vascular endothelial growth factor) aptamer	Improved stability of aptamer as a result of PEGylation	Macular degeneration, neovascular age-related	2004
Mircera®/Methoxy polyethylene glycol-epoetin beta (Hoffman-La Roche)	Chemically synthesized ESA (erythropoiesis-stimulating agent)	Improved stability of aptamer as a result of PEGylation	Anemia associated with chronic kidney disease	2007
Neulasta®/pegfilgrastim (Amgen)	PEGylated G-CSF protein	Improved stability of protein through PEGylation	Neutropenia, Chemotherapy induced	2002
Pegasys® (Genentech)	PEGylated IFN alpha-2a protein	Improved stability of protein through PEGylation	Hepatitis B; Hepatitis C	2002
PegIntron® (Merck)	PEGylated IFN alpha-2b protein	Improved stability of protein through PEGylation	Hepatitis C	2001
Renagel®[sevelamer hydrochloride]/ Renagel®[sevelamer carbonate] (Sanofi)	Poly(allylamine hydrochloride)	Increase circulation and therapeutic delivery	Chronic kidney disease	2000
Somavert®/pegvisomant (Pfizer)	PEGylated HGH receptor antagonist	Improved stability of protein through PEGylation	Acromegaly	2003
Oncaspar®/pegaspargase (Enzon Pharmaceuticals)	Polymer-protein conjugate (PEGylated L-asparaginase)	Improved stability of protein through PEGylation	Acute lymphoblastic leukemia	1994
Krystexxa®/pegloticase (Horizon)	Polymer-protein conjugate (PEGylated porcine-like uricase)	Improved stability of protein through PEGylation; introduction of unique mammalian protein	Chronic gout	2010
Plegridy® (Biogen)	Polymer-protein conjugate (PEGylated IFN beta-1a)	Improved stability of protein through PEGylation	Multiple Sclerosis	2014
ADYNOVATE (Baxalta)	Polymer-protein conjugate (PEGylated factor VIII)	Improved stability of protein through PEGylation	Hemophilia	2015
liposome formulations combined with drugs or biologics				
DaunoXome® (Galen)	Liposomal Daunorubicin	Increased delivery to tumour site; lower systemic toxicity arising from side-effects	Kaposi's Sarcoma	1996
DepoCyt® (Sigma-Tau)	Liposomal Cytarabine	Increased delivery to tumour site; lower systemic toxicity arising from side-effects	Lymphomatous meningitis	1996
Marqibo® (Onco TCS)	Liposomal Vincristine	Increased delivery to tumour site; lower systemic toxicity arising from side-effects	Acute Lymphoblastic Leukemia	2012
Onivyde® (Merrimack)	Liposomal Irinotecan	Increased delivery to tumour site; lower systemic toxicity arising from side-effects	Pancreatic Cancer	2015
AmBisome® (Gilead Sciences)	Liposomal Amphotericin B	Reduced nephrotoxicity	Fungal/protozoal infections	1997
	Liposomal Morphine sulphate	Extended release	Analgesia (post-operative)	2004

Table 1.3 (continued)

Name	Material Description	Nanoparticle Advantage	Indication(s)	Year(s) approved
DepoDur® (Pacira Pharmaceuticals)				
Visudyne® (Bausch and Lomb)	Liposomal Verteporfin	Increased delivery to site of diseased vessels; photosensitive release	Macular degeneration, wet age-related; myopia; ocular histoplasmosis	2000
Doxil®/Caelyx™ (Janssen)	Liposomal doxorubicin	Improved delivery to site of disease; decrease in systemic toxicity of free drug.	Kaposi's Sarcoma; Ovarian cancer; multiple myeloma	1995 2005 2008
Abelcet® (Sigma-tau)	Liposomal Amphotericin B lipid complex	Reduced toxicity	Fungal infections	1995
Curosurf®/Poractant alpha (Chiesi farmaceutici)	Liposome-proteins SP-B and SP-C	Increased delivery for smaller volume; reduced doxicity	pulmonary surfactant for Respiratory Distress Syndrome	1999
Micellar nanoparticles combined with drugs or biologics				
Estrasorb™ (Novavax)	Micellar Estradiol	Controlled delivery of therapeutic	Menopausal therapy	2003
Protein nanoparticles combined with drugs or biologics				
Abraxane®/ABI-007 (Celgene)	Albumin-bound paclitaxel nanoparticles	Improved solubility; improved delivery to tumor	Breast cancer NSCLC Pancreatic cancer	2005 2012 2013
Ontak® (Eisai Inc)	Engineered Protein combining IL-2 and diphtheria toxin	Targeted T-cell specificity; lysosomal escape	Cutaneous T-Cell Lymphoma	1999
Nanocrystals				
Emend® (Merck)	Aprepitant	Surface area allows faster absorption and increases bioavailability	Antiemetic	2003
Tricor® (Lupin Atlantis)	Fenofibrate	Increases bioavailability simplifies administration	Hyperlipidemia	2004
Rapamune® (Wyeth Pharmaceuticals)	Sirolimus	Increased bioavailability	Immunosuppressant	2000
Megace ES® (Par Pharmaceuticals)	Megestrol acetate	Reduced dosing	Anti-anorexic	2001
Avinza® (Pfizer)	Morphine sulfate	Increased drug loading and bioavailability; extended release	Psychostimulant	2002 (2015)
Focalin XR® (Novartis)	Dexamethyl-phenidate HCl	Increased drug loading and bioavailability	Psychostimulant	2005
Ritalin LA® (Novartis)	Methylphenidate HCl	Increased drug loading and bioavailability	Psychostimulant	2002
Zanaflex® (Acorda)	Tizanidine HCl	Increased drug loading and bioavailability	Muscle relaxant	2002
Vitoss® (Stryker)	Calcium phosphate	Mimics bone structure allowing cell adhesion and growth	Bone substitute	2003
Ostim® (Heraeus Kulzer)	Hydroxyapatite	Mimics bone structure allowing cell adhesion and growth	Bone substitute	2004
OsSatura® (IsoTis Orthobiologics)	Hydroxyapatite	Mimics bone structure allowing cell adhesion and growth	Bone substitute	2003
NanOss® (Rti Surgical)	Hydroxyapatite	Mimics bone structure allowing cell adhesion and growth	Bone substitute	2005
EquivaBone® (Zimmer Biomet)	Hydroxyapatite	Mimics bone structure	Bone substitute	2009
Invega® Sustenna® (Janssen Pharms)	Paliperidone Palmitate	Allows slow release of injectable low solubility drug	Schizophrenia Schizoaffective Disorder	2009 2014
Ryanodex® (Eagle Pharmaceuticals)	Dantrolene sodium	Faster administration at higher doses	Malignant hypothermia	2014
Inorganic and metallic nanoparticles				
Nanotherm® (MagForce)	Iron oxide		Glioblastoma	2010

Table 1.3 (continued)

Name	Material Description	Nanoparticle Advantage	Indication(s)	Year(s) approved
		Allows cell uptake and introduces superparamagnetism		
Feraheme™/ferumoxytol (AMAG pharmaceuticals)	Ferumoxytol SPION with polyglucose sorbitol carboxymethylether	Magnetite suspension allows for prolonged steady release, decreasing number of doses	Deficiency anemia iron deficiency in chronic kidney disease (CKD)	2009
Venofer® (Luitpold Pharmaceuticals)	Iron sucrose	Allows increased dose	iron deficiency in chronic kidney disease (CKD)	2000
Ferlecit® (Sanofi Aventis)	Sodium ferric gluconate	Allows increased dose	iron deficiency in chronic kidney disease (CKD)	1999
INFeD® (Sanofi Aventis)	Iron dextran (low MW)	Allows increased dose	iron deficiency in chronic kidney disease (CKD)	1957
DexIron®/Dexferrum® (Sanofi Aventis)	Iron dextran (high MW)	Allows increased dose	iron deficiency in chronic kidney disease (CKD)	1957
Feridex®/Endorem® (AMAG pharmaceuticals)	SPION coated with dextran	Superparamagnetic character	Imaging agent	1996 (2008)
GastroMARK™; umirem® (AMAG pharmaceuticals)	SPION coated with silicone	Superparamagnetic character	Imaging agent	2001 (2009)

Table 1.4. Different active pharmaceutical ingredient (API) molecules incorporated into different particle systems for pulmonary applications¹¹⁵

Disease/API category	Type of particles
Anti-asthma/anti-inflammatory	
Beclomethasone	Lipid NC
Budesonide	SLN, liposomes
Curcumin	SLN, polymeric NP
Indomethacin	Lipid NP
Fluticasone	Dried NP
Pirfenidone	Polymeric NP
Anti-cancer	
Cisplatin	Dried NP
Methotrexate	Polymeric NP
Paclitaxel	Polymeric NP
Silibinin	SLN
Anti-oxidants	
Antioxidants- multiple types	Liposomes, polymeric NP, SLM
Lung infections	
Amikacin	Liposomes, SLN
Amphotericin B (Ambisome®)	Liposomes (parenteral)
Anti-tuberculosis drugs	SLN, polymeric NP, Liposomes
Ciprofloxacin	Liposomes
Moxifloxacin-Ofloxacin	Dried NP, MP
Tobramycin-Clarithromycin-Vancomycin	Spray dried NP, MP
Voriconazole	Polymeric NP
Tacrolimus	Lipid NP
Itraconazole	Lipid NC, dried NP
Proteins, peptides and macromolecules	
Calcitonin	Polymeric liposomes
Heparin	Polymeric NP
Insulin	SLN
Exendin-4	Polymeric NP
Pulmonary arterial hypertension/Congestive heart failure	
Iloprost	Liposomes
Sildenafil	Polymeric NP, SLN
Carvedilol	Polymeric NP
Surfactant, gene and antibody delivery	
siRNA/gene	Polymeric NP
Surfactant therapy	Liposomes
DNA vaccine	Polymeric liposomes
IgG1	Self-assembly NP

SLN, solid lipid nanoparticles; SLM, solid lipid microparticles; NP, nanoparticles; NC, nanocarriers.

1.3.2 Aerosol Delivery Approaches

Inhaled particles deposit along the respiratory tract. Many factors affect where the particles deposit along the respiratory tract following inhalation, of which the aerodynamic diameter (D_a) of particles is the primary determinant.¹¹⁶ Generally, large particles with $D_a > 5 \mu\text{m}$ mainly deposit in the oropharyngeal region and will be subsequently exhaled or swallowed, while particles with D_a ranging from $1 \mu\text{m}$ to $5 \mu\text{m}$ can be delivered to the trachea, bronchi, bronchiole and alveolar region, where they can potentially display therapeutical effectiveness. Even smaller particles with $D_a < 1 \mu\text{m}$ are likely to remain in the airstream and be exhaled in following breathing cycles.⁷¹

However, large particles exhibit relatively lower cell availability compared to small particles.⁹⁷ To achieve both high deposition in deep lungs and high bioavailability for the cells, small particles are usually delivered via aerosolisation in a dry powder formulation or a nebulised liquid formulation into the lungs.¹¹⁷⁻¹¹⁸ Pressurised metered Dose Inhaler (pMDI), dry powder inhaler and nebuliser are the most widely used devices for aerosolisation (Table 1.5). DPI is considered the most convenient device, which is chemically stable and patient friendly. Drug or drug-loaded carriers are required to be formulated into dry powders by freeze drying or spray drying process before aerosolisation.¹¹⁹⁻¹²¹ It has potential advantages for the drug and particle systems, which can keep long-term stability and sterility in a dry state. Therapeutics not amenable to the drying process or unstable in a dry state cannot be delivered via dry powder inhalation.¹²² Most dry powders exhibit a large polydisperse aerosol population and lack control over the resulting particle shape,¹²³ while recently more studies have explored precise control of D_a by designing dry powders in a uniform microparticle shape, size, and morphology.¹²⁴ Table 1.6 shows some examples of pulmonary delivery of nanoparticles using dry powder carriers.

Nebulisation of drug solution or drug-loaded particle suspension is another commonly used approach.¹²⁵ Nebulisers have been used for long time since the mid-1800s. Aerosol droplets are generated from drug solution or drug-loaded particle suspension by ultrasonic, air-jet or other types of nebulisers. Compared to dry powder formulations, nebulisation of particle suspension can avoid freeze drying or spray drying process and provide finely controlled particle size, allowing the modulation of the bio-nano interactions between the drug-loaded particles and the biological environments where they deposit.¹²⁶ However, it requires that the nebulised particle suspension must be physically and chemically stable during the nebulisation

process. Table 1.7 shows some examples of pulmonary delivery of nanoparticle suspensions using nebulisation.

A pMDI device is more portable than a nebuliser and each dose is administered in less than a minute. The pMDI formulation normally consists of a liquid formulation of the active pharmaceutical ingredient (API), a high vapor pressure propellant and surfactants, and other excipients to reduce drug-aerosol aggregation. The pMDI releases a metered dose of liquid drug at a high pressure, resulting in heterogeneous droplets containing drugs from the actuator mouthpiece.

Table 1.5. Comparison of nebuliser, pMDI and DPI¹²⁷

Device	Mechanism	Characteristics	Inconveniences
Nebulizer	air-jet or ultrasonic nebulization	vibrating mesh technology generates aerosol droplets from liquids	long inhalation times cleaning times frequent administration
pMDI	generates aerosol droplets from a drug suspension in volatile liquids (propellant)	unit dosing inexpensive correct size to deposit in the lung	propellant requirement lung deposition <60% coordination difficulties
DPI	dry powder	store drug in dry state: stability and sterility small portable devices short administration avoids coordination problems	requires high inspiratory effort

Table 1.6. Examples of pulmonary delivery of nanoparticles using dry powder carriers¹⁰⁷

Nanoparticle type	Nanoparticle size (nm)	Carrier particle	Carrier particle size (μm)	Active ingredient	Method for the preparation of carrier
Hydroxypropylmethyl cellulose phthalate (HPMCP)	51.6	Lactose	0.6–9.3	Pranlukast	Spray drying and freeze drying
Carboxylate-modified polystyrene (PS) and Nyacol 9950 colloidal silica	PS: 25, 170, 1000 Nyacol: 100	Large porous particles (LPP), nanoparticles attached to each other	4±0.2	–	Spray drying
Gelatin and <i>iso</i> -butyl cyanoacrylate	173, 242	Lactose	2.50–2.60	–	Spray drying
Chitosan	388, 419	Lactose	2–3	Insulin	Spray drying
<i>Iso</i> -butyl cyanoacrylate	244	Effervescent carrier powder	2.17	Ciprofloxacin	Spray drying

Table 1.7. Examples of pulmonary delivery of nanoparticle suspensions using nebulisation¹⁰⁷

Nanoparticle type	Nanoparticle size (nm)	Nebulization device	Active ingredient	<i>In vivo</i> models
DEAPA-PVAL-g-PLGA ^a	76.2–213.6	Pari® LC Star and Optineb®	–	–
Solid lipid nanoparticles (SLN)	200	Ultrasonic nebulizer	^{99m} Tc	Rat
PLG ^b	186–290	Compressor-nebulizer system	Rifampin, isoniazide, pyrazinamide	Guinea pig
Solid lipid nanoparticles (SLN)	Not provided	Compressor-nebulizer system	Rifampin, isoniazide, pyrazinamide	Guinea pig
Surface modified PLGA ^c with chitosan	650	Ultrasonic nebulizer	Calcitonin	Guinea pig
Itraconazole nanocrystals	300–800	Aeroneb Pro micropump nebulizer	Itraconazole	Mice

1.4 Biological Barriers in the Lungs

As a major defence organ that protects the body by filtering out foreign substances from entering the systemic circulation, the lungs have developed highly efficient particle clearance mechanisms, including mucociliary clearance, macrophage clearance, and metabolic degradation as shown in Figure 1.6. Particles that are delivered to the middle or lower airways via aerosolisation, are faced with metabolic degradation, pulmonary absorption, or elimination, dependent on the physicochemical properties of particles and the deposition sites.¹²⁸⁻¹²⁹ The fate of the inhaled particles can be altered by some structural or surface modification.¹³⁰ For example, unmodified dendrimers were found to be rapidly absorbed into the bloodstream with limited retention time in the lungs, while those modified with poly(ethylene glycol) polymers (PEG) resulted in much more accumulation in the lungs leading to a different biodistribution.¹³¹

These clearance mechanisms have an important role in determining the retention time of particles in the lungs. Therefore, it is necessary to study and understand the interactions between particles and the lung physiological environment, in order to design drug delivery carriers that can overcome the biological barriers, or even utilise the defence mechanisms. Mucociliary clearance and macrophage clearance, which are the primary defences in the airway and alveolar region, respectively, will be discussed in this section.

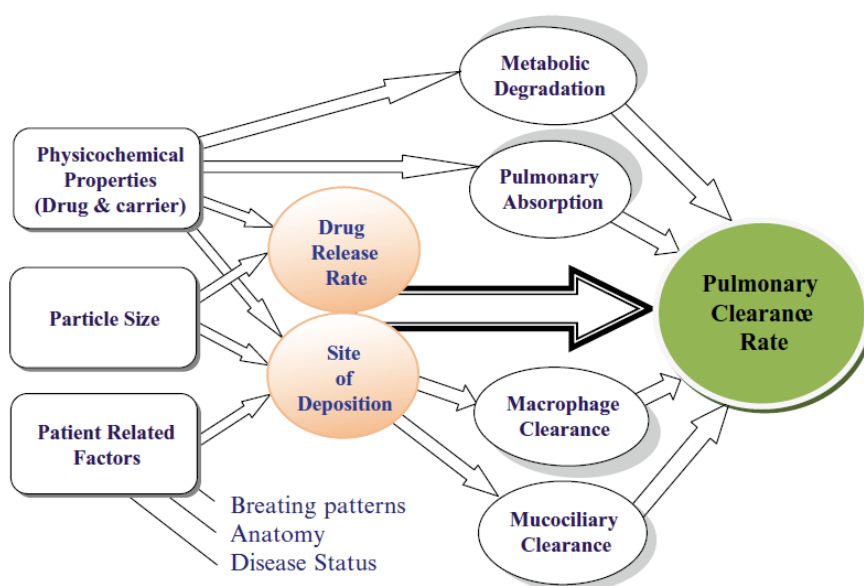


Figure 1.6. Factors affecting drug metabolism, adsorption and clearance in the lungs. The figure is reproduced from reference 4.

1.4.1 Mucociliary Clearance

Mucociliary clearance is the primary clearance mechanism in the airways, serving to entrap foreign particulates in the mucus layer before they move into the deeper regions of the lungs. Particles are swept out of the lungs by the mucociliary escalator.⁴ Airway mucus is a highly hydrated gel-like biological mixture composed of cross-linked mucin glycoproteins, polypeptides, cellular debris, lipids, salts, DNA and other free proteins produced by goblet cells and secretory cells covering the epithelial surface.¹³² Structurally, airway mucus consists of two main layers: a superficial gel layer produced by the goblet cells, composed of glycoproteins, lipids, antibodies and immune cells to destroy any bacteria and viruses; and a periciliary layer (PCL), also called as a sol layer, which enables the gel mucus that locates at the tips of the cilia to be transported by the ciliary (Figure 1.7).¹³³⁻¹³⁴ The mucociliary clearance mechanism can eliminate foreign particles to protect the body, but it also accelerates the clearance of inhaled drug-loaded particles, decrease their retention time in the lungs leading to reduced delivery efficacy. To overcome the mucus barrier, different strategies have been explored to modulate the surface properties of nanoengineered particles and subsequent bio-nano interactions. More details will be discussed in section 1.5.1.

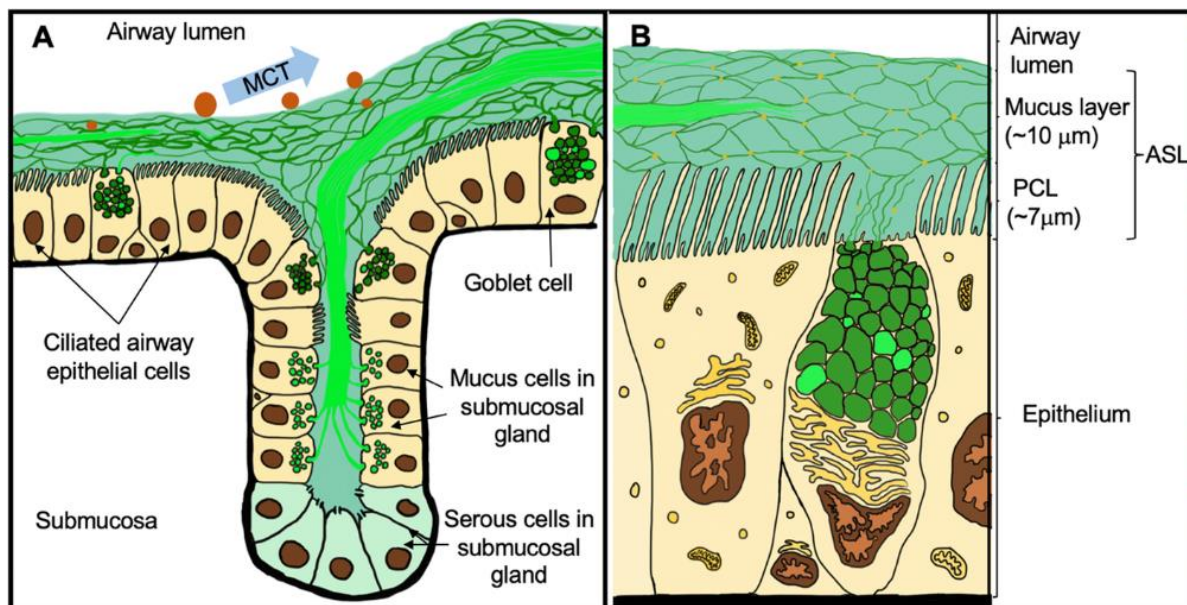


Figure 1.7. (A) general schematic representation of mucus secretion from goblet cells and submucosal glands; (B) schematic representation of the generally accepted structure of airway surface liquid. The figure is adapted from reference 134.

1.4.2 Macrophage Clearance

Particles that escape from mucociliary clearance and land in lower alveolar region can be scavenged by alveolar macrophages, which is a key defence against infections in the deep lungs.¹³⁵ Alveolar macrophages are a heterogeneous family of phagocytic cells which are derived from monocytes, existing in large numbers in the lungs and have phagocytic, antigen processing and immunomodulatory functions.¹³⁶ Normally, there are 12–14 alveolar macrophages in each alveolus serving as a scavenger to keep the alveolus free from foreign substances to maintain health and sterility.¹³⁷ Alveolar macrophages play a very important role in inflammatory activation. Once the particles are recognised and engulfed by the alveolar macrophages, other immune responses are immediately activated.¹³⁸ The neutrophils are quickly recruited to the lungs in response to microbes in which they perform phagocytic functions and kill the invading pathogen by producing proteases, reactive oxygen species (ROS) and reactive nitrogen species (RNS).¹³⁹ This can further lead to enhanced neutrophil recruitment with the release of inflammatory cytokines (IL-6, TNF α , IL-1) and other chemical signals (Figure 1.8).¹⁴⁰ Due to macrophage clearance, the retention time of inhaled particles in alveoli is significantly reduced, resulting in increased dose frequency, which could cause increased side effects and limit patient compliance. Although some studies have reported that the alveolar macrophages mostly uptake particles with the diameters between 1.5 μm and 3 μm , and smaller particles may potentially escape from the alveolar macrophage clearance achieving longer retention time,⁴ the exact mechanism of alveolar macrophage clearance beyond particle uptake, transportation, and elimination in the alveolar region is still not well understood, and there is a significant need to study these mechanisms for the design and development of more effective delivery systems.

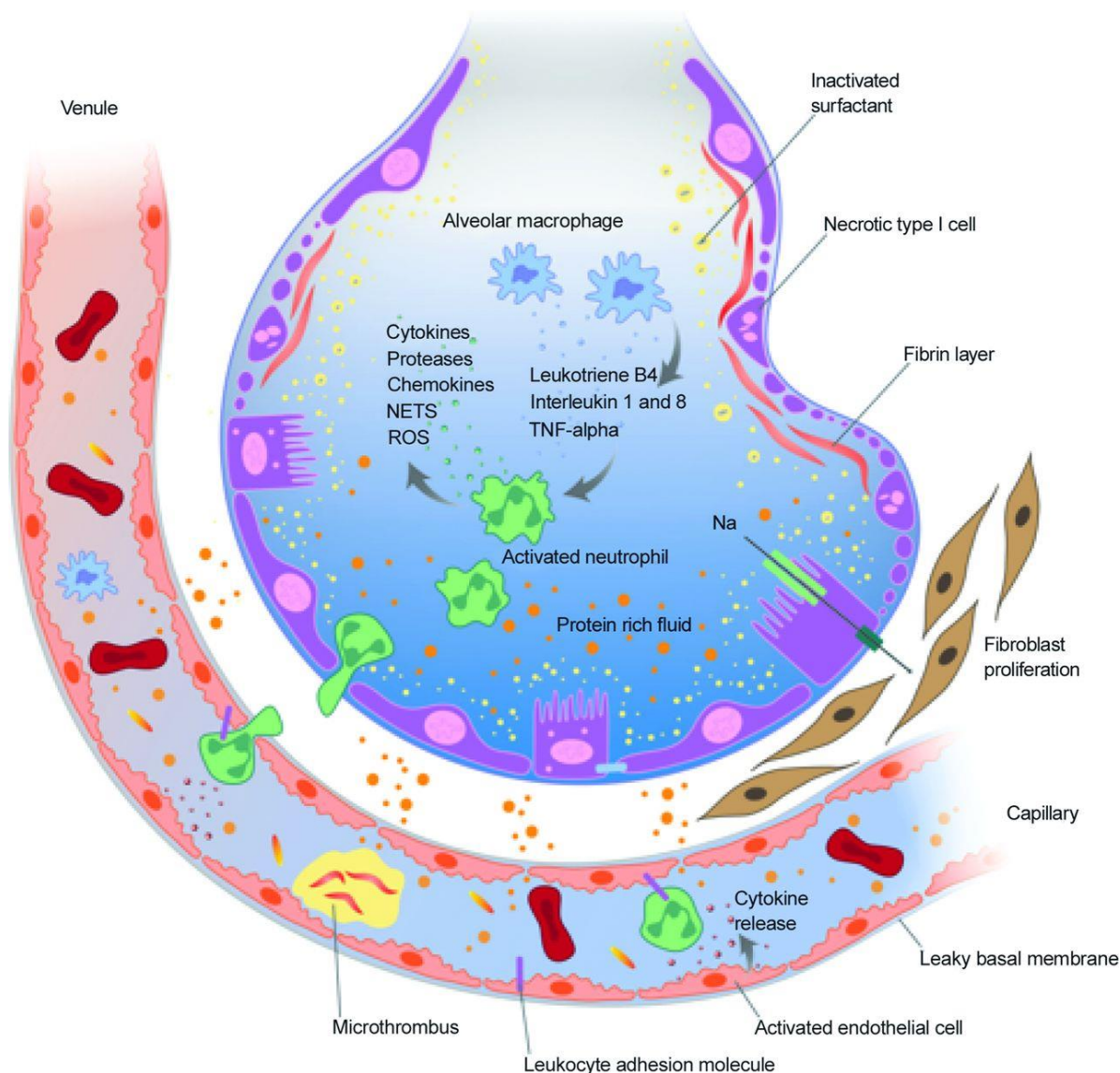


Figure 1.8. Schematic illustration of the immune responses in the alveoli. The figure is reproduced from reference 140.

1.5 Interactions between Particle Systems and Biological Environments

In the past decades, with the rapid development of nanotechnology and an increasing number of novel materials, a major challenge of drug delivery has come to understand how nanoengineered materials interact with complex physiological systems.^{103, 141-143} Studies have transitioned from functional design of particles to investigating particle behaviours both *in vitro*, *ex vivo* and *in vivo* (Figure 1.9).¹⁰³ In this section, the interactions between particle systems and lung physiological environments including particle–mucus interactions and the effect of

pulmonary surfactant on particle properties and cellular interactions will be described to provide important insight into the development of more effective particle systems.

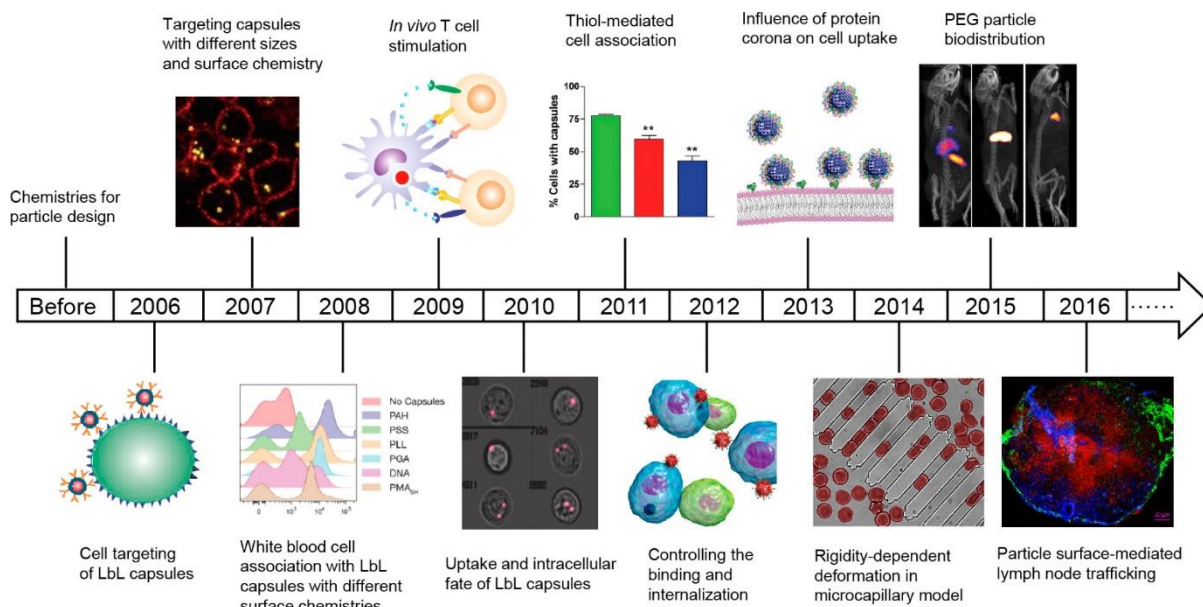


Figure 1.9. Timeline illustration of some examples of bio-nano interactions studied using particles assembled through templating methods in the Caruso group. The figure is adapted from reference 103.

1.5.1 Interactions between Particles and Mucus

As mentioned in 1.4.1, the mucociliary clearance in the airway can accelerate the clearance of inhaled drug-loaded particles and decrease their retention time in the lungs leading to reduced delivery efficacy.¹⁴⁴ It is of vital importance to understand the interactions between mucus and particle systems in order to design more effective particle systems capable of overcoming or even utilizing the mucus barrier for better therapeutic outcome.

Airway mucus is a highly hydrated gel-like biological mixture composed of cross-linked mucin glycoproteins, polypeptides, cellular debris, lipids, salts, DNA and other free proteins produced by goblet cells and secretory cells covering the epithelial surface. Mucins are large molecules with a typical molecular weight ranging from 0.5 MDa to 40 MDa forming a cross-linked network with an average mesh diameter from tens of nanometer to hundreds of nanometer.¹⁴⁵ Due to the size filtering, only particles smaller than the mesh space can diffuse through the

mucus layer.¹⁴⁶ In addition to particle size, particle surface properties played an extremely important role in particle–mucus interactions.¹⁴⁷ Manipulating the particles with mucoadhesion property (termed mucoadhesive particles) such as modification with chitosan, hyaluronic acid or thiol groups that can establish bonding and interactions with negatively charged glycosylated or cysteine-rich hydrophobic domains of mucin fibres, has been commonly applied to improve the drug absorption by increasing the residence time at the targeted mucosal site (Figure 1.10).¹⁴⁸ Others have developed mucus-penetrating particles with modified inert surface property in order to reduce particle immobilisation in mucus and allow further penetration of the particles through the mucus layer to interact with the underlying cells (Figure 1.10).¹⁴⁹⁻¹⁵² One of the commonly used techniques is coating the material with a densely packed layer of poly(ethylene glycol) (PEG), a ‘stealth’ polymer, resulting in a more hydrophilic surface and a large excluded hydrated cloud, which strongly repels the binding of biological components and effectively suppresses the subsequent clearance of particles.^{40, 149, 153} It was also reported that in the presence of both mucoadhesive particles and mucus-penetrating particles, high concentrations of mucoadhesive particles induced disruption of mucus, resulting in enlarged mucus mesh pores to increase the diffusion of mucus-penetrating particles (Figure 1.11).¹⁵⁴

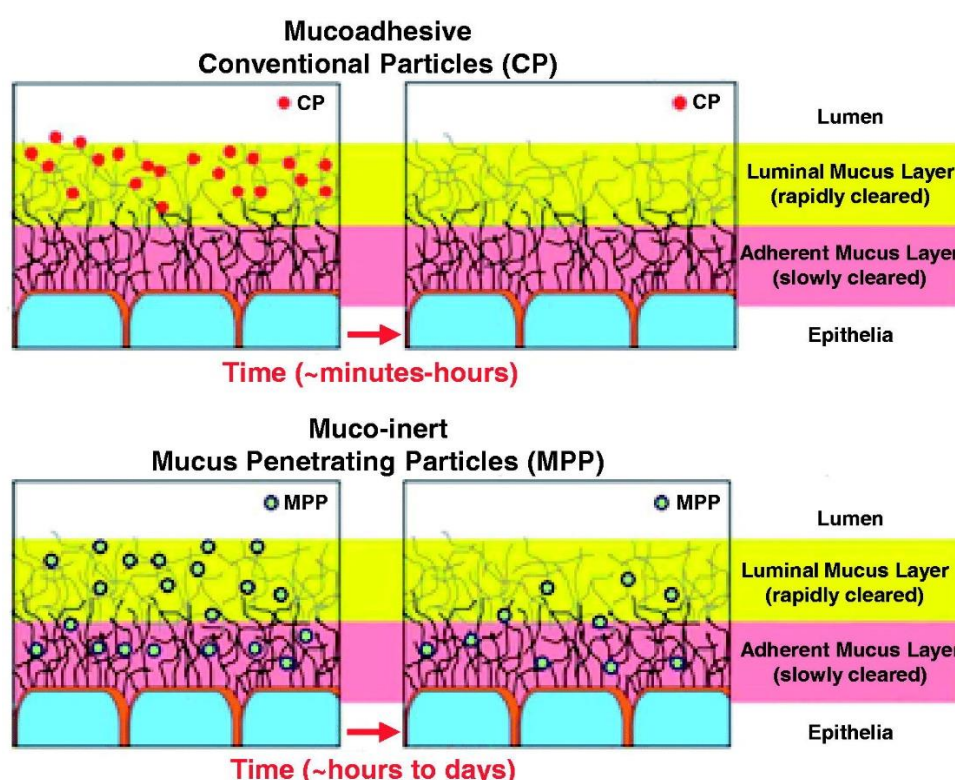


Figure 1.10. Schematic illustration of the fate of conventional mucoadhesive particles (CP) and mucus-penetrating particles (MPP) after administration to a mucosal surface. CP are mostly immobilised in the luminal mucus layer, while MPP tend to penetrate through the luminal

mucus layer and enter the underlying adherent mucus layer. The figure is adapted from reference 132.

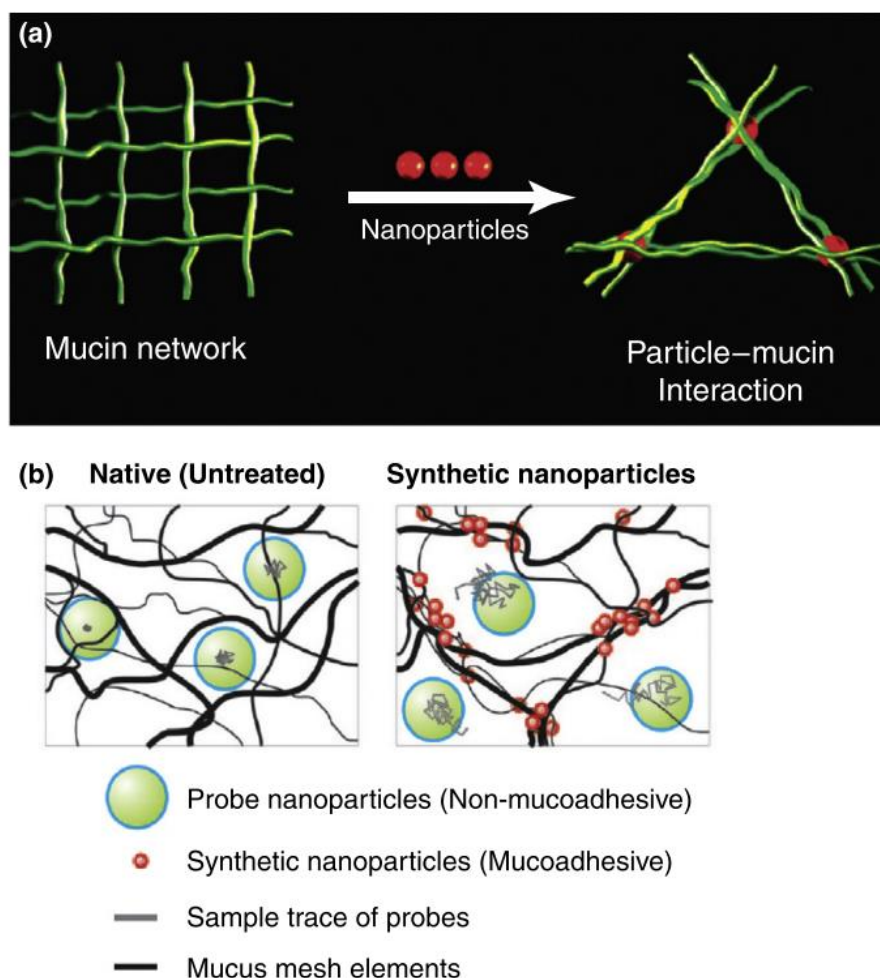


Figure 1.11. (a) Mucus disruption induced by particles. A mucus fibre network is depicted on the left with the introduction of particles leading to their entanglement with mucus resulting in a change of the fibre network. The figure is reproduced from reference 155. (b) The potential effects of mucoadhesive particles on the mucus structure. Mucoadhesive particles can increase mucus pore sizes by bundling mucin fibres with adhesive interactions. The figure is reproduced from reference 154.

Preparation of mucus is a crucial step for all particle–mucus interaction studies. There are various models of mucus from the simplest *ex vivo* model to the closest *in vivo* models, including simple mucin,¹⁵⁶ artificial mucus,¹⁵⁷ and natural mucus from animals (e.g., horse and

pig) or human.^{151, 158-159} The use of pathological mucus and mucus produced by specialised cell lines for *in vitro* transcellular crossing or cell uptake experiments have been reported.¹⁴⁵ Natural mucus is the most ideal model but it is usually difficult to collect from an individual, and the composition and thus the physicochemical properties may vary from different batches and sources. Mucin solutions, or artificial mucus prepared from mucin with the addition of other components such as DNA and salts, are more simple models than natural mucus, with ease of access and relative consistency. However, due to the disruption of mucin structure during the preparation and purification process of mucin, even if using the same concentration of mucin as that in natural mucus and with the addition of other components of mucus, differences can still be observed compared with natural mucus. Researchers should be careful when choosing a mucus model.

There are many methods and techniques to evaluate the interactions between particles and mucus *in vitro* including zeta-potential and particle size measurements, Transwell diffusion system (Figure 1.12), quartz crystal microbalance with dissipation monitoring (QCM-D) (Figure 1.13), multiple particle tracking (MPT) (Figure 1.14), and air-liquid interface (ALI) cell culture (Figure 1.15).¹⁶⁰ Briefly, entrapment of particles by mucus results in a change in particle size and zeta potential, which can be used to evaluate particle–mucus interactions and the mucus-penetrating behaviour of the particles.¹⁵⁷ Furthermore, interactions between particles and mucus can be qualitatively and quantitatively detected by measuring the deposition of particles on mucus-modified sensor via QCM-D analysis.¹⁶¹⁻¹⁶² Transwell diffusion system is another useful model to determine particle diffusion through mucus layer. Permeation of different particles, through different mucus samples, under different environmental conditions (e.g., pH and salt concentration), can be examined by using this model.^{157, 163} In addition, MPT, as a technique that can be used for dynamic measurements of the motion of individual particles in a heterogeneous matrix, is capable of assessing the interactions between particles and mucin fibres or the steric entrapment of particles within the mucin mesh network by calculating the particle diffusion via in-depth analysis of the time-resolved particle trajectories.^{151, 164} Finally, ALI culture, an *in vitro* cell culture model consisting of mixed cell types including goblet cells, basal cells, cilia epithelial cells, and noncilia epithelial cells, offers mimic *in vivo* conditions of the airway epithelium and has proven to be a very useful model for the studies on drug metabolism and particle permeation in respiratory system.¹⁶⁵⁻¹⁶⁷

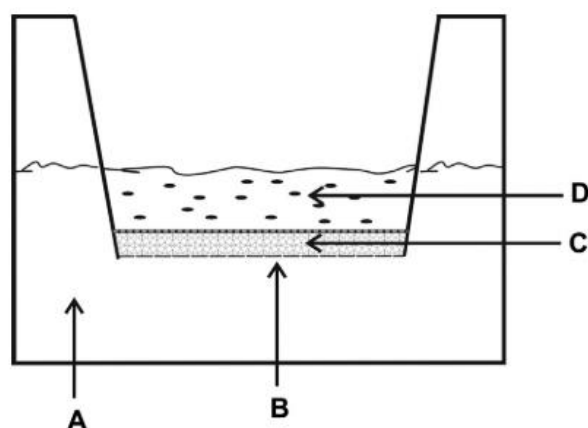


Figure 1.12. A schematic illustration of a Transwell diffusion system containing A: acceptor compartment; B: semipermeable membrane; C: mucus layer; D: donor compartment. The figure is reproduced from reference 168.

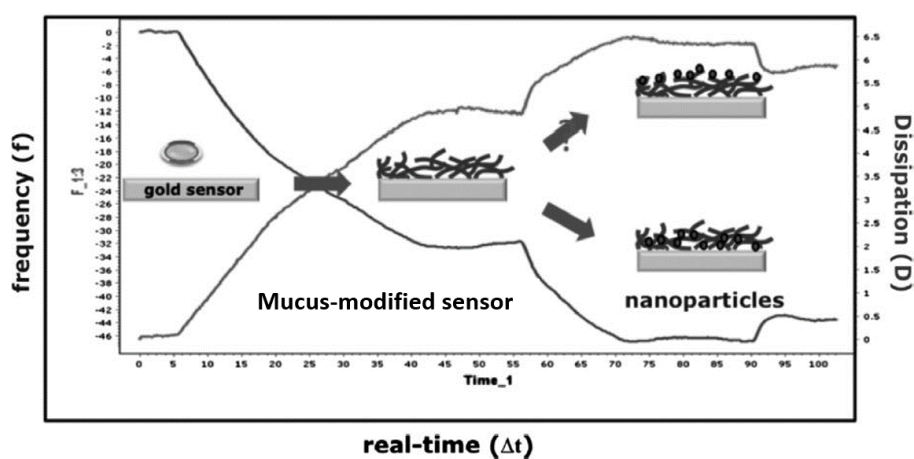


Figure 1.13. Scheme of the experiment setup to study particle–mucus interactions by QCM-D. The figure is adapted from reference 160.

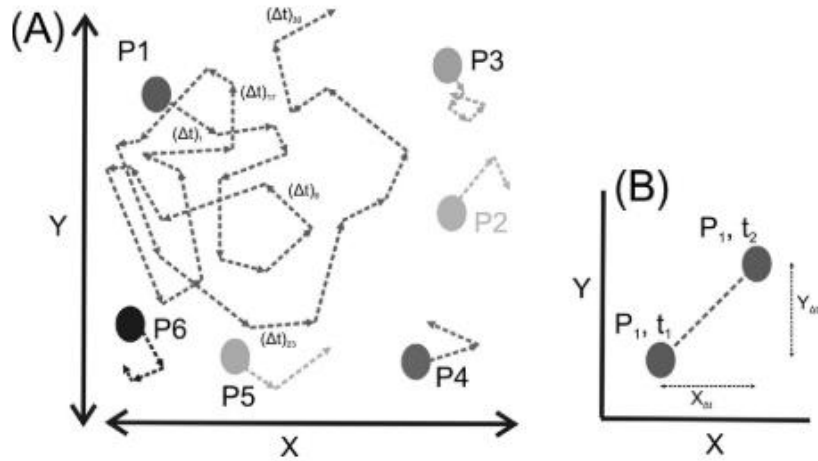


Figure 1.14. The movement of each particle (P) was captured by video as individual particle trajectories (A) over a minimum of 30 consecutive frames with the criterion that any individual particle tracked must display a continuous presence in the X–Y plane throughout the respective 30 sequential frames. For example, P1 in (A) above tracked from (Δt_1) to (Δt_{30}) . The distance moved in the X–Y trajectory by each particle over a time interval (Δt) , i.e. camera frame rate, is expressed as a squared displacement (SD) (B), $SD = (X_{\Delta t})^2 + (Y_{\Delta t})^2$ (Δt n = 1–30). The figure is adapted from reference 160.

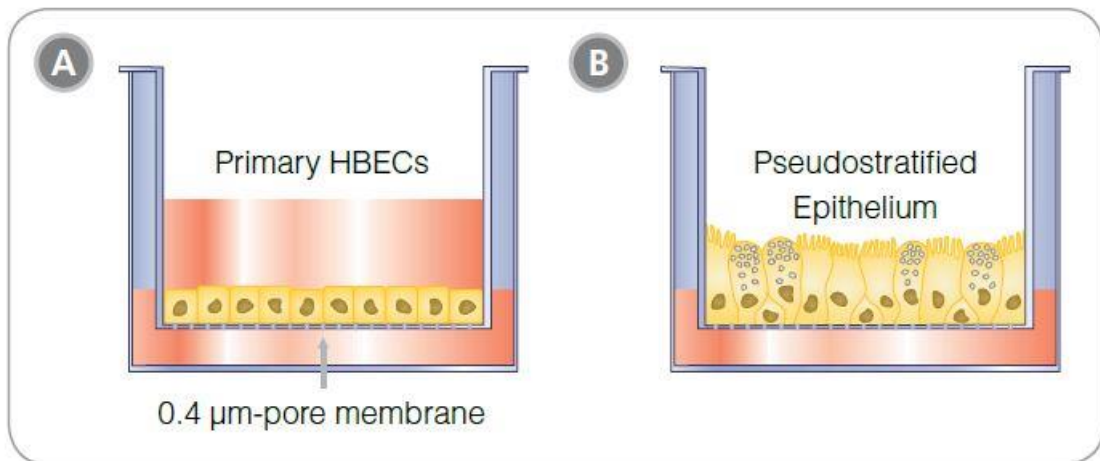


Figure 1.15. Schematic illustration of (A) submerged and (B) air-liquid interface culture of primary human bronchial epithelial cells grown on porous culture inserts. The figure is adapted from reference 169.

1.5.2 Pulmonary Surfactant and Pulmonary Corona

The interactions of nanoengineered particles with biological fluids upon entering a physiological environment, such as the adsorption of biomacromolecules and the resultant formation of a biomolecular corona on particle surfaces, endow the particles with a ‘biological identity’ that might be different from their original ‘synthetic identity’, which could in turn alter their interactions with cells and the eventual fate in the biological system (Figure 1.16).¹⁷⁰⁻¹⁷⁴ The biomolecular corona is generally composed of two parts with an inner tightly bound near-monolayer termed ‘hard corona’ and an outer layer termed ‘soft corona’ in which components are associated more loosely by lower affinity with a higher exchanging speed.¹⁷⁵ The formation of a biomolecular corona is influenced by both the particle physicochemical properties including size, shape, surface chemistry and elasticity, and the distinct composition of the surrounding physiological environments.¹⁷⁶⁻¹⁷⁷ As the interactions between particles and biological fluids are a highly dynamic process with continuous adsorption or desorption of components onto or off from the particle surface, the components associated with particles are likely to be replaced leading to an altered composition of biomolecular corona during the transportation of particles from one biological environment to another.¹⁷⁸⁻¹⁷⁹

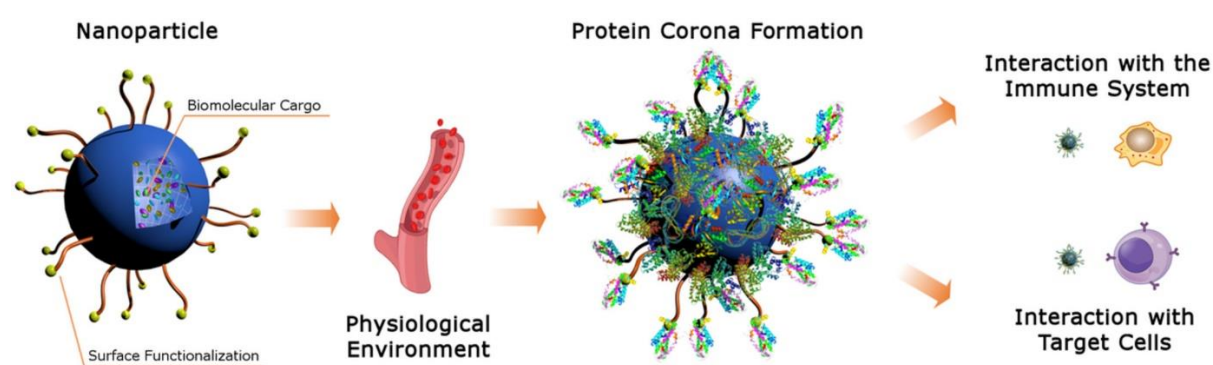


Figure 1.16. Formation of protein corona on particle surface and its effect on immunological identity and targeting ability of nanoparticles. The figure is adapted from reference 180.

Pulmonary surfactant layer covering the epithelial cells in deep lungs is a complex biological aqueous mixture consisting of high contents of surfactant specific proteins, lipids, and other components, constituting the important line of body defence as the most outer section of the air-blood barrier.¹⁸¹ Four pulmonary surfactant associated proteins (SP-A, -B, -C, and -D) have been identified in pulmonary surfactant and have various biological functions.¹⁸² The two

highly hydrophobic SP-B and SP-C are responsible for modulation and dynamisation of the behaviour of the lipid layers, while the two hydrophilic proteins, SP-A and SP-D, are involved in the innate immune response. Therapeutics or particles for systemic circulation via pulmonary delivery will encounter the lung surfactant environment in the deep lungs before entering the blood circulation system.¹⁸² The surface association of the components in pulmonary surfactant will result in a complex mixed corona around particles, which is likely to be different from the conventionally studied protein corona formed in blood plasma, and can potentially affect the downstream biological interactions in blood (Figure 1.17).¹⁸³

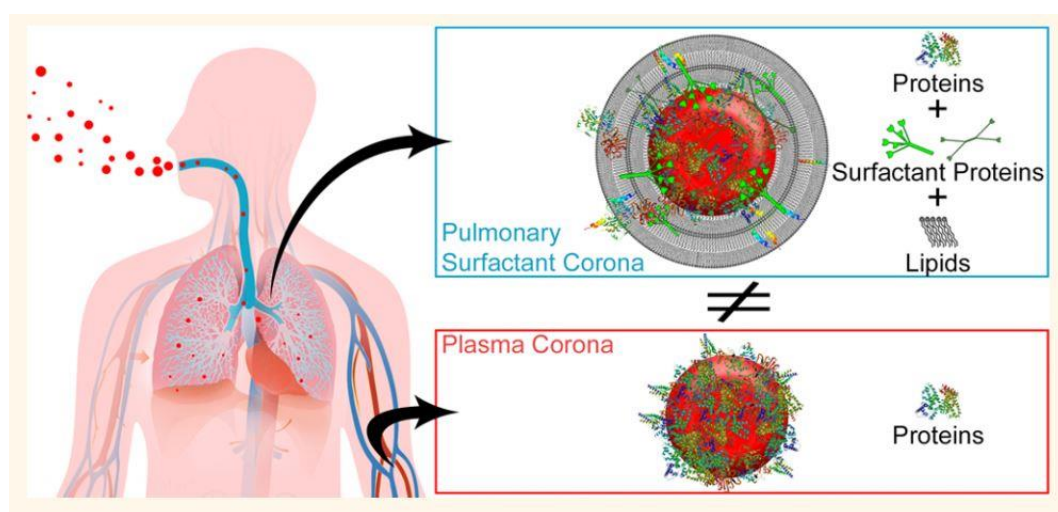


Figure 1.17. Schematic illustration of plasma corona and pulmonary surfactant corona. The figure is reproduced from reference 181.

1.6 Scope of Thesis

As described in the literature review above, pulmonary drug delivery is an promising delivery route for either local lung targeting or systemic delivery. However, the clearance and degradation mechanisms in the lungs especially the mucociliary clearance and the alveolar macrophage clearance may accelerate the elimination of inhaled particles, leading to suboptimal efficacy of therapeutic delivery. Therefore, it is important to understand the interactions of particle systems with the lung physiological environment, including the mucus layer, biological components in lung fluid and specific cells (*e.g.*, alveolar macrophages) for improved lung delivery efficiency and desirable biological outcomes. It is hypothesized that by tuning the particle physicochemical properties, the biological behaviour of particles can be

regulated. This is the motivation and the major scientific question of this PhD thesis. The thesis is set in a structure from particle design to exploring biomedical applications (pulmonary delivery), bridged by studies on particle–biological system interactions. The interaction of engineered particles with mucus, and the influence of the pulmonary corona on particle properties and performances are investigated by using presumably mucoadhesive and mucus-penetrating polymer particles fabricated via mesoporous silica templating. The potential for drug encapsulation and intracellular delivery for lung disease therapies is also demonstrated by using promising carriers.

A variety of instrumentations and techniques were used in this thesis for the characterisation of materials, and evaluation of their properties and performance in biological environments. An overview of the main instrumentations and techniques utilised in this thesis is described in Chapter 2.

Poly (ethylene glycol) (PEG) as a type of low-fouling material is widely used for particle surface modification to suppress the rapid clearance of particles from biological systems. The stealth behaviour of PEGylated particles is governed by the physicochemical properties of particles. PEG molecular weight and PEG coating density have been reported to affect the biological interactions of PEG-based particles. However, little attention has been paid to PEG molecular architecture. In Chapter 3, the influence of the architecture of PEG building blocks on the formation and the mechanical properties of assembled PEG-based particles is systemically described. The impact on particle–immune cell interactions in a complex biological environment (e.g., human blood) and the biomolecular corona were also studied, providing a better understanding on how the physicochemical properties of particles modulate bio-nano interactions and physiological responses.

As the bio-nano interactions can be significantly influenced by the physicochemical properties of particles, manipulating the particles with different surface properties can potentially alter their behaviours in a biological system. In Chapter 4, the interaction between mucus and polymer particles with different surface properties is described. The behaviour of mucoadhesive particles and mucus-penetrating particles in mucus forms a preliminary understanding of mucociliary clearance in the lungs.

When particles encounter the surfactant environment in deep lungs, the surface association of the components in pulmonary surfactant will result in a complex pulmonary corona around particles, resulting in an altered particle identity, which will potentially influence the

subsequent particle–cell interactions. Despite the numerous investigations on the interaction of particles with blood plasma, less attention has been paid to lung biological fluid, including the formation of a pulmonary corona. There is little known about the interplay of the pulmonary corona and plasma protein corona on particle–cell interactions during the delivery of particles via different administration routes. Chapter 5 investigated the effects of pulmonary corona on alveolar macrophage clearance of polymer particles. The interplay of plasma protein corona and pulmonary corona on the composition of the mixed protein corona and further macrophage uptake of particles was also studied. The composition of protein coronas on different particle systems was identified by mass spectrometry.

Drug immobilisation and intracellular delivery by using promising carriers (polyphenol-based capsules) are subsequently explored in Chapter 6 to demonstrate the potential application of particle-based therapies against lung infections.

Overall, this thesis covers the engineering of particle systems for pulmonary delivery, including studies on their bio-nano interactions in complex biological environments and the application of particle-based therapeutics as potential therapies against lung infections. The conclusions and outlook are presented in Chapter 7.

1.7 References

1. Weibel, E. R., Morphological basis of alveolar-capillary gas exchange. *Physiol Rev* **1973**, 53 (2), 419-95.
2. Muller, L.; Lehmann, A. D.; Johnston, B. D.; Blank, F.; Wick, P.; Fink, A.; Rothen-Rutishauser, B., Inhalation Pathway as a Promising Portal of Entry: What Has to Be Considered in Designing New Nanomaterials for Biomedical Application? *Handbook of Nanotoxicology, Nanomedicine and Stem Cell Use in Toxicology* **2014**, 205-221.
3. Patton, J. S.; Byron, P. R., Inhaling medicines: delivering drugs to the body through the lungs. *Nat Rev Drug Discov* **2007**, 6 (1), 67.
4. El-Sherbiny, I. M.; Villanueva, D. G.; Herrera, D.; Smyth, H. D. C., Overcoming Lung Clearance Mechanisms for Controlled Release Drug Delivery. *Adv Del Sci Technol* **2011**, 101-126.
5. Patra, J. K.; Das, G.; Fraceto, L. F.; Campos, E. V. R.; Rodriguez-Torres, M. D. P.; Acosta-Torres, L. S.; Diaz-Torres, L. A.; Grillo, R.; Swamy, M. K.; Sharma, S.; Habtemariam,

- S.; Shin, H. S., Nano based drug delivery systems: recent developments and future prospects. *J Nanobiotechnology* **2018**, 16 (1), 71.
6. Allen, T. M.; Cullis, P. R., Drug delivery systems: entering the mainstream. *Science* **2004**, 303 (5665), 1818-22.
 7. Peer, D.; Karp, J. M.; Hong, S.; Farokhzad, O. C.; Margalit, R.; Langer, R., Nanocarriers as an emerging platform for cancer therapy. *Nat Nanotechnol* **2007**, 2 (12), 751-760.
 8. Kamaly, N.; Xiao, Z.; Valencia, P. M.; Radovic-Moreno, A. F.; Farokhzad, O. C., Targeted polymeric therapeutic nanoparticles: design, development and clinical translation. *Chem Soc Rev* **2012**, 41 (7), 2971-3010.
 9. Lombardo, D.; Kiselev, M. A.; Caccamo, M. T., Smart Nanoparticles for Drug Delivery Application: Development of Versatile Nanocarrier Platforms in Biotechnology and Nanomedicine. *J Nanomater* **2019**.
 10. Ince, L. M.; Pariollaud, M.; Gibbs, J. E., Lung physiology and defense. *Current Opinion in Physiology* **2018**, 5, 9-15.
 11. Patwa, A.; Shah, A., Anatomy and physiology of respiratory system relevant to anaesthesia. *Indian J Anaesth* **2015**, 59 (9), 533-41.
 12. Barnes, P. J.; Blasi, F.; Ward, B.; Reeves, E.; Rabe, K. F., Respiratory diseases in the world: one voice "united for lung health". *Eur Respir J* **2014**, 43 (1), 3-5.
 13. Schraufnagel, D., The world respiratory diseases report. *Int J Tuberc Lung Dis* **2013**, 17 (12), 1517.
 14. Harding, E., WHO global progress report on tuberculosis elimination. *Lancet Respir Med* **2020**, 8 (1), 19.
 15. Yorgancioglu, A.; Cruz, A. A.; Bousquet, J.; Khaltayev, N.; Mendis, S.; Chuchalin, A.; Bateman, E. D.; Camargos, P.; Chavannes, N. H.; Bai, C.; Deleanu, D.; Kolek, V.; Kuna, P.; Laurendi, G.; Masjedi, M. R.; Mele, S.; Mihaltan, F.; Pinto, J. R.; Samolinski, B.; Scalera, G.; Sooronbaev, T.; Tageldin, M. A.; Tuyetlan, T.; Yusuf, O.; Akdis, C.; Baigenzhin, A.; Cagnani, C. B.; Fletcher, M.; Gemicioglu, B.; Muhammed, Y.; Sagra, H.; To, T.; Wagner, A. H., The Global Alliance against Respiratory Diseases (GARD) Country Report. *Prim Care Respir J* **2014**, 23 (1), 98-101.
 16. Docnesia, Anatomy & physiology of respiration, <https://docnesia.com/en/anatomifisiologi-respirasi/> (accessed 15 May 2020).
 17. Cuitandokter, Human respiratory system, <https://cuitandokter.com/respiration-in-humans-human-respiratory-system-parts-and/> (accessed 15 May 2020).

18. Kadyk, L. C.; DeWitt, N. D.; Gomperts, B., Proceedings: Regenerative Medicine for Lung Diseases: A CIRM Workshop Report. *Stem Cells Transl Med* **2017**, *6* (10), 1823-1828.
19. Huang, J.; Cheng, Y.; Wu, Y.; Shi, X.; Du, Y.; Deng, H., Chitosan/tannic acid bilayers layer-by-layer deposited cellulose nanofibrous mats for antibacterial application. *Int J Biol Macromol* **2019**, *139*, 191-198.
20. Leung, C. C.; Porcel, J. M.; Takahashi, K.; Restrepo, M. I.; Lee, P.; Wainwright, C., Year in review 2013: Lung cancer, respiratory infections, tuberculosis, cystic fibrosis, pleural diseases, bronchoscopic intervention and imaging. *Respirology* **2014**, *19* (3), 448-60.
21. Takahashi, K.; Porcel, J. M.; Lee, P.; Leung, C. C., Year in review 2014: Lung cancer, pleural diseases, respiratory infections and tuberculosis, bronchoscopic intervention and imaging. *Respirology* **2015**, *20* (4), 674-83.
22. Lee, P.; Leung, C. C.; Restrepo, M. I.; Takahashi, K.; Song, Y.; Porcel, J. M., Year in review 2015: Lung cancer, pleural diseases, respiratory infections, bronchiectasis and tuberculosis, bronchoscopic intervention and imaging. *Respirology* **2016**, *21* (5), 961-7.
23. Restrepo, M. I.; Chalmers, J. D.; Song, Y.; Mallow, C.; Hewlett, J.; Maldonado, F.; Yarmus, L., Year in review 2016: Respiratory infections, acute respiratory distress syndrome, pleural diseases, lung cancer and interventional pulmonology. *Respirology* **2017**, *22* (3), 602-611.
24. Delgado, B. J.; Bajaj, T., Physiology, Lung Capacity. In *StatPearls*, Treasure Island (FL), 2020.
25. Gou, L. Y.; Niu, F. Y.; Wu, Y. L.; Zhong, W. Z., Differences in driver genes between smoking-related and non-smoking-related lung cancer in the Chinese population. *Cancer* **2015**, *121 Suppl 17*, 3069-79.
26. Brescianini, S.; Fagnani, C.; Aquilini, E.; Annesi-Maesano, I.; Stazi, M. A., Early respiratory infections: the role of passive smoking in gene-environment interaction. *Eur J Public Health* **2016**, *26* (3), 401-403.
27. Yang, S. I.; Lee, E.; Jung, Y. H.; Choi, K. Y.; Kang, M. J.; Yu, H. S.; Lee, C. M.; Shin, Y. H.; Ahn, K.; Kim, K. W.; Hong, S. J.; Grp, C. S., Effect Of Prenatal Exposure To Indoor PM2.5 and Environmental Tobacco Smoke Affecting Lower Respiratory Tract Infection Was Modified By ROS Genes: Cocoa Study. *J Allergy Clin Immun* **2014**, *133* (2), Ab285-Ab285.
28. Lung foundation, Lung health, <https://lungfoundation.com.au/patients-carers/lung-health/what-is-lung-disease/> (accessed 15 May 2020).
29. Casas Herrera, A.; Montes de Oca, M.; Lopez Varela, M. V.; Aguirre, C.; Schiavi, E.; Jardim, J. R.; Team, P., COPD Underdiagnosis and Misdiagnosis in a High-Risk Primary Care

Population in Four Latin American Countries. A Key to Enhance Disease Diagnosis: The PUMA Study. *PLoS One* **2016**, *11* (4), e0152266.

30. de Oca, M. M.; Maria, V. L. V. B.; Acuna, A.; Schiavi, E.; Rey, M. A.; Jardim, J.; Casas, A.; Tokumoto, A.; Duque, C. A. T.; Ramirez-Venegas, A.; Garcia, G.; Stirbulov, R.; Camelier, A.; Bergna, M.; Cohen, M.; Guzman, S.; Sanchez, E., ALAT-2014 Chronic Obstructive Pulmonary Disease (COPD) Clinical Practice Guidelines: Questions and Answers. *Archivos De Bronconeumologia* **2015**, *51* (8), 403-416.

31. Montuschi, P.; Ciabattini, G., Bronchodilating drugs for chronic obstructive pulmonary disease: current status and future trends. *J Med Chem* **2015**, *58* (10), 4131-64.

32. Eisner, M. D.; Anthonisen, N.; Coultas, D.; Kuenzli, N.; Perez-Padilla, R.; Postma, D.; Romieu, I.; Silverman, E. K.; Balmes, J. R.; Hlth, E. O., An Official American Thoracic Society Public Policy Statement: Novel Risk Factors and the Global Burden of Chronic Obstructive Pulmonary Disease. *Am J Resp Crit Care* **2010**, *182* (5), 693-718.

33. Fabbri, L. M.; Hurd, S. S.; Comm, G. S., Global strategy for the diagnosis, management and prevention of COPD: 2003. *Eur Respir J* **2003**, *22* (1), 1-2.

34. Lambrecht, B. N.; Hammad, H., The immunology of asthma. *Nat Immunol* **2015**, *16* (1), 45-56.

35. Asher, I.; Pearce, N., Global burden of asthma among children. *Int J Tuberc Lung D* **2014**, *18* (11), 1269-1278.

36. Adcock, I. M.; Caramori, G.; Chung, K. F., New targets for drug development in asthma. *Lancet* **2008**, *372* (9643), 1073-87.

37. Halwani, R.; Sultana Shaik, A.; Ratemi, E.; Afzal, S.; Kenana, R.; Al-Muhsen, S.; Al Faraj, A., A novel anti-IL4Ralpha nanoparticle efficiently controls lung inflammation during asthma. *Exp Mol Med* **2016**, *48* (10), e262.

38. MacKenzie, T.; Gifford, A. H.; Sabadosa, K. A.; Quinton, H. B.; Knapp, E. A.; Goss, C. H.; Marshall, B. C., Longevity of Patients With Cystic Fibrosis in 2000 to 2010 and Beyond: Survival Analysis of the Cystic Fibrosis Foundation Patient Registry. *Annals of Internal Medicine* **2014**, *161* (4), 233-+.

39. Davis, P. B., Cystic fibrosis since 1938. *Am J Resp Crit Care* **2006**, *173* (5), 475-482.

40. Craparo, E. F.; Porsio, B.; Sardo, C.; Giammona, G.; Cavallaro, G., Pegylated Polyaspartamide-Polylactide-Based Nanoparticles Penetrating Cystic Fibrosis Artificial Mucus. *Biomacromolecules* **2016**, *17* (3), 767-77.

41. Alton, E. W. F. W.; Middleton, P. G.; Caplen, N. J.; Smith, S. N.; Steel, D. M.; Munkonge, F. M.; Jeffery, P. K.; Geddes, D. M.; Hart, S. L.; Williamson, R.; Fasold, K. I.;

- Miller, A. D.; Dickinson, P.; Stevenson, B. J.; Mclachlan, G.; Dorin, J. R.; Porteous, D. J., Noninvasive Liposome-Mediated Gene Delivery Can Correct the Ion-Transport Defect in Cystic-Fibrosis Mutant Mice (Vol 5, Pg 135, 1993). *Nat Genet* **1993**, 5 (3), 312-312.
42. Suk, J. S.; Boylan, N. J.; Trehan, K.; Tang, B. C.; Schneider, C. S.; Lin, J. M. G.; Boyle, M. P.; Zeitlin, P. L.; Lai, S. K.; Cooper, M. J.; Hanes, J., N-acetylcysteine Enhances Cystic Fibrosis Sputum Penetration and Airway Gene Transfer by Highly Compacted DNA Nanoparticles. *Mol Ther* **2011**, 19 (11), 1981-1989.
43. Oakland, M.; Sinn, P. L.; McCray, P. B., Advances in Cell and Gene-based Therapies for Cystic Fibrosis Lung Disease. *Mol Ther* **2012**, 20 (6), 1108-1115.
44. Shi, T.; McAllister, D. A.; O'Brien, K. L.; Simoes, E. A. F.; Madhi, S. A.; Gessner, B. D.; Polack, F. P.; Balsells, E.; Acacio, S.; Aguayo, C.; Alassani, I.; Ali, A.; Antonio, M. et al, Global, regional, and national disease burden estimates of acute lower respiratory infections due to respiratory syncytial virus in young children in 2015: a systematic review and modelling study. *Lancet* **2017**, 390 (10098), 946-958.
45. Taneja, J.; Malik, A.; Malik, A.; Rizvi, M.; Agarwal, M., Acute Lower Respiratory Tract Infections in Children. *Indian Pediatrics* **2009**, 46 (6), 509-511.
46. Butler, C. C.; Hood, K.; Kelly, M. J.; Goossens, H.; Verheij, T.; Little, P.; Melbye, H.; Torres, A.; Molstad, S.; Godycki-Cwirko, M.; Almirall, J.; Blasi, F.; Schaberg, T.; Edwards, P.; Rautakorpi, U. M.; Hupkova, H.; Wood, J.; Nuttall, J.; Coenen, S., Treatment of acute cough/lower respiratory tract infection by antibiotic class and associated outcomes: a 13 European country observational study in primary care. *J Antimicrob Chemother* **2010**, 65 (11), 2472-2478.
47. Obodai, E.; Odoom, J. K.; Adiku, T.; Goka, B.; Wolff, T.; Biere, B.; Schweiger, B.; Reiche, J., The significance of human respiratory syncytial virus (HRSV) in children from Ghana with acute lower respiratory tract infection: A molecular epidemiological analysis, 2006 and 2013-2014. *Plos One* **2018**, 13 (9).
48. Yoshihara, K.; Le, M. N.; Okamoto, M.; Wadagni, A. C. A.; Nguyen, H. A.; Toizumi, M.; Pham, E.; Suzuki, M.; Nguyen, A. T. T.; Oshitani, H.; Ariyoshi, K.; Moriuchi, H.; Hashizume, M.; Dang, D. A.; Yoshida, L. M., Association of RSV-A ON1 genotype with Increased Pediatric Acute Lower Respiratory Tract Infection in Vietnam. *Sci Rep* **2016**, 6.
49. Butler, C. C.; Kelly, M. J.; Hood, K.; Schaberg, T.; Melbye, H.; Serra-Prat, M.; Blasi, F.; Little, P.; Verheij, T.; Molstad, S.; Godycki-Cwirko, M.; Edwards, P.; Almirall, J.; Torres, A.; Rautakorpi, U. M.; Nuttall, J.; Goossens, H.; Coenen, S., Antibiotic prescribing for

discoloured sputum in acute cough/lower respiratory tract infection. *Eur Respir J* **2011**, 38 (1), 119-125.

50. Wood, J.; Butler, C. C.; Hood, K.; Kelly, M. J.; Verheij, T.; Little, P.; Torres, A.; Blasi, F.; Schaberg, T.; Goossens, H.; Nuttall, J.; Coenen, S., Antibiotic prescribing for adults with acute cough/lower respiratory tract infection: congruence with guidelines. *Eur Respir J* **2011**, 38 (1), 112-118.

51. Denholm, R.; van der Werf, E. T.; Hay, A. D., Use of antibiotics and asthma medication for acute lower respiratory tract infections in people with and without asthma: retrospective cohort study. *Resp Res* **2020**, 21 (1).

52. Mizgerd, J. P., Mechanisms of disease: Acute lower respiratory tract infection. *New Engl J Med* **2008**, 358 (7), 716-727.

53. Hansen, L. S.; Lykkegaard, J.; Thomsen, J. L.; Hansen, M. P., Acute lower respiratory tract infections: Symptoms, findings and management in Danish general practice. *Eur J Gen Pract* **2020**, 26 (1), 14-20.

54. Barta, J. A.; Powell, C. A.; Wisnivesky, J. P., Global Epidemiology of Lung Cancer. *Ann Glob Health* **2019**, 85 (1).

55. Miranda-Filho, A.; Pineros, M.; Bray, F., The descriptive epidemiology of lung cancer and tobacco control: a global overview 2018. *Salud Publica Mex* **2019**, 61 (3), 219-229.

56. Zabczyk, M.; Krolczyk, G.; Czyzewicz, G.; Plens, K.; Prior, S.; Butenas, S.; Undas, A., Altered fibrin clot properties in advanced lung cancer: strong impact of cigarette smoking. *Med Oncol* **2019**, 36 (4), 37.

57. Schuller, H. M., The impact of smoking and the influence of other factors on lung cancer. *Expert Rev Respir Med* **2019**, 13 (8), 761-769.

58. Lipfert, F. W.; Wyzga, R. E., Longitudinal relationships between lung cancer mortality rates, smoking, and ambient air quality: a comprehensive review and analysis. *Crit Rev Toxicol* **2019**, 49 (9), 790-818.

59. Couraud, S.; Zalcman, G.; Milleron, B.; Morin, F.; Souquet, P. J., Lung cancer in never smokers--a review. *Eur J Cancer* **2012**, 48 (9), 1299-311.

60. Gaughan, E. M.; Cryer, S. K.; Yeap, B. Y.; Jackman, D. M.; Costa, D. B., Family history of lung cancer in never smokers with non-small-cell lung cancer and its association with tumors harboring EGFR mutations. *Lung Cancer* **2013**, 79 (3), 193-7.

61. Zarogoulidis, P.; Chatzaki, E.; Porpodis, K.; Domvri, K.; Hohenforst-Schmidt, W.; Goldberg, E. P.; Karamanos, N.; Zarogoulidis, K., Inhaled chemotherapy in lung cancer: future concept of nanomedicine. *Int J Nanomed* **2012**, 7, 1551-1572.

62. Kim, I.; Byeon, H. J.; Kim, T. H.; Lee, E. S.; Oh, K. T.; Shin, B. S.; Lee, K. C.; Youn, Y. S., Doxorubicin-loaded highly porous large PLGA microparticles as a sustained-release inhalation system for the treatment of metastatic lung cancer. *Biomaterials* **2012**, 33 (22), 5574-5583.
63. Bach, P. B.; Kelley, M. J.; Tate, R. C.; McCrory, D. C., Screening for lung cancer: a review of the current literature. *Chest* **2003**, 123 (1 Suppl), 72S-82S.
64. Hoppentocht, M.; Hagedoorn, P.; Frijlink, H. W.; de Boer, A. H., Developments and strategies for inhaled antibiotic drugs in tuberculosis therapy: a critical evaluation. *Eur J Pharm Biopharm* **2014**, 86 (1), 23-30.
65. Mortensen, N. P.; Durham, P.; Hickey, A. J., The role of particle physico-chemical properties in pulmonary drug delivery for tuberculosis therapy. *J Microencapsul* **2014**, 31 (8), 785-95.
66. Goyal, A. K.; Garg, T.; Bhandari, S.; Rath, G., Advancement in pulmonary drug delivery systems for treatment of tuberculosis. In *Nanostructures for Drug Delivery*, Elsevier: 2017; pp 669-695.
67. Lam, S. J.; Wong, E. H.; Boyer, C.; Qiao, G. G., Antimicrobial polymeric nanoparticles. *Progress in Polymer Science* **2017**.
68. Lam, S. J.; O'Brien-Simpson, N. M.; Pantarat, N.; Sulistio, A.; Wong, E. H.; Chen, Y. Y.; Lenzo, J. C.; Holden, J. A.; Blencowe, A.; Reynolds, E. C.; Qiao, G. G., Combating multidrug-resistant Gram-negative bacteria with structurally nanoengineered antimicrobial peptide polymers. *Nat Microbiol* **2016**, 1 (11), 16162.
69. Shirbin, S. J.; Insua, I.; Holden, J. A.; Lenzo, J. C.; Reynolds, E. C.; O'Brien-Simpson, N. M.; Qiao, G. G., Architectural Effects of Star-Shaped "Structurally Nanoengineered Antimicrobial Peptide Polymers" (SNAPPs) on Their Biological Activity. *Adv Healthc Mater* **2018**, 7 (21), e1800627.
70. Shah, N.; Shah, V.; Chivate, N., Pulmonary drug delivery: a promising approach. *J. Appl Pharm Sci* **2012**, 2 (2).
71. Mangal, S.; Gao, W.; Li, T.; Zhou, Q. T., Pulmonary delivery of nanoparticle chemotherapy for the treatment of lung cancers: challenges and opportunities. *Acta Pharmacol Sin* **2017**, 38 (6), 782-797.
72. Esmailpour, N.; Hogger, P.; Rabe, K. F.; Heitmann, U.; Nakashima, M.; Rohdewald, P., Distribution of inhaled fluticasone propionate between human lung tissue and serum in vivo. *Eur Respir J* **1997**, 10 (7), 1496-1499.

73. Leach, C. L.; Davidson, P. J.; Hasselquist, B. E.; Boudreau, R. J., Lung deposition of hydrofluoroalkane-134a beclomethasone is greater than that of chlorofluorocarbon fluticasone and chlorofluorocarbon beclomethasone - A cross-over study in healthy volunteers. *Chest* **2002**, 122 (2), 510-516.
74. Savla, R.; Minko, T., Nanotechnology approaches for inhalation treatment of fibrosis. *J Drug Target* **2013**, 21 (10), 914-925.
75. Kuzmov, A.; Minko, T., Nanotechnology approaches for inhalation treatment of lung diseases. *J Control Release* **2015**, 219, 500-518.
76. Miragoli, M.; Ceriotti, P.; Iafisco, M.; Vacchiano, M.; Salvarani, N.; Alogna, A.; Carullo, P.; Ramirez-Rodriguez, G. B.; Patricio, T.; Esposti, L. D.; Rossi, F.; Ravanetti, F.; Pinelli, S.; Alinovi, R.; Erreni, M.; Rossi, S.; Condorelli, G.; Post, H.; Tampieri, A.; Catalucci, D., Inhalation of peptide-loaded nanoparticles improves heart failure. *Sci Transl Med* **2018**, 10 (424).
77. Zhong, Q.; Bielski, E. R.; Rodrigues, L. S.; Brown, M. R.; Reineke, J. J.; da Rocha, S. R., Conjugation to Poly(amidoamine) Dendrimers and Pulmonary Delivery Reduce Cardiac Accumulation and Enhance Antitumor Activity of Doxorubicin in Lung Metastasis. *Mol Pharm* **2016**, 13 (7), 2363-75.
78. Germershaus, O.; Schultz, I.; Luhmann, T.; Beck-Broichsitter, M.; Hogger, P.; Meinel, L., Insulin-like growth factor-I aerosol formulations for pulmonary delivery. *Eur J Pharm Biopharm* **2013**, 85 (1), 61-8.
79. Mendivil, C. O.; Teeter, J. G.; Finch, G. L.; Schwartz, P. F.; Riese, R. J.; Brain, J. D., Trough insulin levels in bronchoalveolar lavage following inhaled human insulin (Exubera) in patients with diabetes mellitus. *Diabetes Technol Ther* **2012**, 14 (1), 50-8.
80. Patton, J. S.; Bukar, J. G.; Eldon, M. A., Clinical pharmacokinetics and pharmacodynamics of inhaled insulin. *Clin Pharmacokinet* **2004**, 43 (12), 781-801.
81. Quattrin, T.; Belanger, A.; Bohannon, N. J. V.; Schwartz, S. L.; Grp, E. P. I. S., Efficacy and safety of inhaled insulin (exubera) compared with subcutaneous insulin therapy in patients with type 1 diabetes - Results of a 6-month, randomized, comparative trial. *Diabetes Care* **2004**, 27 (11), 2622-2627.
82. Patton, J. S., Mechanisms of macromolecule absorption by the lungs. *Adv Drug Deliv Rev* **1996**, 19 (1), 3-36.
83. Malam, Y.; Loizidou, M.; Seifalian, A. M., Liposomes and nanoparticles: nanosized vehicles for drug delivery in cancer. *Trends Pharmacol Sci* **2009**, 30 (11), 592-9.

84. Beltran-Gracia, E.; Lopez-Camacho, A.; Higuera-Ciapara, I.; Velazquez-Fernandez, J. B.; Vallejo-Cardona, A. A., Nanomedicine review: clinical developments in liposomal applications. *Cancer Nanotechnol* **2019**, *10* (1).
85. Tanner, P.; Baumann, P.; Enea, R.; Onaca, O.; Palivan, C.; Meier, W., Polymeric vesicles: from drug carriers to nanoreactors and artificial organelles. *Acc Chem Res* **2011**, *44* (10), 1039-49.
86. Ke, W. D.; Li, J. J.; Mohammed, F.; Wang, Y. H.; Tou, K.; Liu, X. Y.; Wen, P. Y.; Kinoh, H.; Anraku, Y.; Chen, H. B.; Kataoka, K.; Ge, Z. S., Therapeutic Polymersome Nanoreactors with Tumor-Specific Activable Cascade Reactions for Cooperative Cancer Therapy. *ACS Nano* **2019**, *13* (2), 2357-2369.
87. Chudasama, V.; Maruani, A.; Caddick, S., Recent advances in the construction of antibody-drug conjugates. *Nat Chem* **2016**, *8* (2), 114-9.
88. Lee, J. J.; Choi, H. J.; Yun, M.; Kang, Y.; Jung, J. E.; Ryu, Y.; Kim, T. Y.; Cha, Y. J.; Cho, H. S.; Min, J. J.; Chung, C. W.; Kim, H. S., Enzymatic prenylation and oxime ligation for the synthesis of stable and homogeneous protein-drug conjugates for targeted therapy. *Angew Chem Int Ed Engl* **2015**, *54* (41), 12020-4.
89. Kedar, U.; Phutane, P.; Shidhaye, S.; Kadam, V., Advances in polymeric micelles for drug delivery and tumor targeting. *Nanomedicine* **2010**, *6* (6), 714-29.
90. Hill, L. K.; Frezzo, J. A.; Katyal, P.; Hoang, D. M.; Gironda, Z. B.; Xu, C.; Xie, X.; Delgado-Fukushima, E.; Wadghiri, Y. Z.; Montclare, J. K., Protein-Engineered Nanoscale Micelles for Dynamic F-19 Magnetic Resonance and Therapeutic Drug Delivery. *ACS Nano* **2019**, *13* (3), 2969-2985.
91. Kesharwani, P.; Jain, K.; Jain, N. K., Dendrimer as nanocarrier for drug delivery. *Prog Polym Sci* **2014**, *39* (2), 268-307.
92. Wang, L.; Shi, C.; Wang, X.; Guo, D.; Duncan, T. M.; Luo, J., Zwitterionic Janus Dendrimer with distinct functional disparity for enhanced protein delivery. *Biomaterials* **2019**, *215*, 119233.
93. Yan, Y.; Lai, Z. W.; Goode, R. J.; Cui, J.; Bacic, T.; Kamphuis, M. M.; Nice, E. C.; Caruso, F., Particles on the move: intracellular trafficking and asymmetric mitotic partitioning of nanoporous polymer particles. *ACS Nano* **2013**, *7* (6), 5558-67.
94. Yan, Y.; Such, G. K.; Johnston, A. P.; Lomas, H.; Caruso, F., Toward therapeutic delivery with layer-by-layer engineered particles. *ACS Nano* **2011**, *5* (6), 4252-7.
95. Yan, Y.; Bjornmalm, M.; Caruso, F., Assembly of Layer-by-Layer Particles and Their Interactions with Biological Systems. *ChemMater* **2014**, *26* (1), 452-460.

96. Majumder, J.; Taratula, O.; Minko, T., Nanocarrier-based systems for targeted and site specific therapeutic delivery. *Adv Drug Deliv Rev* **2019**, *144*, 57-77.
97. Albanese, A.; Tang, P. S.; Chan, W. C., The effect of nanoparticle size, shape, and surface chemistry on biological systems. *Annu Rev Biomed Eng* **2012**, *14*, 1-16.
98. Song, D.; Cui, J.; Ju, Y.; Faria, M.; Sun, H.; Howard, C. B.; Thurecht, K. J.; Caruso, F., Cellular Targeting of Bispecific Antibody-Functionalized Poly(ethylene glycol) Capsules: Do Shape and Size Matter? *ACS Appl Mater Interfaces* **2019**, *11* (32), 28720-28731.
99. Cui, J.; Bjornmalm, M.; Ju, Y.; Caruso, F., Nanoengineering of Poly(ethylene glycol) Particles for Stealth and Targeting. *Langmuir* **2018**, *34* (37), 10817-10827.
100. Guo, P.; Liu, D.; Subramanyam, K.; Wang, B.; Yang, J.; Huang, J.; Auguste, D. T.; Moses, M. A., Nanoparticle elasticity directs tumor uptake. *Nat Commun* **2018**, *9* (1), 130.
101. Cui, J.; Alt, K.; Ju, Y.; Gunawan, S. T.; Braunger, J. A.; Wang, T. Y.; Dai, Y.; Dai, Q.; Richardson, J. J.; Guo, J.; Bjornmalm, M.; Hagemeyer, C. E.; Caruso, F., Ligand-Functionalized Poly(ethylene glycol) Particles for Tumor Targeting and Intracellular Uptake. *Biomacromolecules* **2019**.
102. Engineering of Biomaterials for Drug Delivery Systems: Beyond Polyethylene Glycol. *Woodh Publ Ser Biom* **2018**, 1-389.
103. Cui, J.; Richardson, J. J.; Bjornmalm, M.; Faria, M.; Caruso, F., Nanoengineered Templated Polymer Particles: Navigating the Biological Realm. *Acc Chem Res* **2016**, *49* (6), 1139-48.
104. Babu, A.; Templeton, A. K.; Munshi, A.; Ramesh, R., Nanoparticle-Based Drug Delivery for Therapy of Lung Cancer: Progress and Challenges. *J Nanomater* **2013**.
105. Anselmo, A. C.; Mitragotri, S., Nanoparticles in the clinic. *Bioeng Transl Med* **2016**, *1* (1), 10-29.
106. Wang, A. Z.; Langer, R.; Farokhzad, O. C., Nanoparticle Delivery of Cancer Drugs. *Annu Rev Med* **2012**, *63*, 185-198.
107. Azarmi, S.; Roa, W. H.; Lobenberg, R., Targeted delivery of nanoparticles for the treatment of lung diseases. *Adv Drug Deliv Rev* **2008**, *60* (8), 863-75.
108. Chow, A. H.; Tong, H. H.; Chattopadhyay, P.; Shekunov, B. Y., Particle engineering for pulmonary drug delivery. *Pharm Res* **2007**, *24* (3), 411-37.
109. Chono, S.; Tanino, T.; Seki, T.; Morimoto, K., Efficient drug delivery to alveolar macrophages and lung epithelial lining fluid following pulmonary administration of liposomal ciprofloxacin in rats with pneumonia and estimation of its antibacterial effects. *Drug Dev Ind Pharm* **2008**, *34* (10), 1090-1096.

110. Tagami, T.; Ando, Y.; Ozeki, T., Fabrication of liposomal doxorubicin exhibiting ultrasensitivity against phospholipase A(2) for efficient pulmonary drug delivery to lung cancers. *Int J Pharmaceut* **2017**, *517* (1-2), 35-41.
111. Zarogoulidis, P.; Chatzaki, E.; Porpodis, K.; Domvri, K.; Hohenforst-Schmidt, W.; Goldberg, E. P.; Karamanos, N.; Zarogoulidis, K., Inhaled chemotherapy in lung cancer: future concept of nanomedicine. *Int J Nanomedicine* **2012**, *7*, 1551-72.
112. El-Sherbiny, I. M.; El-Baz, N. M.; Yacoub, M. H., Inhaled nano- and microparticles for drug delivery. *Glob Cardiol Sci Pract* **2015**, *2015*, 2.
113. Beck-Broichsitter, M.; Merkel, O. M.; Kissel, T., Controlled pulmonary drug and gene delivery using polymeric nano-carriers. *J Control Release* **2012**, *161* (2), 214-24.
114. Bobo, D.; Robinson, K. J.; Islam, J.; Thurecht, K. J.; Corrie, S. R., Nanoparticle-based medicines: a review of FDA-approved materials and clinical trials to date. *Pharm Res-Dordr* **2016**, *33* (10), 2373-2387.
115. Paranjpe, M.; Muller-Goymann, C. C., Nanoparticle-mediated pulmonary drug delivery: a review. *Int J Mol Sci* **2014**, *15* (4), 5852-73.
116. Byron, P. R., Prediction of drug residence times in regions of the human respiratory tract following aerosol inhalation. *J Pharm Sci* **1986**, *75* (5), 433-8.
117. Willis, L.; Hayes, D., Jr.; Mansour, H. M., Therapeutic liposomal dry powder inhalation aerosols for targeted lung delivery. *Lung* **2012**, *190* (3), 251-62.
118. Fu, T. T.; Cong, Z. Q.; Zhao, Y.; Chen, W. Y.; Liu, C. Y.; Zheng, Y.; Yang, F. F.; Liao, Y. H., Fluticasone propionate nanosuspensions for sustained nebulization delivery: An in vitro and in vivo evaluation. *Int J Pharm* **2019**, *572*, 118839.
119. Mathpal, D.; Garg, T.; Rath, G.; Goyal, A. K., Development and Characterization of Spray Dried Microparticles for Pulmonary Delivery of Antifungal Drug. *Current Drug Delivery* **2015**, *12* (4), 464-471.
120. Kaur, R.; Garg, T.; Malik, B.; Gupta, U. D.; Gupta, P.; Rath, G.; Goyal, A. K., Development and characterization of spray-dried porous nanoaggregates for pulmonary delivery of anti-tubercular drugs. *Drug Delivery* **2016**, *23* (3), 882-887.
121. Vranic, E.; Sirbubalo, M.; Tucak, A.; Hadziabdic, J.; Rahic, O.; Elezovic, A., Development of Inhalable Dry Gene Powders for Pulmonary Drug Delivery by Spray-Freezing-Drying. *Ifmbe Proc* **2020**, *73*, 533-537.
122. Vehring, R., Pharmaceutical particle engineering via spray drying. *Pharm Res* **2008**, *25* (5), 999-1022.

123. Yang, Y.; Bajaj, N.; Xu, P.; Ohn, K.; Tsifansky, M. D.; Yeo, Y., Development of highly porous large PLGA microparticles for pulmonary drug delivery. *Biomaterials* **2009**, *30* (10), 1947-1953.
124. Wilson, E. M.; Luft, J. C.; DeSimone, J. M., Formulation of High-Performance Dry Powder Aerosols for Pulmonary Protein Delivery. *Pharm Res* **2018**, *35* (10), 195.
125. Respaud, R.; Marchand, D.; Pelat, T.; Tchou-Wong, K. M.; Roy, C. J.; Parent, C.; Cabrera, M.; Guillemain, J.; Mac Loughlin, R.; Levacher, E.; Fontayne, A.; Douziech-Eyrolles, L.; Junqua-Moullet, A.; Guilleminault, L.; Thullier, P.; Guillot-Combe, E.; Vecellio, L.; Heuze-Vourch, N., Development of a drug delivery system for efficient alveolar delivery of a neutralizing monoclonal antibody to treat pulmonary intoxication to ricin. *J Control Release* **2016**, *234*, 21-32.
126. Ju, Y.; Cortez-Jugo, C.; Chen, J.; Wang, T. Y.; Mitchell, A. J.; Tsantikos, E.; Bertleff-Zieschang, N.; Lin, Y. W.; Song, J.; Cheng, Y.; Mettu, S.; Rahim, M. A.; Pan, S.; Yun, G.; Hibbs, M. L.; Yeo, L. Y.; Hagemeyer, C. E.; Caruso, F., Engineering of Nebulized Metal-Phenolic Capsules for Controlled Pulmonary Deposition. *Adv Sci (Weinh)* **2020**, *7* (6), 1902650.
127. Geller, D. E., Comparing clinical features of the nebulizer, metered-dose inhaler, and dry powder inhaler. *Respir Care* **2005**, *50* (10), 1313-21; discussion 1321-2.
128. Patton, J. S.; Brain, J. D.; Davies, L. A.; Fiegel, J.; Gumbleton, M.; Kim, K.-J.; Sakagami, M.; Vanbever, R.; Ehrhardt, C., The particle has landed—characterizing the fate of inhaled pharmaceuticals. *J Aerosol Med Pulm Drug Deliv* **2010**, *23* (S2), S-71-S-87.
129. Chung, K. F.; Seiffert, J.; Chen, S.; Theodorou, I. G.; Goode, A. E.; Leo, B. F.; McGilvery, C. M.; Hussain, F.; Wiegman, C.; Rossios, C.; Zhu, J.; Gong, J.; Tariq, F.; Yufit, V.; Monteith, A. J.; Hashimoto, T.; Skepper, J. N.; Ryan, M. P.; Zhang, J.; Tetley, T. D.; Porter, A. E., Inactivation, Clearance, and Functional Effects of Lung-Instilled Short and Long Silver Nanowires in Rats. *ACS Nano* **2017**, *11* (3), 2652-2664.
130. Xiong, Y.; Gao, W.; Xia, F.; Sun, Y.; Sun, L.; Wang, L.; Ben, S.; Turvey, S. E.; Yang, H.; Li, Q., Peptide-Gold Nanoparticle Hybrids as Promising Anti-Inflammatory Nanotherapeutics for Acute Lung Injury: In Vivo Efficacy, Biodistribution, and Clearance. *Adv Healthc Mater* **2018**, *7* (19), e1800510.
131. Ryan, G. M.; Kaminskas, L. M.; Kelly, B. D.; Owen, D. J.; McIntosh, M. P.; Porter, C. J. H., Pulmonary Administration of PEGylated Polylysine Dendrimers: Absorption from the Lung versus Retention within the Lung Is Highly Size-Dependent. *Mol Pharmaceut* **2013**, *10* (8), 2986-2995.

132. Lai, S. K.; Wang, Y. Y.; Hanes, J., Mucus-penetrating nanoparticles for drug and gene delivery to mucosal tissues. *Adv Drug Deliv Rev* **2009**, 61 (2), 158-71.
133. Ma, J.; Rubin, B. K.; Voynow, J. A., Mucins, Mucus, and Goblet Cells. *Chest* **2018**, 154 (1), 169-176.
134. Atanasova, K. R.; Reznikov, L. R., Strategies for measuring airway mucus and mucins. *Resp Res* **2019**, 20 (1).
135. Lehnert, B. E., Pulmonary and thoracic macrophage subpopulations and clearance of particles from the lung. *Environ Health Perspect* **1992**, 97, 17-46.
136. Geiser, M., Update on Macrophage Clearance of Inhaled Micro- and Nanoparticles. *J Aerosol Med Pulm Drug Deliv* **2010**, 23 (4), 207-217.
137. Stone, K. C.; Mercer, R. R.; Gehr, P.; Stockstill, B.; Crapo, J. D., Allometric Relationships of Cell Numbers and Size in the Mammalian Lung. *Am J Resp Cell Mol* **1992**, 6 (2), 235-243.
138. Beck-Schimmer, B.; Schwendener, R.; Pasch, T.; Reyes, L.; Booy, C.; Schimmer, R. C., Alveolar macrophages regulate neutrophil recruitment in endotoxin-induced lung injury. *Resp Res* **2005**, 6.
139. Craig, A.; Mai, J.; Cai, S. S.; Jeyaseelan, S., Neutrophil Recruitment to the Lungs during Bacterial Pneumonia. *Infect Immun* **2009**, 77 (2), 568-575.
140. Willson, D. F., Aerosolized Surfactants, Anti-Inflammatory Drugs, and Analgesics. *Respir Care* **2015**, 60 (6), 774-90; discussion 790-3.
141. Nel, A. E.; Madler, L.; Velegol, D.; Xia, T.; Hoek, E. M. V.; Somasundaran, P.; Klaessig, F.; Castranova, V.; Thompson, M., Understanding biophysicochemical interactions at the nano-bio interface. *Nat Mater* **2009**, 8 (7), 543-557.
142. Pulido-Reyes, G.; Leganes, F.; Fernandez-Pinas, F.; Rosal, R., Bio-nano interface and environment: A critical review. *Environ Toxicol Chem* **2017**, 36 (12), 3181-3193.
143. Cai, K. M.; Wang, A. Z.; Yin, L. C.; Cheng, J. J., Bio-nano interface: The impact of biological environment on nanomaterials and their delivery properties. *J Control Release* **2017**, 263, 211-222.
144. García-Díaz, M.; Birch, D.; Wan, F.; Nielsen, H. M., The role of mucus as an invisible cloak to transepithelial drug delivery by nanoparticles. *Adv Drug Deliv Rev* **2017**.
145. Groo, A. C.; Lagarce, F., Mucus models to evaluate nanomedicines for diffusion. *Drug Discov Today* **2014**, 19 (8), 1097-108.

146. Murgia, X.; Pawelzyk, P.; Schaefer, U. F.; Wagner, C.; Willenbacher, N.; Lehr, C. M., Size-Limited Penetration of Nanoparticles into Porcine Respiratory Mucus after Aerosol Deposition. *Biomacromolecules* **2016**, *17* (4), 1536-42.
147. Wu, L.; Shan, W.; Zhang, Z.; Huang, Y., Engineering nanomaterials to overcome the mucosal barrier by modulating surface properties. *Adv Drug Deliv Rev* **2018**, *124*, 150-163.
148. Sosnik, A.; das Neves, J.; Sarmiento, B., Mucoadhesive polymers in the design of nano-drug delivery systems for administration by non-parenteral routes: a review. *Prog Polym Sci* **2014**, *39* (12), 2030-2075.
149. Huckaby, J. T.; Lai, S. K., PEGylation for enhancing nanoparticle diffusion in mucus. *Adv Drug Deliv Rev* **2018**, *124*, 125-139.
150. Khutoryanskiy, V. V., Beyond PEGylation: Alternative surface-modification of nanoparticles with mucus-inert biomaterials. *Adv Drug Deliv Rev* **2018**, *124*, 140-149.
151. Schneider, C. S.; Xu, Q.; Boylan, N. J.; Chisholm, J.; Tang, B. C.; Schuster, B. S.; Henning, A.; Ensign, L. M.; Lee, E.; Adstamongkonkul, P., Nanoparticles that do not adhere to mucus provide uniform and long-lasting drug delivery to airways following inhalation. *Sci Adv* **2017**, *3* (4), e1601556.
152. Walker, D.; Kasdorf, B. T.; Jeong, H. H.; Lieleg, O.; Fischer, P., Enzymatically active biomimetic micropropellers for the penetration of mucin gels. *Sci Adv* **2015**, *1* (11), e1500501.
153. Walkey, C. D.; Olsen, J. B.; Guo, H.; Emili, A.; Chan, W. C., Nanoparticle size and surface chemistry determine serum protein adsorption and macrophage uptake. *J Am Chem Soc* **2012**, *134* (4), 2139-47.
154. Wang, Y. Y.; Lai, S. K.; So, C.; Schneider, C.; Cone, R.; Hanes, J., Mucoadhesive Nanoparticles May Disrupt the Protective Human Mucus Barrier by Altering Its Microstructure. *Plos One* **2011**, *6* (6).
155. McGill, S. L.; Smyth, H. D. C., Disruption of the Mucus Barrier by Topically Applied Exogenous Particles. *Mol Pharmaceut* **2010**, *7* (6), 2280-2288.
156. Poinard, B.; Kamaluddin, S.; Tan, A. Q. Q.; Neoh, K. G.; Kah, J. C. Y., Polydopamine Coating Enhances Mucopenetration and Cell Uptake of Nanoparticles. *ACS Appl Mater Interfaces* **2019**, *11* (5), 4777-4789.
157. Porsio, B.; Craparo, E. F.; Mauro, N.; Giammona, G.; Cavallaro, G., Mucus and Cell-Penetrating Nanoparticles Embedded in Nano-into-Micro Formulations for Pulmonary Delivery of Ivacaftor in Patients with Cystic Fibrosis. *ACS Appl Mater Interfaces* **2018**, *10* (1), 165-181.

158. Macierzanka, A.; Mackie, A. R.; Bajka, B. H.; Rigby, N. M.; Nau, F.; Dupont, D., Transport of particles in intestinal mucus under simulated infant and adult physiological conditions: impact of mucus structure and extracellular DNA. *PLoS One* **2014**, 9 (4), e95274.
159. Hirose, R.; Nakaya, T.; Naito, Y.; Daidoji, T.; Watanabe, Y.; Yasuda, H.; Konishi, H.; Itoh, Y., Viscosity is an important factor of resistance to alcohol-based disinfectants by pathogens present in mucus. *Sci Rep* **2017**, 7 (1), 13186.
160. Griessinger, J.; Dunnhaupt, S.; Cattoz, B.; Griffiths, P.; Oh, S.; Borros i Gomez, S.; Wilcox, M.; Pearson, J.; Gumbleton, M.; Abdulkarim, M.; Pereira de Sousa, I.; Bernkop-Schnurch, A., Methods to determine the interactions of micro- and nanoparticles with mucus. *Eur J Pharm Biopharm* **2015**, 96, 464-76.
161. Oh, S.; Wilcox, M.; Pearson, J. P.; Borros, S., Optimal design for studying mucoadhesive polymers interaction with gastric mucin using a quartz crystal microbalance with dissipation (QCM-D): Comparison of two different mucin origins. *Eur J Pharm Biopharm* **2015**, 96, 477-83.
162. Feiler, A. A.; Sahlholm, A.; Sandberg, T.; Caldwell, K. D., Adsorption and viscoelastic properties of fractionated mucin (BSM) and bovine serum albumin (BSA) studied with quartz crystal microbalance (QCM-D). *J Colloid Interface Sci* **2007**, 315 (2), 475-81.
163. Poinard, B.; Kamaluddin, S.; Tan, A. Q. Q.; Neoh, K. G.; Kah, J. C. Y., Polydopamine Coating Enhances Mucopenetration and Cell Uptake of Nanoparticles. *Acs Appl Mater Inter* **2019**, 11 (5), 4777-4789.
164. Ensign, L. M.; Tang, B. C.; Wang, Y. Y.; Tse, T. A.; Hoen, T.; Cone, R.; Hanes, J., Mucus-penetrating nanoparticles for vaginal drug delivery protect against herpes simplex virus. *Sci Transl Med* **2012**, 4 (138), 138ra79.
165. Wu, N. H.; Yang, W.; Beineke, A.; Dijkman, R.; Matrosovich, M.; Baumgartner, W.; Thiel, V.; Valentin-Weigand, P.; Meng, F.; Herrler, G., The differentiated airway epithelium infected by influenza viruses maintains the barrier function despite a dramatic loss of ciliated cells. *Sci Rep* **2016**, 6, 39668.
166. Xia, Y. C.; Radwan, A.; Keenan, C. R.; Langenbach, S. Y.; Li, M.; Radojicic, D.; Londrigan, S. L.; Gualano, R. C.; Stewart, A. G., Glucocorticoid insensitivity in virally infected airway epithelial cells is dependent on transforming growth factor- β activity. *PLoS pathogens* **2017**, 13 (1), e1006138.
167. Lin, H.; Li, H.; Cho, H. J.; Bian, S.; Roh, H. J.; Lee, M. K.; Kim, J. S.; Chung, S. J.; Shim, C. K.; Kim, D. D., Air-liquid interface (ALI) culture of human bronchial epithelial cell

monolayers as an in vitro model for airway drug transport studies. *J Pharm Sci* **2007**, 96 (2), 341-50.

168. Friedl, H.; Dunnhaupt, S.; Hintzen, F.; Waldner, C.; Parikh, S.; Pearson, J. P.; Wilcox, M. D.; Bernkop-Schnurch, A., Development and Evaluation of a Novel Mucus Diffusion Test System Approved by Self-Nanoemulsifying Drug Delivery Systems. *J Pharm Sci* **2013**, 102 (12), 4406-4413.

169. Stemcell, Air-liquid interface culture, <https://www.stemcell.com/air-liquid-interface-culture-respiratory-research-lp.html> (accessed 15 May 2020).

170. Ke, P. C.; Lin, S.; Parak, W. J.; Davis, T. P.; Caruso, F., A Decade of the Protein Corona. *ACS Nano* **2017**, 11 (12), 11773-11776.

171. Monopoli, M. P.; Aberg, C.; Salvati, A.; Dawson, K. A., Biomolecular coronas provide the biological identity of nanosized materials. *Nat Nanotechnol* **2012**, 7 (12), 779-86.

172. Yan, Y.; Gause, K. T.; Kamphuis, M. M.; Ang, C. S.; O'Brien-Simpson, N. M.; Lenzo, J. C.; Reynolds, E. C.; Nice, E. C.; Caruso, F., Differential roles of the protein corona in the cellular uptake of nanoporous polymer particles by monocyte and macrophage cell lines. *ACS Nano* **2013**, 7 (12), 10960-70.

173. Giulimondi, F.; Digiacomo, L.; Pozzi, D.; Palchetti, S.; Vulpis, E.; Capriotti, A. L.; Chiozzi, R. Z.; Lagana, A.; Amenitsch, H.; Masuelli, L.; Mahmoudi, M.; Screpanti, I.; Zingoni, A.; Caracciolo, G., Interplay of protein corona and immune cells controls blood residency of liposomes. *Nat Commun* **2019**, 10 (1), 3686.

174. Saha, K.; Rahimi, M.; Yazdani, M.; Kim, S. T.; Moyano, D. F.; Hou, S.; Das, R.; Mout, R.; Rezaee, F.; Mahmoudi, M.; Rotello, V. M., Regulation of Macrophage Recognition through the Interplay of Nanoparticle Surface Functionality and Protein Corona. *ACS Nano* **2016**, 10 (4), 4421-30.

175. Docter, D.; Westmeier, D.; Markiewicz, M.; Stolte, S.; Knauer, S. K.; Stauber, R. H., The nanoparticle biomolecule corona: lessons learned - challenge accepted? *Chem Soc Rev* **2015**, 44 (17), 6094-6121.

176. Dai, Q.; Guo, J.; Yan, Y.; Ang, C. S.; Bertleff-Zieschang, N.; Caruso, F., Cell-Conditioned Protein Coronas on Engineered Particles Influence Immune Responses. *Biomacromolecules* **2017**, 18 (2), 431-439.

177. Jiang, Y.; Huo, S.; Mizuhara, T.; Das, R.; Lee, Y. W.; Hou, S.; Moyano, D. F.; Duncan, B.; Liang, X. J.; Rotello, V. M., The Interplay of Size and Surface Functionality on the Cellular Uptake of Sub-10 nm Gold Nanoparticles. *ACS Nano* **2015**, 9 (10), 9986-93.

178. Lundqvist, M.; Stigler, J.; Cedervall, T.; Berggard, T.; Flanagan, M. B.; Lynch, I.; Elia, G.; Dawson, K., The evolution of the protein corona around nanoparticles: a test study. *ACS Nano* **2011**, 5 (9), 7503-9.
179. Dell'Orco, D.; Lundqvist, M.; Oslakovic, C.; Cedervall, T.; Linse, S., Modeling the time evolution of the nanoparticle-protein corona in a body fluid. *PLoS One* **2010**, 5 (6), e10949.
180. Digiacomo, L.; Pozzi, D.; Palchetti, S.; Zingoni, A.; Caracciolo, G., Impact of the protein corona on nanomaterial immune response and targeting ability. *Wiley Interdiscip Rev Nanomed Nanobiotechnol* **2020**, e1615.
181. Raesch, S. S.; Tenzer, S.; Storck, W.; Rurainski, A.; Selzer, D.; Ruge, C. A.; Perez-Gil, J.; Schaefer, U. F.; Lehr, C. M., Proteomic and Lipidomic Analysis of Nanoparticle Corona upon Contact with Lung Surfactant Reveals Differences in Protein, but Not Lipid Composition. *ACS Nano* **2015**, 9 (12), 11872-85.
182. Ruge, C. A.; Schaefer, U. F.; Herrmann, J.; Kirch, J.; Canadas, O.; Echaide, M.; Perez-Gil, J.; Casals, C.; Muller, R.; Lehr, C. M., The interplay of lung surfactant proteins and lipids assimilates the macrophage clearance of nanoparticles. *PLoS One* **2012**, 7 (7), e40775.
183. Whitwell, H.; Mackay, R.-M.; Elgy, C.; Morgan, C.; Griffiths, M.; Clark, H.; Skipp, P.; Madsen, J., Nanoparticles in the lung and their protein corona: the few proteins that count. *Nanotoxicology* **2016**, 10 (9), 1385-1394.

Chapter 2

Instrumentation and Techniques

Chapter 2. Instrumentation and Techniques	51
2.1 Aim.....	53
2.2 Instrumentation and Techniques	53
2.2.1 Differential Interference Contrast (DIC) Microscopy	53
2.2.2 Fluorescence Microscopy	54
2.2.3 Confocal Laser Scanning Microscopy (CLSM)	57
2.2.4 Transmission Electron Microscopy (TEM).....	59
2.2.5 Scanning Electron Microscopy (SEM).....	60
2.2.6 Atomic Force Microscopy (AFM).....	61
2.2.7 Ultraviolet–Visible (UV–vis) Spectroscopy.....	63
2.2.8 Fluorescence Spectroscopy.....	64
2.2.9 Dynamic Light Scattering (DLS)	65
2.2.10 Microelectrophoresis	66
2.2.11 Quartz Crystal Microbalance with Dissipation Monitoring (QCM-D)	68
2.2.12 Flow Cytometry	70
2.2.13 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)	71
2.2.14 Mass Spectrometry	72
2.2.15 Nebulisation.....	75
2.3 References	75

2.1 Aim

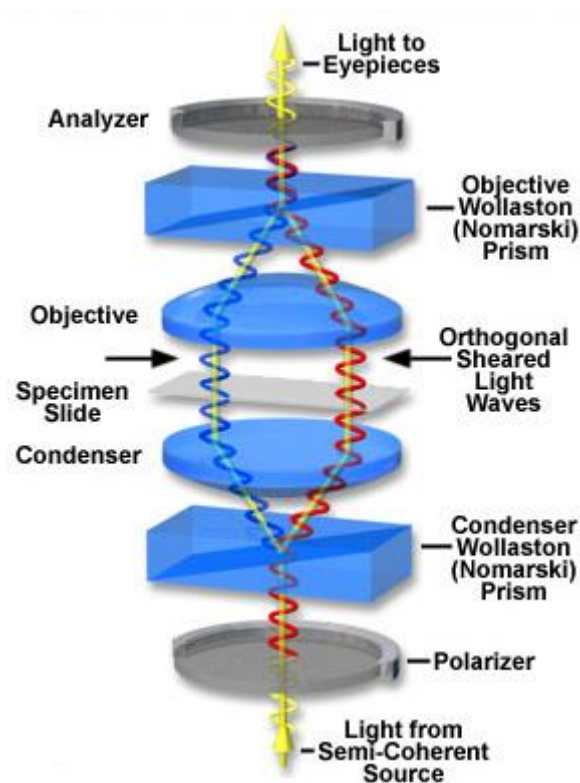
This chapter provides an overview of the major instrumentation and techniques used in this thesis, including basic theoretical principles, general operation procedures, and their application in material characterisation. Specific instruments and detailed experimental protocols are presented in the experimental section of respective chapters.

2.2 Instrumentation and Techniques

2.2.1 Differential Interference Contrast (DIC) Microscopy

DIC microscopy is an optical microscopy technique that is typically used to enhance the contrast of unstained and transparent specimens to achieve improved visualisation in bright field mode using a beam-shearing interface system. Different from a conventional bright-field microscope in which visible light illuminates the samples and traverses a set of optical lenses, resulting in a magnified image of the samples, in a DIC microscope, light produced by the light source (illuminator) is polarised at 45° after passing through a polarising filter. The polarised light is then separated into two beams polarised at 90° relative to each other after entering the first Nomarski (modified Wollaston) prism. The two beams (a sampling beam and a reference beam) are focused by the condenser and then traverse two adjacent points in the sample that are approximately $0.2\ \mu\text{m}$ apart from each other. The two beams encounter different optical path lengths due to different refractive indexes or thicknesses, leading to a change in phase of one beam relative to the other. The two beams are subsequently focused by an objective lens and go through the second Nomarski prism, leading to a recombined light polarised at 135° . The combination of the two beams results in an interference that enhances the contrast of the image by brightening or darkening the image at some points based on the different optical paths (Scheme 2.1).¹

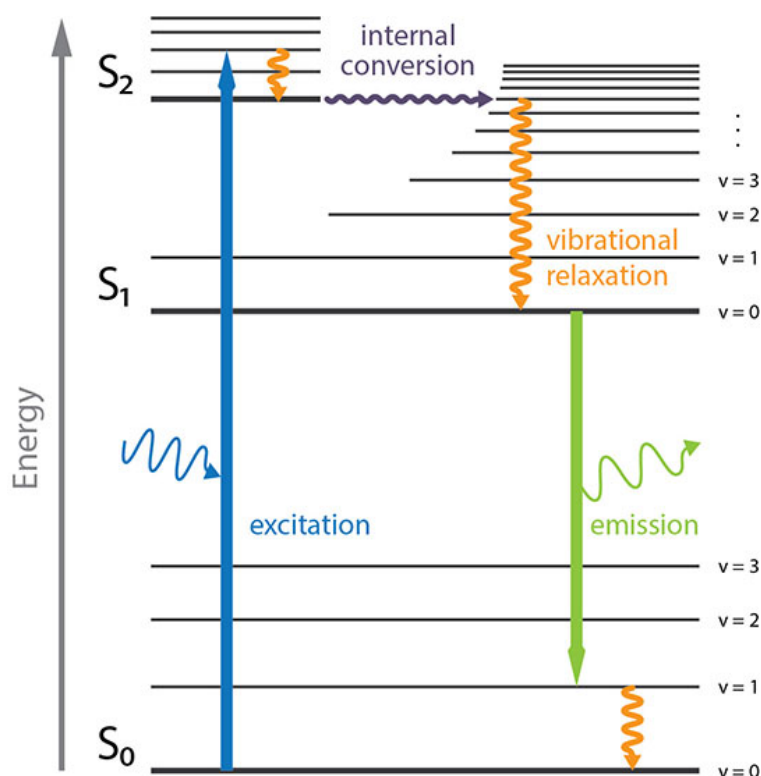
In this thesis, a DIC microscope was used for the imaging of polyphenol-based capsules (Chapter 6).



Scheme 2.1. Simplified illustration of the basic components and principle of a DIC microscope. The figure is reproduced from reference 2.

2.2.2 Fluorescence Microscopy

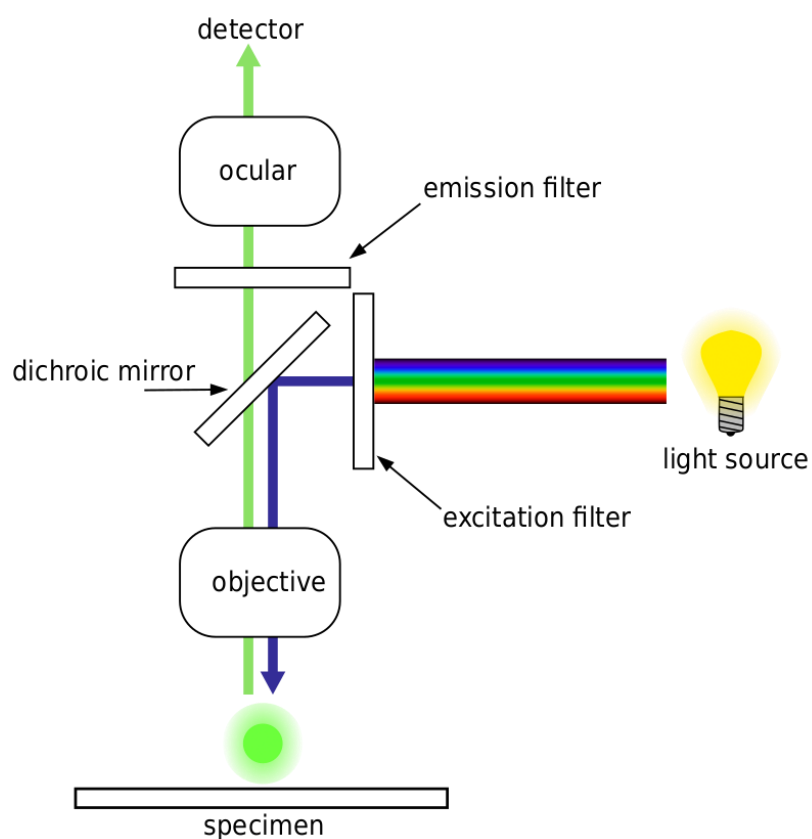
Fluorescence microscopy is an optical microscopy technique that is used for the imaging of fluorescent samples either with auto-fluorescence or fluorescent labelling, following a similar operating principle to bright-field microscopy. When a fluorophore is excited upon absorption of photons from an excitation light, the electrons jump to the highest excited energy state (S_2) from the ground state (S_0). However, owing to instability at the high-energy level, the fluorophore returns to a lower excited state (S_1) by internal conversion and vibrational relaxation. Further relaxation from S_1 to the ground state (S_0) results in the emission of fluorescence (Scheme 2.2). The loss of energy during the short excited state lifetime results in a lower energy and a longer wavelength of the emitted light when compared with that of the excited light, allowing distinction between the two of light irradiation.³⁻⁴



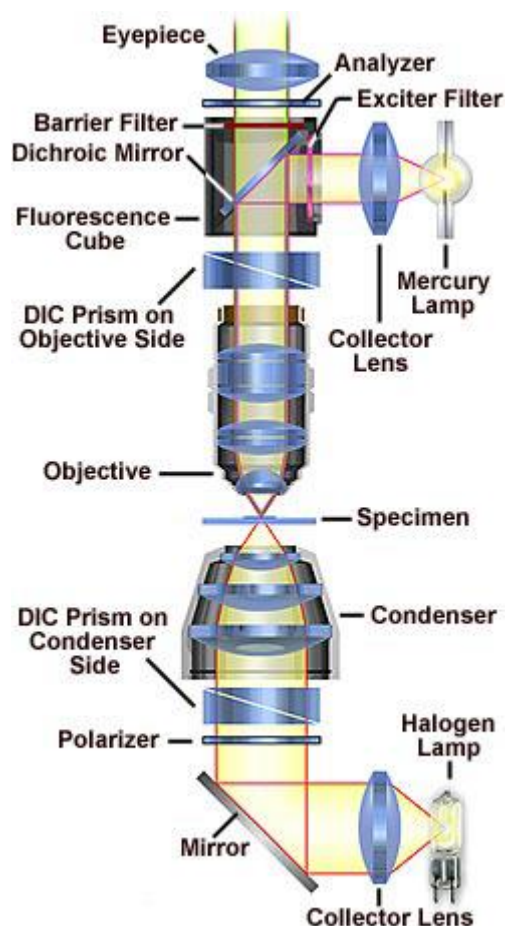
Scheme 2.2. Simplified Jablonski diagram of fluorescence photoluminescence. An electron is excited by an excitation light, followed by internal conversion and vibrational relaxation to the lowest excited state (S_1) owing to instability at the high-energy state. Further relaxation to the ground state (S_0) results in the emission of fluorescence light. The figure is reproduced from reference 3.

A typical fluorescence microscope comprises a light source, an emission filter, a dichroic mirror, an excitation filter, and a set of lenses. The sample is illuminated by a light with a specific excitation wavelength of the fluorophores associated with the sample; this is achieved by filtering the light generated from the light source (e.g., xenon-arc lamp or mercury-vapor lamp) with a set of emission filter and dichroic mirror. The fluorophore is excited after absorbing the excitation light, resulting in the emission of fluorescence light, as illustrated in Scheme 2.2.⁵ The emission light is then separated from the excitation light by passing through the dichroic mirror and emission filter, which are used to block the incident excitation light, and finally passes through a set of lenses for focus and magnification of the image (Scheme 2.3). A digital image is obtained by capturing the light with a digital camera, and multi-colour images resulting from various fluorophores can be produced by overlaying several mono-colour images.⁵

In this thesis, a combined DIC/fluorescence microscope, as illustrated in Scheme 2.4, was used for the imaging of fluorescently labelled polymer particles (Chapters 3–5) and polyphenol-based capsules (Chapter 6).



Scheme 2.3. Simplified illustration of the basic components and principle of a fluorescence microscope. The figure is reproduced from reference 5.



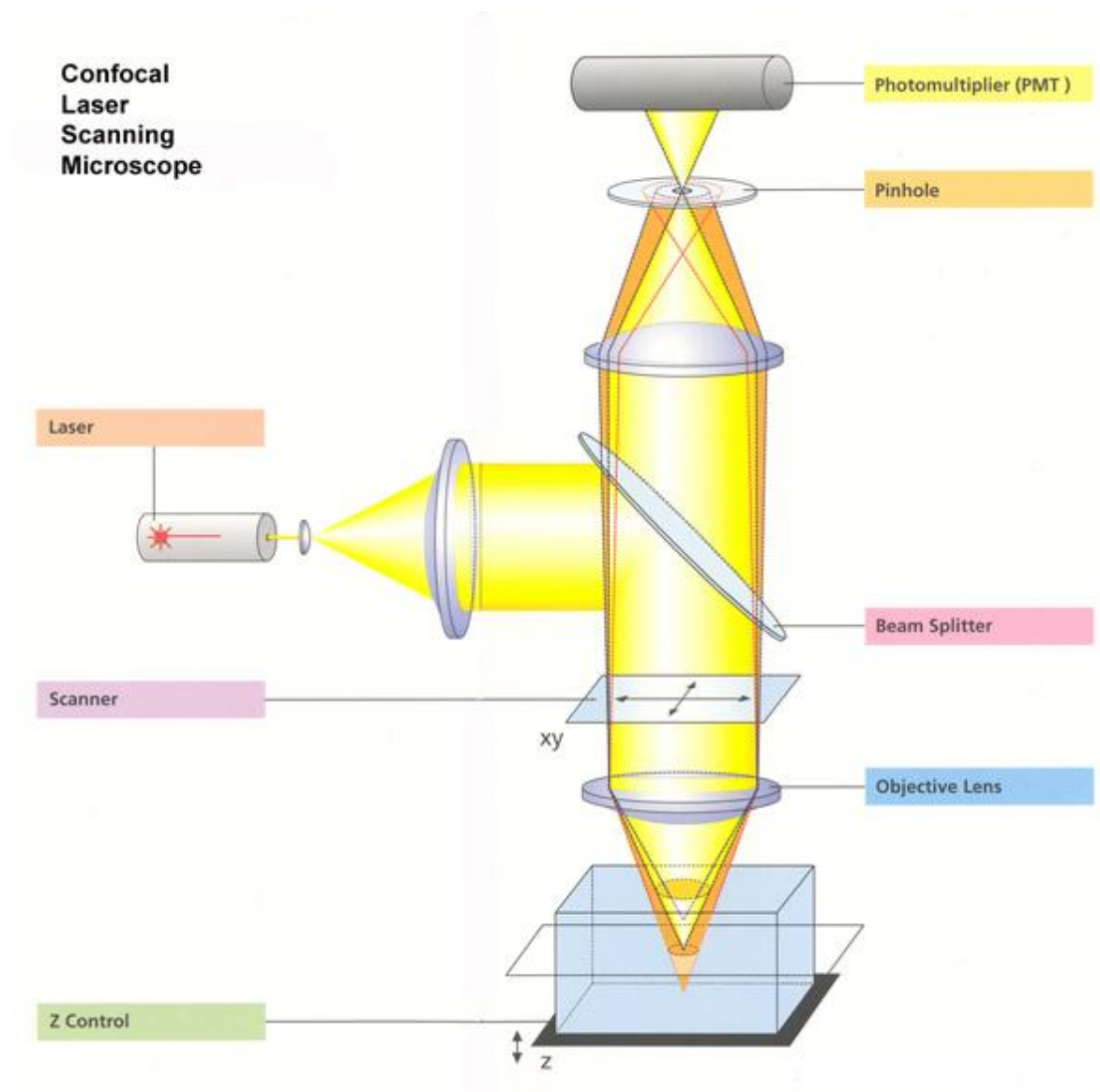
Scheme 2.4. Simplified illustration of the basic components and principle of a DIC/fluorescence microscope configuration. The figure is reproduced from reference 6.

2.2.3 Confocal Laser Scanning Microscopy (CLSM)

CLSM is an advanced optical imaging technique for detecting fluorescent molecules in three dimensions. A higher resolution and better focus than that achieved by conventional fluorescence microscopy can be attained by using a spatial pinhole between the photon detector and dichroic mirror (Scheme 2.5) to exclude out-of-focus light in image formation.⁷ In a conventional microscope, light passes through the sample as deep as it can penetrate, whereas in a confocal laser scanning microscope, a smaller beam of light is focused to a particular depth level and thus only the focused plane of the sample is imaged. A three-dimensional (3D) structure of the sample can be constructed by scanning the sample at different depths to gather multiple two-dimensional (2D) images. CLSM is particularly useful and extensively used in life sciences, especially for the imaging of particle–cell interactions including the cellular binding and uptake of particles. In addition, the permeability of capsules can be easily

determined by using CLSM due to the lower amount of out-of-focus light relative to that obtained from a conventional fluorescence microscope.

In this thesis, the imaging of particle–cell interactions (Chapters 3–6) and the capsule permeability measurements (Chapter 6) were conducted using CLSM.

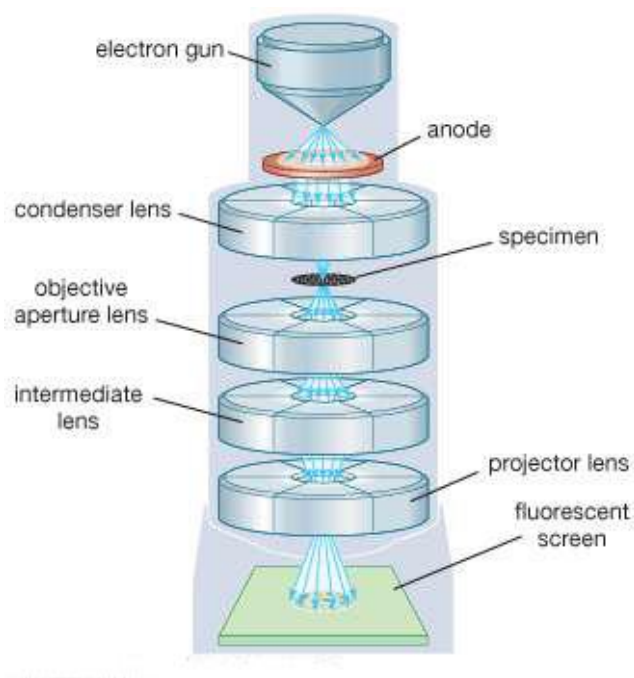


Scheme 2.5. Simplified illustration of the setup of a confocal laser scanning microscope. The figure is adapted from reference 8.

2.2.4 Transmission Electron Microscopy (TEM)

TEM follows a similar working principle to that of light microscopy but can achieve significantly higher magnification and resolution by using a beam of electrons (instead of light) transmitting through a specimen.⁹ A typical transmission electron microscope comprises an electron emission source (electron gun), a set of electromagnetic lenses, and an electron sensor for imaging such as a fluorescent screen (Scheme 2.6). High-energy electron beams generated from an electron gun which is connected to a high voltage source (typically ~100–300 kV) at the top of the microscope are accelerated by an electric potential and subsequently focused by a set of electrostatic and electromagnetic lenses onto the sample after passing through the vacuum in the column of the microscope. TEM samples are generally prepared as ultrathin sections (<100 nm thick) or by dropping a suspension on a grid to reduce the reflection of electrons, thus allowing for the transmission of electrons.¹⁰ The amount of transmitted electrons is different for different parts of the sample based on the thickness, density, and structure of each part. The transmitted electrons hit a fluorescent screen at the bottom of the microscope and are projected for visualisation. A ‘shadow image’ of the sample with distinct darkness and brightness in different parts based on the amount of transmitted electrons is generated containing detailed structure information of the sample.¹¹

In this thesis, the morphology and structure of mesoporous silica particles, various polymer particles (Chapters 3–5), calcium carbonate particles, and polyphenol-based capsules (Chapter 6) were characterised by TEM.

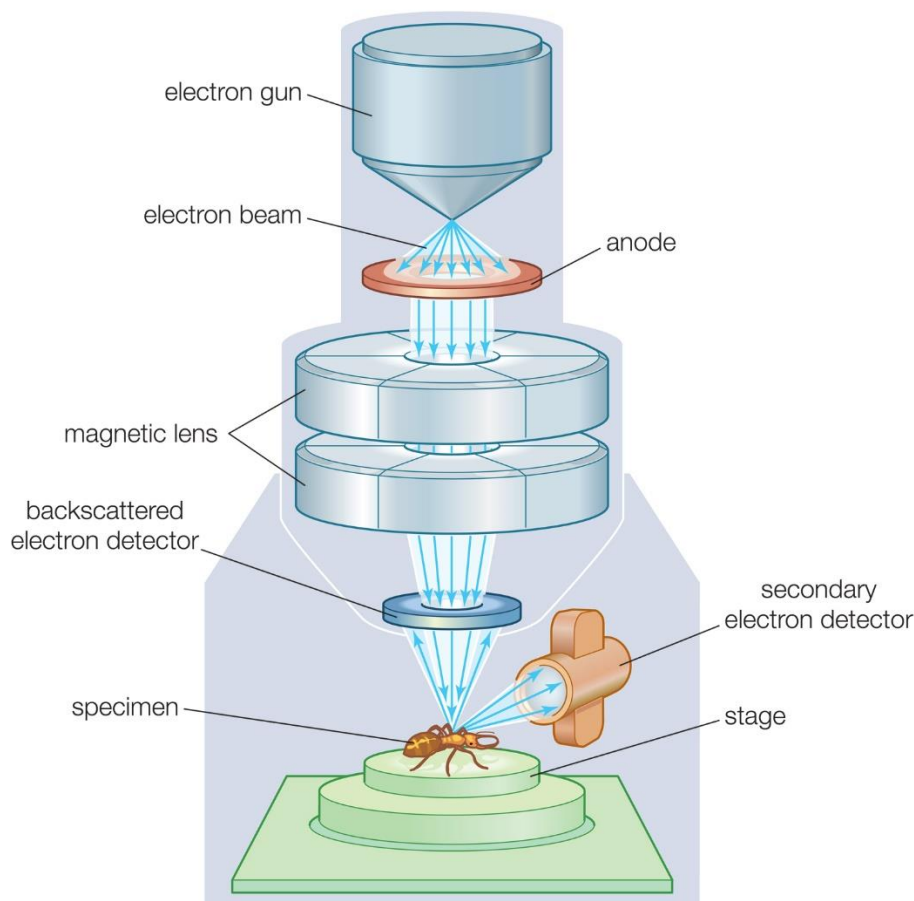


Scheme 2.6. Simplified illustration of the setup of a transmission electron microscope. The figure is reproduced from reference 11.

2.2.5 Scanning Electron Microscopy (SEM)

SEM is a commonly used microscopy technique for analysing the surface morphology of a sample. The setup of a scanning electron microscope is similar to that of a transmission electron microscope and consists of an electron gun, a set of electromagnetic lenses and an electron detector for imaging such as a fluorescent screen (Scheme 2.7). The major difference between SEM and TEM is the type of electrons used to generate the images. An SEM image is produced from the emitted secondary electrons by scanning the sample surface with a focused beam of electrons. In SEM, the electron beams generated from an electron gun and directed by a set of lens are scattered on the surface when interacting with the sample, resulting in the emission of secondary electrons which are subsequently detected by an electron detector and projected for visualisation.¹² The diverse types of secondary electron signals collected, including those differing in intensity, represent the surface morphology and composition information of the sample. SEM can achieve a typical resolution of 1 nm. However, sample pre-treatments are required as SEM analysis requires a conductive sample surface. To achieve this, the sample is typically sputter-coated with gold or platinum to enhance its surface electrical conductivity.

In this thesis, the surface morphology of mesoporous silica particles (Chapter 3–5), calcium carbonate particles and polyphenol-based capsules (Chapter 6) was measured using SEM.



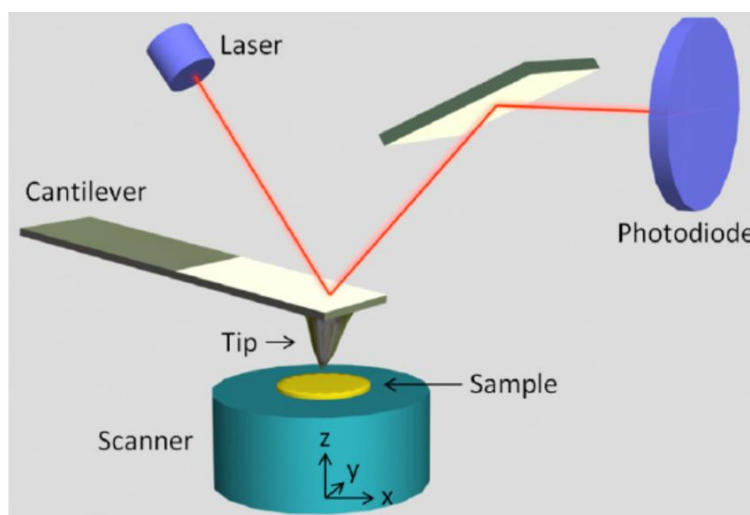
Scheme 2.7. Simplified illustration of the setup of a scanning electron microscope. The figure is reproduced from reference 13.

2.2.6 Atomic Force Microscopy (AFM)

AFM is a type of scanning probe microscopy with extremely high resolution that can be used for topographic imaging (to characterise the 3D structure of a sample surface), force measurements (to determine the mechanical properties, such as the Young's modulus and stiffness, of a sample) and manipulation (for microfabrication of a structure) by 'feeling' or 'touching' the sample surface with a mechanical tip.¹⁴ An atomic force microscope typically consists of (1) a small spring-like cantilever with a sharp tip attached to its free end that acts as a probe to scan the sample surface, (2) a sample stage, (3) a Piezoelectric element to oscillate

cantilever at its eigen frequency, (4) a laser source to generate a laser beam directed onto the top surface of the cantilever, (5) a position-sensitive photodetector to collect the reflected laser for recording deflection and motion of the cantilever, (6) an xyz drive to allow sample movement and (7) a set of mirrors and lenses (Scheme 2.8).¹⁵ The operating principle of an atomic force microscope is based on the interactions between the tip and the sample surface. When the tip approaches the sample surface, the forces between the tip and the sample surface (e.g., mechanical contact force, van der Waals forces, capillary forces, chemical bonding, electrostatic forces, and solvation forces) result in deflection of the cantilever based on Hooke's law. A laser beam generated from the laser source is directed onto the top surface of the cantilever, and the reflected laser is collected by a position-sensitive photodetector while the cantilever scans the sample surface to record the deflection and motion of the cantilever; these changes translate to information of the forces experienced by the cantilever and the surface features of the sample.¹⁴ Generally, two operating modes termed the contact mode and tapping mode are used in AFM measurements. The tapping mode in which the tip is oscillated at a frequency and has little contact with the sample; it is more commonly used than the contact mode owing to reduced damage to the sample.¹⁵

In this thesis, the morphology, structure and thickness of various polymer particles (Chapters 3–5) and polyphenol-based capsules (Chapter 6) were analysed by AFM. The Young's modulus of poly(ethylene glycol) particles (Chapter 3) was evaluated via AFM force measurements.



Scheme 2.8. Typical configuration of an atomic force microscope. The figure is reproduced from reference 15.

2.2.7 Ultraviolet–Visible (UV–vis) Spectroscopy

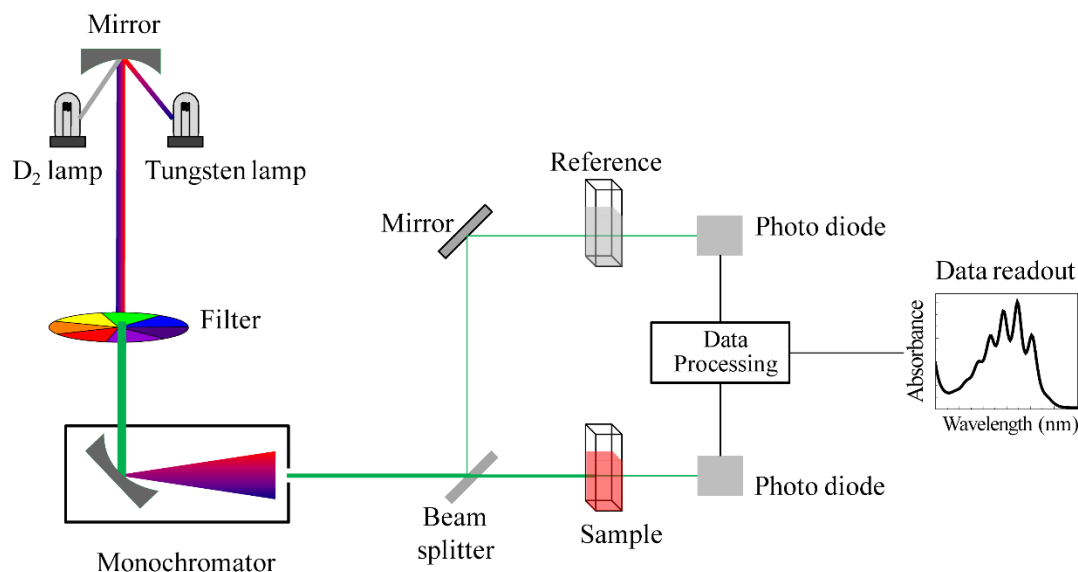
UV–vis spectroscopy is a spectroscopic technique routinely used to qualitatively or quantitatively analyse the chemical information of a specimen based on the absorption of light in the ultraviolet–visible region (wavelength ranging from 200 to 800 nm). In general, the absorption of ultraviolet or visible light by a specimen containing bonding and non-bonding electrons leads to the excitation of these electrons from the ground state to an excited state. The energy gap between the highest occupied molecular orbital and the lowest occupied molecular orbital determines the wavelength of light that can be absorbed, which is related to the molecular structure of the specimen.¹⁶ The absorption varying from different wavelength results in an absorption spectrum of the specimen that can be used for the characterisation of molecular compounds and quantification of the concentration of a specimen.

In a typical UV–vis spectrophotometer, light generated from a light source is diffracted by passing through a diffraction grating monochromator, resulting in monochromatic light at a single wavelength. The monochromatic light then passes through the sample solution in the cuvette resulting in reduced intensity due to the absorption of light by the sample. The transmitted light is then detected and recorded by a detector. The transmittance of the light can be converted into absorbance, which can be used to determine the concentration of the absorbing compound in the sample solution using the Beer–Lambert Law (Equation 2.1):¹⁶

$$A = \log_{10} \left(\frac{I_0}{I} \right) = \varepsilon c L \quad (\text{Equation 2.1})$$

where A is the measured absorbance at a specific wavelength in arbitrary units (a.u.), I_0 is the intensity of the incident light, I is the intensity of the transmitted light, ε ($\text{L mol}^{-1} \text{ cm}^{-1}$) is the extinction coefficient of the absorbing compound, c (mol L^{-1}) is the sample concentration in the solution, and L (cm) is the path length of the light passing through the sample in the cuvette.

In this thesis, UV–vis spectroscopy was used to determine the thiol-modification degree (Chapters 4 and 5), concentration of structurally nanoengineered antimicrobial peptide polymers (SNAPPs) and cell viability (Chapter 6).



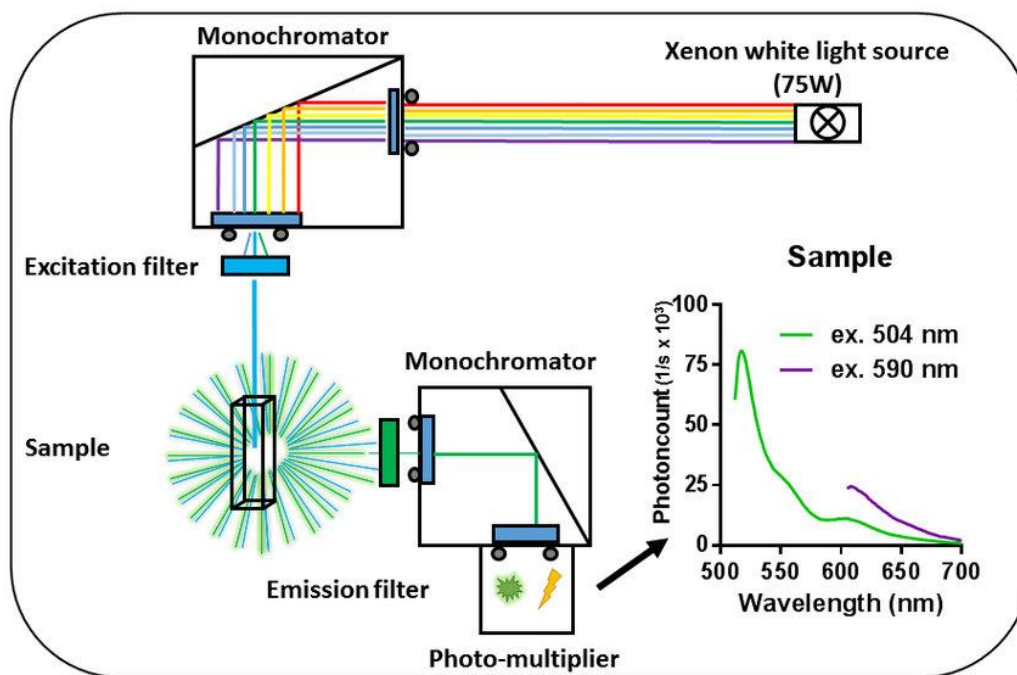
Scheme 2.9. Simplified illustration of a typical UV–vis spectrophotometer. The figure is reproduced from reference 17.

2.2.8 Fluorescence Spectroscopy

Fluorescence spectroscopy is an electromagnetic spectroscopy typically used to analyse the fluorescence of a sample. Complementary to absorption spectroscopy, which analyses the transition of electrons from the ground state to the excited state, fluorescence spectroscopy detects transitions from the excited state to the ground state (Scheme 2.2) by exciting the electrons in the molecules of a certain specimen with a beam of light. The concentration of the compounds with fluorophores in the sample can be determined by analysing the fluorescence intensity.¹⁸ The instrument used for fluorescence spectroscopy measurements is called a fluorometer (Scheme 2.10). The operating principle of a fluorometer includes: (1) selecting a specific excitation wavelength of a certain compound in the sample by diffracting the light generated from a light source with a broad spectrum using a diffraction grating monochromator; (2) excitation of the sample in a cuvette by absorbing the excited light, leading to emission of fluorescence light; (3) selecting the emitted light by passing through a second diffraction grating monochromator that is at 90° to the incident light; and (4) collecting the selected light by a detector that is at 90° to the incident light to eliminate noise signals of the transmitted or reflected incident light. A fluorescence excitation spectrum or fluorescence emission spectrum

can be generated by plotting the fluorescence intensity against a series of measured excitation wavelengths or measured emission wavelengths at a fixed emission wavelength or a fixed excitation wavelength, respectively.

In this thesis, fluorescence spectroscopy was used to determine the amount of the fluorescently labelled drug that was released from polyphenol-based capsules (Chapter 6).



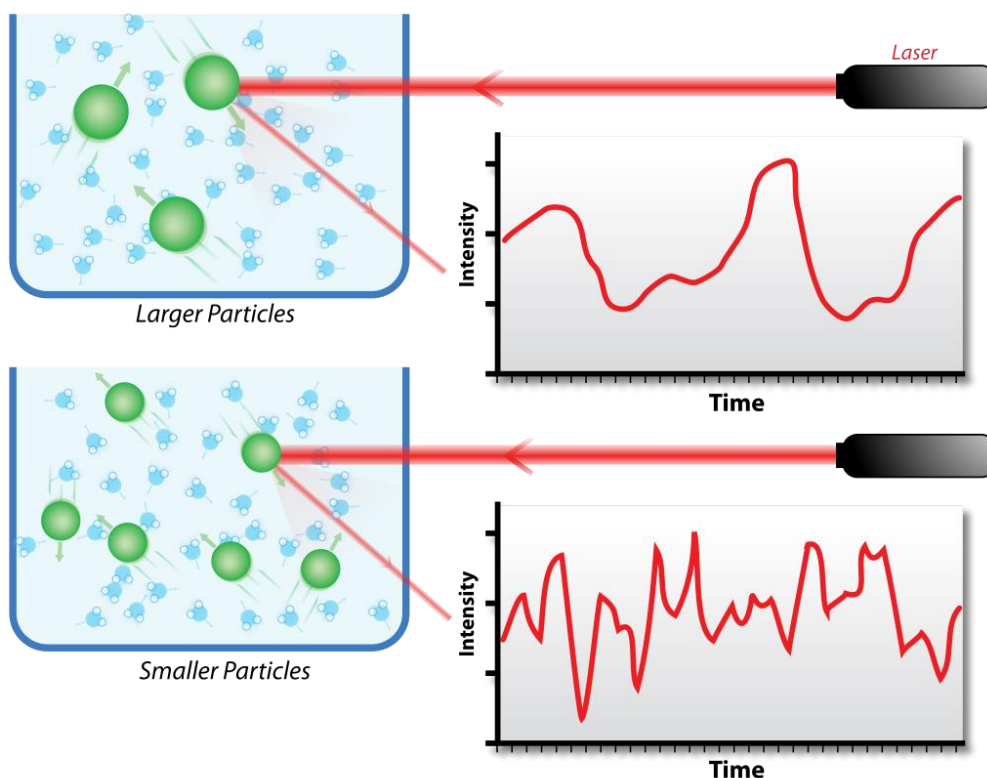
Scheme 2.10. Simplified illustration of a typical fluorometer. The figure is reproduced from reference 19.

2.2.9 Dynamic Light Scattering (DLS)

DLS is a commonly used technique to determine the size distribution of small particles or polymers in aqueous environments (Scheme 2.11). For the measurements, monochromatic light is shone toward the sample after passing through a polariser. The light is scattered in all directions by the sample if the particle or molecule size is smaller than the wavelength of the light. As small particles or small molecules in the solution are undergoing constant Brownian motion, the intensity of the scattered light caused by the samples is constantly changing with time. Particles or molecules with different sizes lead to different fluctuation of the scattered light due to the size-dependent Brownian motion. The scattered light is collected by a

photomultiplier after going through a second polariser. The size distribution of the particles or molecules can be calculated from the fluctuated scattered light.²⁰

In this thesis, DLS was used to measure the size distribution of mesoporous silica particles and various polymer particles (in Chapters 3–5).



Scheme 2.11. Simplified illustration of DLS measurement of two particle systems comprising particles of different sizes. The figure is reproduced from reference 20.

2.2.10 Microelectrophoresis

Microelectrophoresis is a technique used to analyse the electrophoresis of dispersed particles by optical microscopy. The net surface charge of particles in an aqueous environment is difficult to determine directly. Charged particles attract counter ions that can bind strongly to the particle surface, resulting in a ‘Stern Layer’ (Scheme 2.12). Beyond the Stern Layer, there is a relatively loose layer of an ion cloud consisting of both ions and counter ions. At the boundary of this loose region, which is termed the slipping plane, ions do not bind or associate with the particle surface and the solute no longer moves with the particle. The zeta potential is

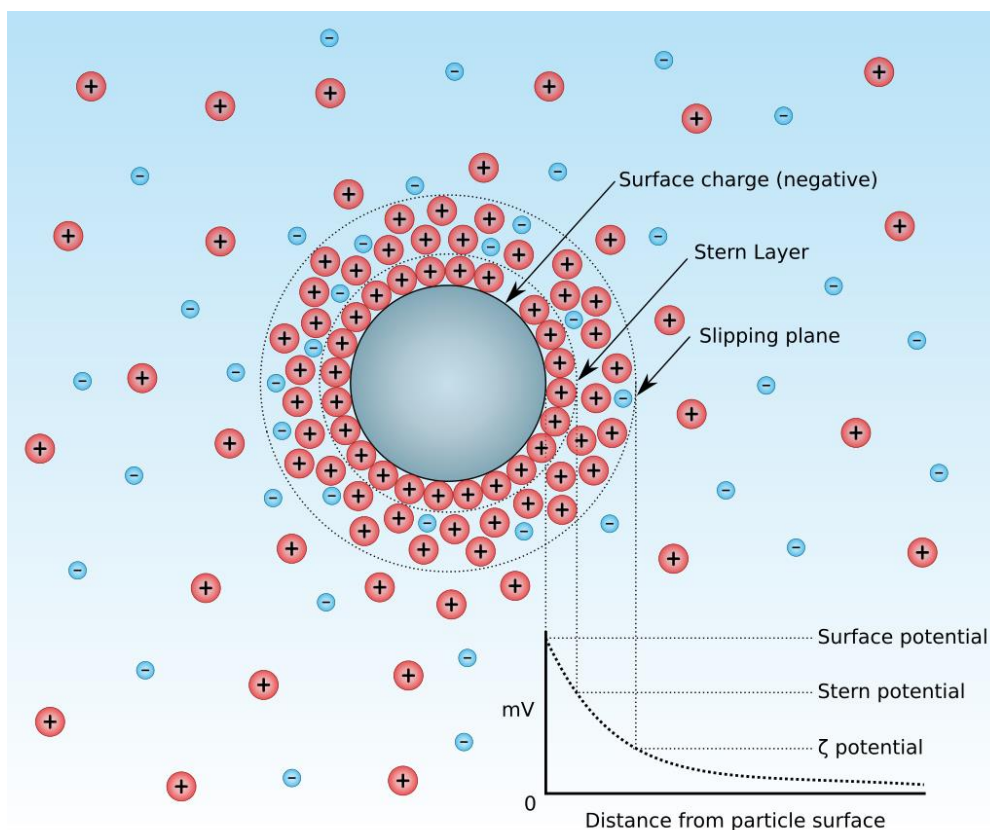
defined as the electrical potential at the slipping plane and is widely used as a reference to the surface charge of a particle. The zeta potential can be strongly influenced by the environment including ionic strength, pH and suspension media.

Electrophoresis measurements are typically performed to measure the zeta potential of a sample. After an electric field is applied across the particle suspension, the charged particles move toward the electrode with an opposite charge at a constant velocity that is relative to the zeta potential of the particles. The migration velocity can be measured by DLS. The scattered light caused by the moving particles is detected and analysed for the calculation of zeta potential by using the Helmholtz–Smoluchowski equation (Equation 2.2):²¹

$$v = \frac{\mu}{E} = \frac{\epsilon_R \epsilon_0 E}{\eta} \zeta \quad (\text{Equation 2.2})$$

where v is the particle velocity, μ is the electrophoretic mobility of the particles, ϵ_R is the relative permittivity of the solution, ϵ_0 is the permittivity of vacuum, E is the electric field strength, η is the dynamic viscosity and ζ is the zeta potential of the moving particles.

In this thesis, zeta potential measurements were performed to determine the surface charges of various polymer particles (Chapters 3 and 5) and polyphenol-based capsules (Chapter 6). The effect of protein corona on the particle surface charge was also investigated (Chapters 3 and 5).



Scheme 2.12. Illustration of the distribution of ions within an electrical double layer around a charged particle in solution. The zeta potential is measured at the slipping plane. The figure is reproduced from reference 22.

2.2.11 Quartz Crystal Microbalance with Dissipation Monitoring (QCM-D)

QCM is a surface characterisation technique that can be used to study the adsorption properties of materials and interactions between biomolecules by measuring changes in mass on a thin quartz crystal sensor which is located between a pair of electrodes. QCM can be used in different conditions including vacuum, gas phase and liquid phase. Upon application of a voltage to the quartz crystal, the latter oscillates at a fundamental frequency that is dependent on the thickness of the crystal. When the test material is deposited on the crystal surface, the resonance frequency decreases due to the increase in mass of the crystal, according to Sauerbrey's equation (Equation 2.3).²³ Thus, the deposition of the material can be quantitatively evaluated by monitoring the resonance frequency of the oscillating crystal.

$$\Delta f = -\frac{2f_0^2}{A\sqrt{\rho_q\mu_q}}\Delta m \quad (\text{Equation 2.3})$$

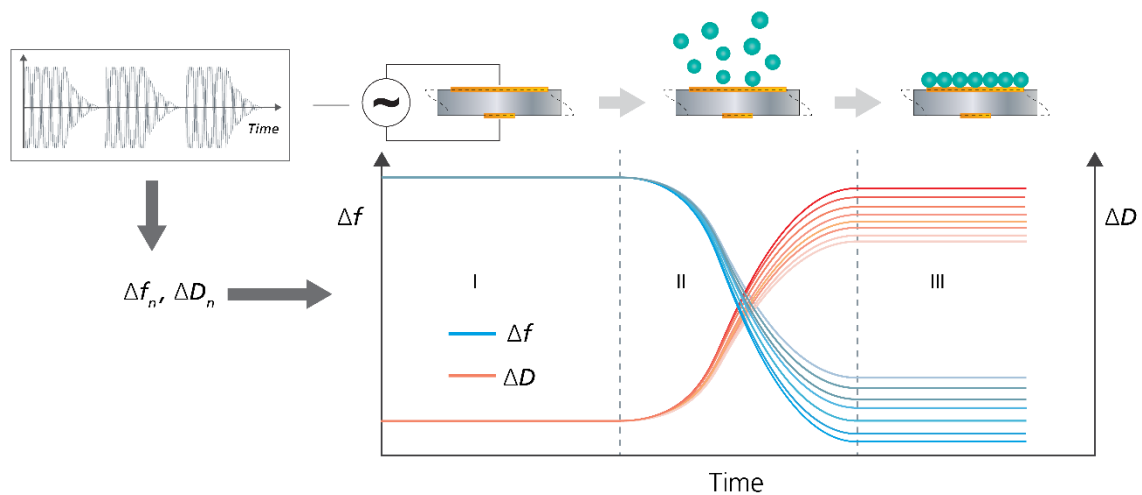
where Δf (Hz) is the measured frequency change, f_0 (Hz) is the resonance frequency, A (cm²) is the area of the piezoelectrically active crystal, ρ_q (2.648 g cm⁻³) is the density of the quartz crystal, μ_q (2.947×10^{11} g cm⁻¹ s⁻²) is the shear modulus of the quartz crystal and Δm (g) is the mass change.

However, Sauerbrey equation assumes that the deposited material is an extension of the crystal and is therefore valid for rigid and evenly distributed deposition only. In addition, the measured resonance frequency shift (Δf) should be smaller than the fundamental resonance frequency (f_0). For analysis of soft or viscoelastic materials, QCM-D is more ideal (Scheme 2.13). QCM-D is based on the ring-down technique and is typically used in interfacial acoustic sensing. Dissipation (D) is a parameter that is used to evaluate the viscoelasticity of the film, as defined as the loss of energy per oscillation period divided by the total energy stored in the system (Equation 2.4).²³ QCM-D is appropriate for in situ, real-time measurements of both mass and viscoelastic changes of films on a quartz crystal surface.

$$D = \frac{E_{\text{loss}}}{2\pi E_{\text{store}}} \quad (\text{Equation 2.4})$$

where D is the dissipation, E_{loss} is the energy loss during the oscillation cycle, and E_{store} is the total energy stored in the oscillator.

In this thesis, the interactions between different particles and mucus layer (Chapter 4) were analysed by QCM-D.

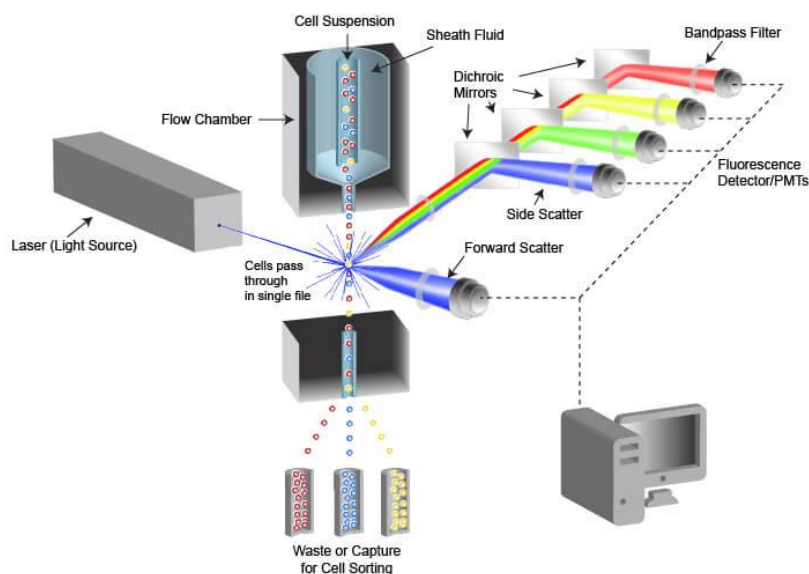


Scheme 2.13. Illustration of the working principle of QCM-D. The figure is adapted from reference 24.

2.2.12 Flow Cytometry

Flow cytometry is a technique that has been widely used in materials science and biological research to detect and measure multiple parameters (e.g., cell size, morphology) of a population of cells and various physical and chemical characteristics (e.g., size, granularity, fluorescence intensity) of particles by using light scattering.²⁵ A flow cytometer typically comprises a fluidics or flow system, an optics system consisting of laser beams and a series of optical devices, and an electronic system to convert the detected light signals into electronic signals.²⁵ In the measurement process, a sample suspension containing cells or particles is injected into the flow cytometer. The cells or particles are individually aligned and pass through a small aperture in the flow chamber and then illuminated by a high-power laser beam, resulting in light scattering and emitted fluorescence light, both of which are filtered and oriented by a series of dichroic mirrors. The scattering signals are finally collected by a forward scatter detector, which allows assessment of particle size, and a side scatter detector, which allows evaluation of morphological complexity of cells or particles. The emitted fluorescence light is collected by photomultipliers that convert the light into electrical signals, which can be amplified and digitised by an external computer system for data analysis.²⁵

In this thesis, flow cytometry is used to determine particle and capsule count and to quantitatively evaluate cell association (Chapters 3, 5 and 6).

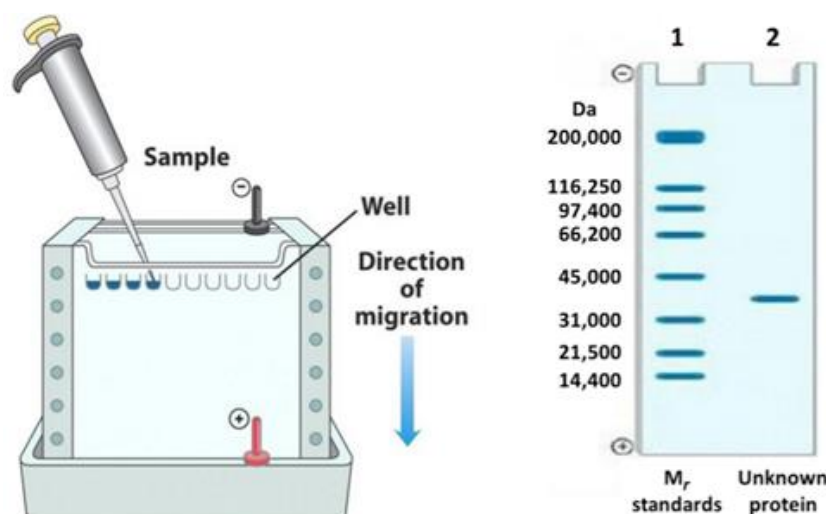


Scheme 2.14. Simplified illustration of a typical flow cytometry system. The figure is reproduced by reference 26.

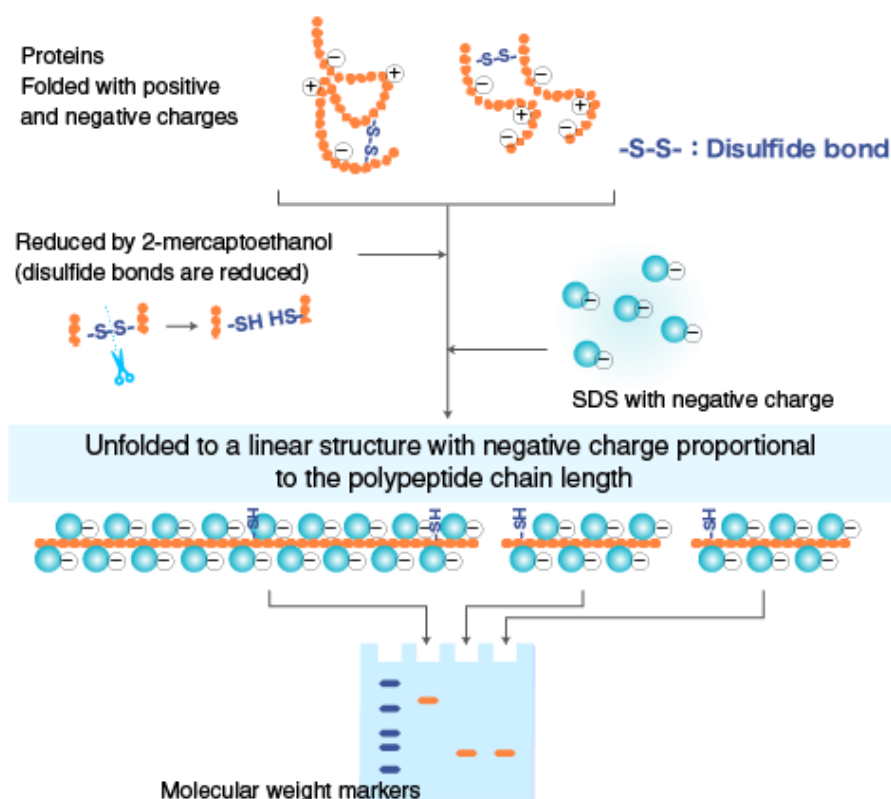
2.2.13 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Gel electrophoresis is a technique that is primarily used for the separation and identification of biomacromolecules (e.g., protein, DNA and RNA) based on their different electrophoretic mobility, which is dependent on their characteristics such as molecular weight, conformation and charge. The basic procedure of gel electrophoresis, as shown in Scheme 2.15, includes loading samples in a gel, sample movement from the cathode to anode upon application of an electric field and staining the gel for visualisation. To eliminate the effect of charge and high-order structures (secondary/tertiary structures), proteins are typically pre-incubated with a chemical denaturant that converts the ordered structured proteins into unstructured chains before conducting gel electrophoresis. This step ensures that the electrophoretic mobility is governed by the molecular weight only.²⁷ SDS is an anionic surfactant that is commonly used to convert proteins into a linear structure with an evenly distributed negative charge (Scheme 2.16). The SDS-treated proteins are loaded onto a gel matrix consisting of polyacrylamide with uniformly sized pores and subsequently allowed to migrate through the gel to the positively charged anode after applying a voltage. This procedure is called SDS-PAGE. Small proteins readily migrate through the mesh of the gel at a higher moving speed, whereas larger proteins migrate more slowly, resulting in the separation of proteins based on molecular weight. The gel is then stained for visualisation and further analysis.

In this thesis, SDS-PAGE with SimplyBlue staining was used to analyse the composition of protein coronas on various particles (Chapters 3 and 5).



Scheme 2.15. Illustration of the setup of gel electrophoresis to separate biomolecules. The figure is adapted from reference 28.



Scheme 2.16. Simplified illustration of SDS-PAGE procedure. The figure is reproduced from reference 29.

2.2.14 Mass Spectrometry

Mass spectrometry is a highly sensitive, analytical technique capable of identifying different molecules in a sample and provides quantitatively analytic data by measuring the mass-to-charge ratio of ions from the sample.³⁰ Mass spectrometry is widely used in food and nutrition sciences, life sciences and environmental sciences. A mass spectrometer typically consists of four main components: an ion source, a mass analyser, a detector and a recording device (Scheme 2.17). The basic operating principle of a mass spectrometry includes the ionisation of sample molecules into negatively charged ions by a variety of methods such as electron ionisation and chemical ionisation, and subsequent separation of ions according to their mass-to-charge ratio by acceleration and deflection processes. Ions with increasing mass reach the detector in a sequential manner.³⁰ The signals are detected and converted into electrical signals resulting in a spectrum that can be processed and analysed.³⁰

The ions are separated by a mass analyser based on their mass-to-charge ratio, according to two laws that determine the dynamics of charged particles in electric and magnetic fields in vacuum, i.e., Lorentz force law (Equation 2.5) and Newton's Second Law (Equation 2.6):³⁰

$$\mathbf{F} = Q(\mathbf{E} + \mathbf{v} \times \mathbf{B}) \quad (\text{Equation 2.5})$$

Lorentz force law, where F is the force applied to the ion, Q is the ion charge, $\mathbf{E}$ is the electric field, $\mathbf{v}$ is the vector cross product of the ion velocity and $\mathbf{B}$ is the magnetic field.

$$\mathbf{F} = m\mathbf{a} \quad (\text{Equation 2.6})$$

Newton's second law of motion in non-relativistic case, where F is the force applied to the ion, m is the mass of the ion and a is acceleration.

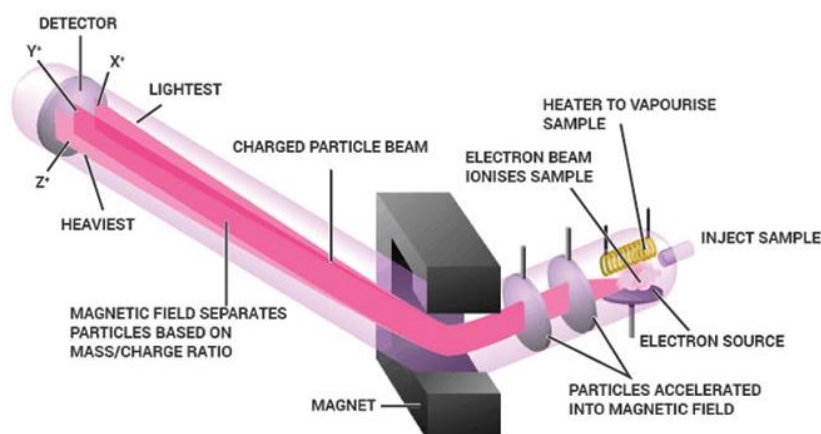
The differential equation generated from these two laws is the classic equation of motion for charged particles (Equation 2.7), which governs the particle's motion in space and time in terms of m/Q , together with the particle's initial conditions.³⁰

$$(m / Q)\mathbf{a} = \mathbf{E} + \mathbf{v} \times \mathbf{B} \quad (\text{Equation 2.7})$$

where m is the mass of the ion, Q is the charge of the ion, a is the acceleration, $\mathbf{E}$ is the electric field, $\mathbf{v}$ is the vector cross product of the ion velocity and $\mathbf{B}$ is the magnetic field.

Mass spectrometry is a useful technique for proteomics analysis. Proteins are pre-treated with chemicals and enzymes, which after digestion generate small fragments of peptides. The resulted peptides are then subjected to mass spectrometry analysis.

In this thesis, mass spectrometry was used for the detection, identification and characterisation of proteins from protein coronas on various particles (Chapter 5).



Scheme 2.17. Simplified illustration of the setup of a mass spectrometer. The figure is reproduced from reference 31.

2.2.15. Nebulisation

Nebulisation is a commonly used approach to deliver drug solution or drug-loaded particle suspension to the lungs, which can avoid freeze drying or spray drying processes common in dry powder production and provide finely controlled particle size, allowing the modulation of the bio-nano interactions between the drug-loaded particles and the biological environment where they deposit.³² Aerosol droplets are generated from drug solution or drug-loaded particle suspension by ultrasonic, air-jet or other types of nebulisers. The basic working principle of an air-jet nebuliser is that the compressed air is forced through a tubing system which is connected to a nozzle, resulting in negative pressure and suction. The liquid in the reservoir is pulled up on a feed tube and then formed as an aerosol.³³ In this thesis, an air-jet nebuliser was used to assess the amenability of particles during the nebulisation process (Chapter 4 and 6).



Scheme 2.18. A picture of a PARI air-jet nebuliser, reproduced from Trek® S Portable Aerosol System, PARI, USA.

2.3 References

1. Rosenberger, H. E. *In Interpretive Techniques for Microstructural Analysis*, 1st ed.; McCall, J. L., French, P. M., Eds.; Springer US: Boston, MA, **1977**; pp 79-104.
2. Olympus Lifescience, Differential Interference Contrast, <https://www.olympus-lifescience.com/en/microscope-resource/primer/techniques/dic/dicoverview/> (accessed 11 May 2020).
3. Enzo Lifesciences, Luminescence, <https://www.enzolifesciences.com/science-center/technotes/2019/december/what-is-the-difference-between-fluorescence-phosphorescence-and-luminescence?/> (accessed 11 May 2020).
4. Thermo Fisher Scientific Inc, Fluorescent Probes, <https://www.thermofisher.com/au/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/fluorescent-probes.html>(accessed 11 May 2020).
5. Wikipedia, Fluorescence microscope, https://en.wikipedia.org/wiki/Fluorescence_microscope (accessed 11 May 2020).
6. Olympus Lifescience, Combination Methods with Differential Interference Contrast (DIC), <https://www.olympus-lifescience.com/en/microscope-resource/primer/techniques/fluorescence/fluorodic/> (accessed 11 May 2020).

7. Inoué, S., *Handbook of Biological Confocal Microscopy*, 3rd ed.; Pawley, J. B., Eds.; Springer: Berlin, **2006**.
8. BiteSize Bio, What is Confocal Laser Scanning Microscopy?, <https://bitesizebio.com/19958/what-is-confocal-laser-scanning-microscopy/> (accessed 11 May 2020).
9. Williams, D. B.; Carter, C. B., *Transmission Electron Microscopy: A Textbook for Materials Science*, 2nd ed.; Williams, D. B., Carter, C. B., Eds.; Springer US: Boston, MA, **2009**.
10. Wikipedia, Ultramicrotomy, <https://en.wikipedia.org/wiki/Ultramicrotomy> (accessed 11 May 2020).
11. Britannica, Transmission electron microscope, <https://www.britannica.com/technology/transmission-electron-microscope> (accessed 11 May 2020).
12. Smith, K. C. A.; C. W. Oatley., *Adv Imaging Electron Phys* **2004**, 133, 111-125.
13. Britannica, Scanning electron microscope, <https://www.britannica.com/technology/scanning-electron-microscope> (accessed 11 May 2020).
14. Binnig, G.; Quate, C. F.; Gerber, Ch., *Phys Rev Lett* **1986**, 56 (9), 930-933.
15. Zeng, G.; Duan, Y., *Atomic Force Microscopy Investigations into Biology: From Cell to Protein*; Frewin, C. L., Eds.; BoD—Books on Demand, **2012**.
16. Perkampus, H.-H., *UV-Vis Spectroscopy and Its Applications*, 1st ed.; Perkampus, H.-H., Ed.; Springer: Berlin, Heidelberg, **1992**.
17. Wikipedia, Ultraviolet–visible spectroscopy, https://en.wikipedia.org/wiki/Ultraviolet%E2%80%93visible_spectroscopy (accessed 11 May 2020).
18. Wikipedia, Fluorescence spectroscopy, https://en.wikipedia.org/wiki/Fluorescence_spectroscopy (accessed 11 May 2020).
19. Meiresonne, N., Alexeeva, S., van der Ploeg, R., & den Blaauwen, T., *Bio-protocol* **2018**, 8, e2697.
20. Wikipedia, Dynamic light scattering, https://en.wikipedia.org/wiki/Dynamic_light_scattering (accessed 11 May 2020).
21. Durand-Vidal, S.; Simonin, J. P.; Turq, P. In *Electrolytes at Interfaces*, 1st ed.; Durand-Vidal, S., Simonin, J. P., Turq, P., Eds.; Springer Netherlands: Dordrecht, **2002**; pp 269-288.

22. Wikipedia, Zeta potential, https://en.wikipedia.org/wiki/Zeta_potential (accessed 11 May 2020).
23. Biolin Scientific, Q-Sense, <https://www.biolinscientific.com/q-sense/> (accessed 11 May 2020).
24. Biolin Scientific, QCM-D, <https://blog.biolinscientific.com/qcm-d-how-does-it-work> (accessed 11 May 2020).
25. Apogee Flow Systems, Products, <https://www.apogee-flow.com/> (accessed 11 May 2020).
26. Cell signal, Flow cytometry overview, https://www.cellsignal.com/contents/_/overview-of-flow-cytometry/flow-cytometry-overview (accessed 11 May 2020).
27. Bio-Rad Laboratories, Protein Electrophoresis Methods, <https://www.bio-rad.com/en-au/applications-technologies/protein-electrophoresis-methods?ID=LUSOW4GRI> (accessed 11 May 2020).
28. Pronalyse, SDS-PAGE service, <https://www.creative-proteomics.com/pronalyse/sds-page-service.html> (accessed 11 May 2020).
29. MBL, SDS-PAGE, <https://ruo.mbl.co.jp/bio/e/support/method/sds-page.html> (accessed 11 May 2020).
30. Wikipedia, Mass spectrometry, https://en.wikipedia.org/wiki/Mass_spectrometry (accessed 11 May 2020).
31. Microbiology Notes, Mass spectrometry, <https://microbenotes.com/mass-spectrometry-ms-principle-working-instrumentation-steps-applications/> (accessed 11 May 2020).
32. Respaud, R.; Marchand, D.; Pelat, T.; Tchou-Wong, K. M.; Roy, C. J.; Parent, C.; Cabrera, M.; Guillemain, J.; Mac Loughlin, R.; Levacher, E.; Fontayne, A.; Douziech-Eyrolles, L.; Junqua-Moulet, A.; Guilleminault, L.; Thullier, P.; Guillot-Combe, E.; Vecellio, L.; Heuze-Vourch, N., Development of a drug delivery system for efficient alveolar delivery of a neutralizing monoclonal antibody to treat pulmonary intoxication to ricin. *J Control Release* **2016**, 234, 21-32.
33. Collins, N. Nebulizer therapy in cystic fibrosis: an overview. *J R Soc Med* **2009**, 102 Suppl 1, 11-17.

Chapter 3

Engineering Stiffness of Poly(ethylene glycol)-Based Particles for Regulating Particle–Immune Cell Interactions

Chapter 3. Engineering Stiffness of Poly(ethylene glycol)-Based Particles for Regulating Particle–Immune Cell Interactions	78
3.1 Aim.....	80
3.2 Introduction	80
3.3 Experimental section	82
3.3.1 Materials	82
3.3.2 Synthesis of Mesoporous Silica (MS) Particles	83
3.3.3 Synthesis of MS@PEG and PEG Particles	84
3.3.4 Particle Characterisation.....	84
3.3.5 Mechanical Property Measurements and Analysis.....	85
3.3.6 Raw 264.7 Cell Association	86
3.3.7 RAW 264.7 Cell Association Imaging by Confocal Microscopy	87
3.3.8 Human Whole Blood Cell Association	87
3.3.9 Human Washed Blood Cell Association	88
3.3.10 Formation of Biomolecular Corona-Coated Particles	88
3.3.11 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)	89
3.4 Results and Discussions	89
3.4.1 Synthesis and Characterisation of MS@PEG and PEG Particles	89
3.4.2 Mechanical Property Analysis.....	92
3.4.3 Cell Association.....	93
3.4.4 Protein Corona.....	99
3.5 Conclusions	101
3.6 Supporting Information	102
3.7 References	114

3.1 Aim

Poly(ethylene glycol) coating (termed PEGylation) exerts a ‘stealth property’ on surfaces, including carriers for drug delivery, to prolong circulation lifetimes by suppressing phagocytosis and subsequent clearance of nanoengineered materials following intravenous administration. PEG replica particles prepared by templated assembly are promising nanomaterials for biomedical applications due to the tunable bio-nano interactions they impart. In this chapter, the influence of the mechanical properties of PEG-based particles on their biological interactions in a clinically relevant sample (i.e., human blood) was investigated. Four types of PEGylated mesoporous silica (MS@PEG) particles (template present) and corresponding PEG particles (template removed) with modulated mechanical properties were fabricated using PEG building blocks with the same arm length but different arm numbers, and their behaviour in human blood especially the interactions with immune cells was studied.

3.2 Introduction

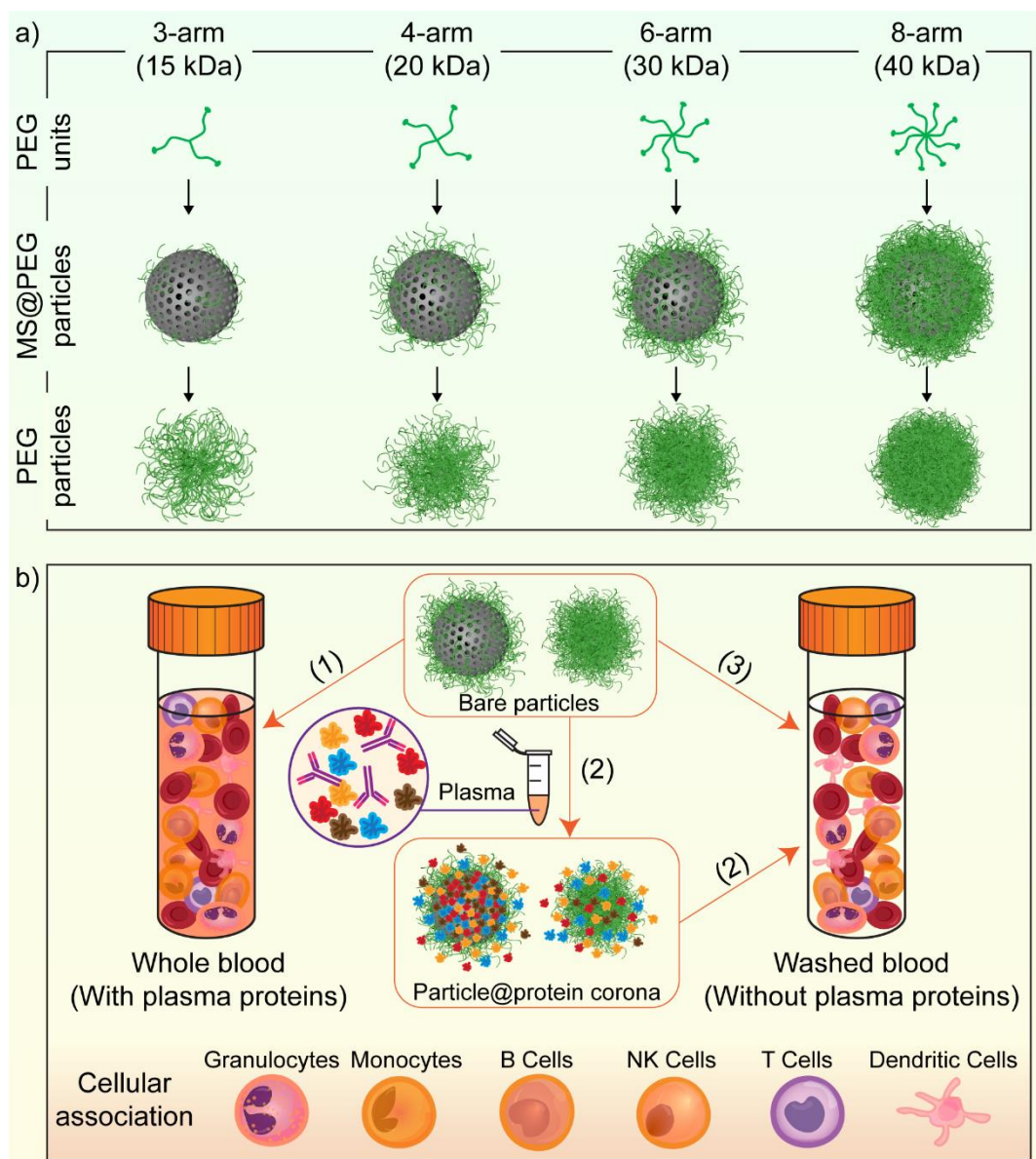
Over the past decades, nanostructured materials with unique properties and functions have been developed as drug delivery carriers with the aim of overcoming biological barriers and the immune defence, prolonging blood circulation time, increasing target accumulation, and thereby improving the delivery efficacy of encapsulated therapeutics.¹⁻⁵ The behaviour of nanostructured materials when entering a biological environment is mostly governed by their physicochemical properties including size, shape, surface chemistry and elasticity.⁶⁻¹⁰ However, the physiological interactions such as the adsorption of biomacromolecules and the resultant formation of a biomolecular corona on the surface endow the material with a ‘biological identity’ that might be different from its original ‘synthetic identity’, which could in turn alter its interactions with the biointerface and determine its fate.¹¹⁻¹⁴

Different strategies have been explored to modulate the surface properties of nanomaterials, with the aim of avoiding uptake by the mononuclear phagocyte system and hence rapid clearance from the bloodstream.¹⁵⁻¹⁷ A commonly used technique is coating the material with a densely packed layer of poly(ethylene glycol) (PEG), a ‘stealth’ polymer, resulting in a more hydrophilic surface and a large excluded hydrated cloud.¹⁸ This type of modification strategy, termed ‘PEGylation’, strongly reduces interparticle attractive forces, thus preventing severe particle aggregation, and repels the binding of biological components, such as plasma proteins,

thereby effectively suppressing phagocytosis and subsequent clearance.¹⁹ Numerous studies have investigated the factors influencing the behaviour of PEGylated particles *in vitro* or *in vivo*, including the influence of PEG molecular weight and PEG surface density on the low fouling behaviour of particles.^{7, 20-25}

Caruso and co-workers have reported the templated assembly of PEG replica particles and demonstrated their potential as platforms for a range of biomedical applications, including drug delivery and tumour targeting.²⁶⁻²⁹ In these study, the molecular weight of PEG building block and the overall particle size significantly influenced particle association with blood cells and biodistribution in mice.²⁶ Moreover, these PEG particles can counterfeit red blood cell as examined by micro-channels due to their extremely ‘soft’ character.²⁷ However, the relationship between the mechanical properties and bio–nano interactions of PEG-based particles (especially with immune cells) has yet to be determined.

Herein, in the chapter, the influence of the mechanical properties of PEG-based particles on their biological interactions in clinical-relevant samples (i.e., human blood) was examined. Four types of PEGylated mesoporous silica (MS@PEG) particles (template present) and corresponding PEG particles (template removed) with modulated mechanical properties were fabricated using PEG building blocks with the same arm length but different arm numbers (Scheme 3.1a), and their behaviour in a complex biological environment (i.e., human whole blood) was studied (Scheme 3.1b). Varying the PEG unit architecture from 3-arm PEG to 4-arm PEG, 6-arm PEG and 8-arm PEG produced particles with different morphology and elasticity. When incubated in human blood, the different MS@PEG and PEG replica particles exhibited different interactions with plasma proteins and specific blood cell populations. This work indicates that the building block architecture can influence the mechanical properties of the assembled particles of the same composition, and subsequently alert their interactions with biological systems.



Scheme 3.1. (a) Schematic illustration of the assembly of MS@PEG (template present) and PEG particles (template removed) using different PEG building blocks (3-arm PEG, M_w 15 kDa; 4-arm PEG, M_w 20 kDa; 6-arm PEG, M_w 30 kDa; and 8-arm PEG, M_w 40 kDa) via MS particle templating. (b) Human blood assays: MS@PEG particles and PEG particles were incubated in (1) human whole blood (with plasma proteins), (2) human blood plasma followed by washed blood (without plasma proteins) or (3) human washed blood (without plasma proteins) and their cellular association with human immune cells was subsequently examined.

3.3 Experimental section

3.3.1 Materials

Tetraethyl orthosilicate (TEOS), cetyltrimethylammonium bromide (CTAB), poly(acrylic acid) (PAA, M_w ~250 kDa, 35 wt% solution in water), ammonium hydroxide solution ($\text{NH}_3 \cdot \text{H}_2\text{O}$, 28–30%), sodium phosphate dibasic (Na_2HPO_4), hydrofluoric acid (HF, 48 wt%), ammonium fluoride (NH_4F), anhydrous dimethyl sulfoxide (DMSO), Dulbecco phosphate-buffered saline (DPBS), paraformaldehyde, formaldehyde, ethylenediaminetetraacetic acid (EDTA) and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (Australia). 3-Arm-poly(ethylene glycol) amine (3-arm-PEG- NH_2 , M_w 15 kDa), 4-arm-poly(ethylene glycol) amine (4-arm-PEG- NH_2 , M_w 20 kDa), 6-arm-poly(ethylene glycol) amine (6-arm-PEG- NH_2 , M_w 30 kDa) and 8-arm-poly(ethylene glycol) amine (8-arm-PEG- NH_2 , M_w 40 kDa) were obtained from JenKem Technology USA Inc. (China). 8-Arm-poly(ethylene glycol) succinimidyl succinate (8-arm-PEG-NHS, hexaglycerol core, M_w 10 kDa) was purchased from Creative PEGWorks (USA). Dulbecco's modified Eagle's medium (DMEM), Alexa Fluor 488 carboxylic acid succinimidyl ester (AF488-NHS), Alexa Fluor 647 carboxylic acid succinimidyl ester (AF647-NHS), RPMI-1640 medium, fetal bovine serum (FBS), wheat germ agglutinin Alexa Fluor 594 conjugate (WGA-594), Hoechst 33342, NuPAGE LDS sample buffer, NuPAGE sample reducing agent, SeeBlue Plus2 pre-stained protein standard, and SimplyBlue safe stain were provided by Life Technologies (Australia). BioRad 4–20% Mini-PROTEAN TGX gel and Tris/glycine/sodium dodecyl sulfate running buffer were purchased from BioRad (CA, USA). The antibodies CD66b BV421 (G10F5), CD19 BUV395 (HIB19), CD3 AF700 (SP34-2), CD14 APC-H7 (MΦP9), CD56 PE (B159) and HLA-DR PE-CF594 (G46-6) were purchased from BD Biosciences. The antibody lineage cocktail 1 fluorescein isothiocyanate (Lin-1 FITC) was obtained from BioLegend (USA).

High-purity water used in all experiments with a resistivity greater than 18.2 $\text{M}\Omega$ cm was generated from a three-stage Millipore Milli-Q plus 185 purification system (Millipore Corporation).

3.3.2 Synthesis of Mesoporous Silica (MS) Particles

MS particles were synthesised according to a previously reported method.²⁶ Briefly, 1.1 g of CTAB was completely dissolved in 50 mL of water under constant stirring. Then, 4.3 g of PAA solution (35 wt% in water) was added to the above solution. When the solution turned clear after ca. 20 min, 3.5 mL of ammonium hydroxide solution (28–30%) was added under vigorous stirring. Then, 4.46 mL of TEOS was added to the above milky suspension after stirring for 20

min. The suspension was further stirred for 15 min and finally transferred into a Teflon-sealed autoclave allowing 48 h reaction between 80 and 90 °C. After washing with water and ethanol, and subsequent drying at 80 °C, the synthesised MS particles were finally calcined at 550 °C for 30 h for the removal of organic materials.

3.3.3 Synthesis of MS@PEG and PEG Particles

MS@PEG and PEG Particles were fabricated by using a modified published method.²⁶ For MS@3-arm PEG and 3-arm PEG particles, 480 μL of 3-arm-PEG-NH₂ solution (5 mg mL⁻¹ in 100 mM, pH 8.0 phosphate buffer) was added to approximately 6 mg of MS particles for overnight incubation with constant shaking. 3-Arm-PEG-NH₂ was infiltrated into the pores of the MS particles driven by electrostatic interactions. After removing excess polymer by washing with phosphate buffer three times, the particles were subsequently incubated in 400 μL of 8-arm-PEG-NHS solution (2 mg mL⁻¹ in phosphate buffer) for 2 h to form a cross-linked polymer network by the covalent bond between 3-arm-PEG-NH₂ and 8-arm-PEG-NHS. The MS@3-arm PEG particles were labelled with AF488-NHS or AF647-NHS (5 μL , 1 mg mL⁻¹ in anhydrous DMSO) during the cross-linking step. The resultant particles were finally washed three times with water to remove unreacted cross-linker and dye. The 3-arm PEG particles were obtained by dissolving MS templates with a 2 M HF/8 M NH₄F solution (pH ~5). *Caution! HF is highly toxic and extreme care should be taken during handling HF solution.* The resultant 3-arm PEG particles were washed three times with water and resuspended in water until analysis.

4-Arm-PEG-NH₂, 6-arm-PEG-NH₂ and 8-arm-PEG-NH₂ were used as building blocks for the preparation of MS@4-arm PEG or 4-arm PEG particles, MS@6-arm PEG or 6-arm PEG particles and MS@8-arm PEG or 8-arm PEG particles, respectively, as described for the MS@3-arm PEG/3-arm PEG particles above.

3.3.4 Particle Characterisation

Fluorescence microscopy images were taken by using a differential interference contrast (DIC) (U-DICT, Olympus) / inverted fluorescence combined microscope (Olympus IX71) equipped with a 100 $\times$ oil immersion objective. Transmission electron microscopy (TEM) images were taken with an FEI Tecnai TF20 instrument operating at the voltage of 80 kV for the PEG

particles and 120 kV for the MS particles. The TEM samples were prepared by placing a drop of an aqueous particle suspension onto plasma-treated formvar-coated copper grids and subsequent drying in air. Atomic force microscopy (AFM) images were acquired by using a NanoWizard II atomic force microscope (JPK Instruments, Berlin, Germany). An aqueous suspension of particles was placed onto Piranha-precleaned glass slides and dried in air. *Caution! Piranha solution is extremely corrosive and reacts violently with organic materials. It should be handled with great care.* Scanning electron microscopy (SEM) images were taken with a Philips XL30 instrument at an operation voltage of 15 kV. The SEM samples were prepared by placing a drop of the particle suspension onto clean silicon wafers, followed by sputter-coating with gold. The zeta potential of the particles was measured using a Zetasizer Nano-ZS instrument (Malvern Instruments, Malvern, UK). Particle counting and particle fluorescence measurements were carried out by flow cytometry (Apogee Flow). Image analysis was conducted using ImageJ software.

3.3.5 Mechanical Property Measurements and Analysis

Mechanical property measurements of the PEG particles were conducted using a Nanowizard II atomic force microscope (JPK Instruments AG, Berlin, Germany) equipped with a fluorescence microscope (Lecia DMI4000B).

The samples were prepared by placing a drop of concentrated AF488-labelled PEG particle suspension (approximately 10 μL) onto a poly(ethyleneimine)-precoated FluoroDish (50 mm, World Precision Instruments Inc.), which was subsequently filled with Milli-Q water. A monolayer of PEG particles was immobilised at the base of the FluoroDish after settling, which was visualised by fluorescence microscopy (60 $\times$ oil immersion objective) (Figure S3.5b).

The probe used in this experiment was prepared by modifying the cantilever (MLCT, Bruker AFM Probes) by attaching a spherical glass bead ($D = 22\text{ }\mu\text{m}$, Polysciences) using an epoxy resin (Selleys Araldite Super Strength, Selleys) through micromanipulation in an AFM system (Figure S3.5a). After drying the glued glass bead overnight for complete curing, the resultant probe was completely cleaned with ethanol, water, and via an oxygen plasma treatment. The spring constant of the probe was 0.09 N m^{-1} determined using the Hutter–Bechhoefer method.³⁰

For the measurements, the probe was slowly lowered into an aqueous particle suspension avoiding air bubbles and hydrodynamic effects. A clean area on the glass with no particles was

used for calibration. Next, the probe was slowly lowered onto individual immobilised particles ensuring vertical alignment by using the inbuilt micromanipulation stage. The location of individual particles was identified by fluorescence microscopy. Force measurements were performed at an approach velocity of $2 \mu\text{m s}^{-1}$ with a maximum indentation force of approximately 10 nN. Force–deformation curves were obtained from the raw AFM voltage–displacement data processed using JPK data processing software (v.4.4.29). After completing the measurements on a single particle, the location of the particle was assessed using the attached fluorescence microscope. The particle position remained unchanged during the measurements (Figure S3.5b and c). Six repeated measurements were performed for each particle, and at least five different particles were measured and analysed for each sample to obtain representative Young’s modulus values of different PEG particles.

The Hertz theory, which predicts force (F) on a particle as a function of a (small) applied indentation by a spherical probe, was used as follows:³¹

$$F = \frac{4E\sqrt{R_{\text{eff}}}}{3(1-\nu^2)} \delta^{3/2} \quad (\text{Equation 3.1})$$

where $R_{\text{eff}} = \frac{RR_p}{R+R_p}$ is the effective radius of the probe radius R_p and the PEG hydrogel particle radius R . Equation 3.1 is valid when the Young’s modulus of the indenter E_p is greater than the elastic modulus of the PEG hydrogel particle (E). Otherwise, the effective Young’s modulus is used. In the present work, the probe is stiffer than the PEG hydrogel particle hence the equation is valid. Here, F is the measured force as a function of indentation of the probe into the particle (δ) and ν is the Poisson ratio (~ 0.4). The Young’s modulus value of PEG particles was evaluated from the linear range (0 to 200 nm) of the F versus $\delta^{3/2}$ curves. The slope of the force curves ($\frac{4E\sqrt{R_{\text{eff}}}}{3(1-\nu^2)}$) was used to calculate the modulus.

3.3.6 Raw 264.7 Cell Association

RAW 264.7 cells were seeded in a 24-well plate (80 000 cells per well) in RPMI media with 10% (v/v) FBS and treated with AF488-labelled MS@3-arm PEG, MS@4-arm PEG, MS@6-arm PEG, MS@8-arm PEG or the corresponding PEG particles at a particle-to-cell ratio of 100:1 or 1000:1. After 12 or 24 h incubation at 37 °C, the cells were detached from the plate using trypsin and subsequently washed with DPBS three times via centrifugation (300 g, 5

min). The resultant cell pellets were resuspended in DPBS and analysed by flow cytometry (Apogee Flow).

3.3.7 RAW 264.7 Cell Association Imaging by Confocal Microscopy

RAW 264.7 cells were seeded in a 8-well Lab-Tek chambered coverglass slides (Thermo Fisher Scientific, USA) (40 000 cells per well) in RPMI media with 10% (v/v) FBS and treated with AF488-labelled MS@3-arm PEG, MS@4-arm PEG, MS@6-arm PEG, MS@8-arm PEG or the corresponding PEG particles at a particle-to-cell ratio of 100:1 for 12 h at 37 °C. After incubation, the cells were gently washed with DPBS and then fixed with paraformaldehyde (4% in DPBS) for 15 min at room temperature. The fixed cells were then stained with WGA-594 (5 $\mu\text{g mL}^{-1}$) for 5 min and Hoechst 33342 (0.1 mg mL^{-1}) for 5 min at room temperature.

Cell association imaging was carried out using a laser scanning confocal microscope (Nikon Corporation, Japan) equipped with a Plan Apo λ 60 $\times$ 1.4 NA oil immersion objective, and 405, 488, 561, and 640 nm lasers, and analysed by ImageJ software.

3.3.8 Human Whole Blood Cell Association

Human whole blood from a healthy volunteer was collected into sodium heparin vacuettes (Greiner Bio-One) and then aliquoted in 100 μL in polystyrene tubes (Falcon® Round-Bottom Polystyrene Tubes, 5 mL) with 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. Cell numbers were counted with a CELL-DYN Emerald analyser (Abbott) using 20 μL of blood. MS@PEG or PEG particles dispersed in DPBS (approximately 5 μL) were added to the blood for 1 h incubation at 37 °C. The ratio of particles to white blood cells (WBCs, also called leukocytes) was 50:1, 20:1, 10:1, 5:1 for MS@PEG particles, and 200:1, 100:1, 50:1 for PEG particles. After treatment with particles, the red blood cells were lysed using 4 mL of PharmLyse buffer and removed by centrifugation (500 g, 5 min). After washing twice with 4 mL of DPBS (500 g, 7 min), the cells were phenotyped on ice for 30 min with an antibody ‘cocktail’ consisting of Lin-1 FITC (BioLegend), CD66b BV421 (G10F5, BD), CD19 BUV395 (HIB19, BD), CD3 AF700 (SP34-2, BD), CD14 APC-H7 (M Φ P9, BD), CD56 PE (B159, BD) and HLA-DR PE-CF594 (G46-6, BD) in titrated concentrations. The cells were then washed twice with flow

cytometry staining (FACS) wash buffer (DPBS containing 2 mM EDTA and 0.5% w/v BSA, pH 8.0) and centrifuged at 500 g for 7 min to remove free antibodies. After fixation with 100 μ L of 1% w/v formaldehyde in DPBS, the cells were analysed in a flow cytometer (LSRFortessa, BD Biosciences) for particle association determination. The data were analysed using FlowJo V10.

3.3.9 Human Washed Blood Cell Association

Human whole blood collected from a healthy volunteer was centrifuged (900 g, 15 min, no brake) in sodium heparin vacuettes (Greiner Bio-One) directly. The supernatant was collected and transferred into 1.5 mL eppendorf tubes for further centrifugation (1500 g, 10 min) to remove any remaining cells, resulting in human plasma for further experiments. The blood cells remaining in sodium heparin vacuettes were transferred into 50 mL tubes filled with DPBS and centrifuged at 950 g for 10 min with low brake. The supernatant was discarded carefully without disturbing the cell pellets, and this wash step was repeated four more times until the absorbance of the supernatant at 280 nm was virtually 0 as measured using Nanodrop (Thermo Fisher Scientific, Australia), which confirmed the absence of plasma proteins. The cells were finally resuspended in DPBS in a similar cell concentration to that of the whole blood.

The concentrated MS@PEG or PEG particles were preincubated in 50 μ L of DPBS or plasma for 1 h at 37 °C. Approximately 5 μ L of above particle suspension was added to 100 μ L of washed blood at a particle-to-cell ratio of 10:1 for MS@PEG particles or 100:1 for PEG particles. Subsequent steps were the same as those used for the measurements of human whole blood cell association described above.

3.3.10 Formation of Biomolecular Corona-Coated Particles

MS@3-arm PEG, MS@4-arm PEG, MS@6-arm PEG, MS@8-arm PEG or corresponding 3-arm PEG, 4-arm PEG, 6-arm PEG, 8-arm PEG particles (2×10^8 dispersed in DPBS) were incubated in 300 μ L of plasma, which was separated from human whole blood for 1 h at 37 °C with constant shaking (600 rpm). The particles were then washed with DPBS six times resulting in ‘hard’ corona-coated particles for zeta potential and fluorescence intensity measurements.

3.3.11 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The biomolecular corona-coated particles were obtained as described above. The adsorbed proteins were eluted from the particles by incubating particles with NuPAGE LDS sample buffer and NuPAGE sample reducing agent, followed by heating to 70 °C for 10 min. After removal of particles by centrifugation, the supernatant was loaded on 4–20% Mini-PROTEAN TGX gel (BioRad) running at 120 V for 40 min. Diluted plasma was also loaded as control. After washing with RO water, the gel was stained with SimplyBlue overnight and rinsed with RO water three times prior to imaging.

3.4 Results and Discussions

3.4.1 Synthesis and Characterisation of MS@PEG and PEG Particles

MS@PEG and PEG particles were prepared from PEG building blocks with the same arm length but different arm numbers (Figure S3.1a, amine group-functionalised PEG: 3-arm-PEG-NH₂, M_w 15 kDa; 4-arm-PEG-NH₂, M_w 20 kDa; 6-arm-PEG-NH₂, M_w 30 kDa; and 8-arm-PEG-NH₂, M_w 40 kDa) via MS particle templating based on a modified method (denoted as MS@3-arm PEG, MS@4-arm PEG, MS@6-arm PEG, MS@8-arm PEG particles and 3-arm PEG, 4-arm PEG, 6-arm PEG, 8-arm PEG particles, respectively).²⁶ PEG-NH₂ was infiltrated into the porous structure of MS particles driven by electrostatic interactions, followed by cross-linking with succinimidyl carboxyl methyl ester group modified PEG (Figure S3.1b, 8-arm PEG-NHS) via covalent bonding, resulting in MS@PEG particles. The average particle size of the MS templates was 891 ± 83 nm as determined from TEM analysis (Figure S3.2). After MS template removal, PEG replica particles were obtained. Both MS@PEG and PEG particle systems were subsequently labelled with fluorescence dye AF488-NHS or AF647-NHS to enable particle visualisation, imaging and identification.

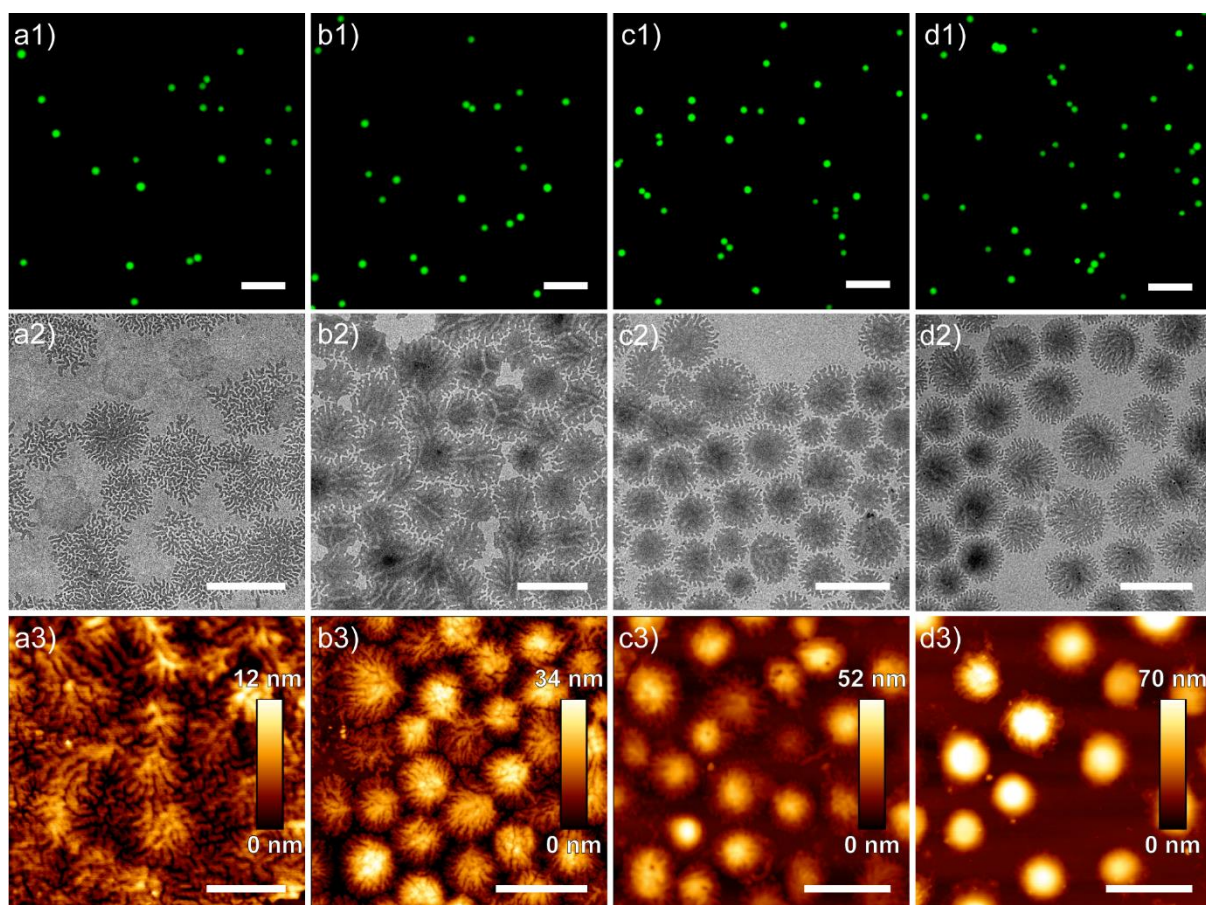


Figure 3.1. Fluorescence microscopy, TEM and AFM images of (a1–a3) 3-arm PEG particles, (b1–b3) 4-arm PEG particles, (c1–c3) 6-arm PEG particles and (d1–d3) 8-arm PEG particles. Scale bars are 10 μm in (a1–d1) and 2 μm in (a2–d2) and (a3–d3).

Figure 3.1 reveals the morphology and structure of 3-arm PEG, 4-arm PEG, 6-arm PEG and 8-arm PEG particles. The fluorescence microscopy images (Figure 3.1a1–d1) show the monodispersity and homogenous fluorescence of all the particles in aqueous solution, with an average diameter of $\sim 1.3 \mu\text{m}$ (Table 3.1), which represents approximately 46% swelling compared with the initial MS template size due to extensive hydration of PEG. TEM (Figure 3.1a2–d2) and AFM (Figure 3.1a3–d3) images show the detailed morphology and structure of PEG particles in the dry state. Particles appeared collapsed after air-drying as a result of dehydration. 3-Arm PEG particles exhibited a loose and dendritic structure, with a thickness of $5.6 \pm 1.2 \text{ nm}$ from analysis of a collapsed particle by AFM (Table 3.1, Figure S3.3), revealing that the cross-linked PEG network was highly porous and not rigid. Increasing the PEG arm number resulted in PEG particles that typically had tighter and denser structures that were less dendritic. Particle thickness (Table 3.1) and fluorescence intensity increased with increasing

PEG arm numbers (Figure S3.4), suggesting the higher mass of polymer material was cross-linked and constructed into particles.

Based on the above results, it appears that the architecture of the PEG building block (multi-arm PEG-NH₂) may influence adsorption into the pores of MS particles and subsequent cross-linking due to the distinct steric effect from the stretched PEG arms, which contributes to the differences in the structure and mechanical properties of the resultant PEG particles.

Table 3.1. Average size and thickness of PEG particles in aqueous solution or in dry state

Sample	Average size in aqueous solution^a (nm)	Thickness in dry state^b (nm)
3-arm PEG	1316 ± 118	5.6 ± 1.2
4-arm PEG	1310 ± 107	22.1 ± 3.4
6-arm PEG	1313 ± 97	36.9 ± 8.3
8-arm PEG	1333 ± 40	55.9 ± 4.1

^a The average size of PEG particles in aqueous solution was analysed from fluorescence microscopy images using ImageJ software. The full width at half maximum of the fluorescence profile across the particles was considered as the particle diameter. Forty particles were analysed for each sample.

^b The air-dried particle thickness was determined from the distance between the highest point on the particle and the substrate surface using AFM. Fifteen particles were analysed for each sample.

Zeta potential measurements (Figure 3.2) show that MS@PEG particles had a less negative surface charge than the MS particles in phosphate buffer (pH 7.4, 5 mM) owing to the neutral PEG coating. This ‘neutralisation effect’ was more obvious with increasing PEG arm number, probably due to the higher PEG coating density and different PEG conformation on the surface masking the negative charge of the MS core in phosphate buffer. PEG replica particles (without MS templates) exhibited near-neutral charge at pH 7.4 irrespective of the PEG arm number.

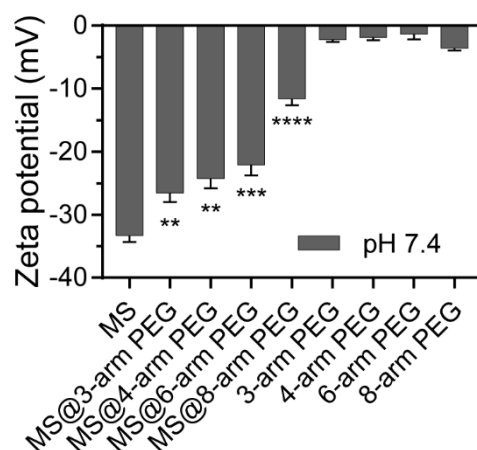


Figure 3.2. Zeta potential of MS particles, MS@PEG particles and PEG particles in phosphate buffer (pH 7.4, 5 mM). Data are shown as the mean $\pm$ standard error of at least three independent experiments (Student *t*-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ versus the column of ‘MS’).

3.4.2 Mechanical Property Analysis

To measure the elastic modulus of the PEG particles, AFM force measurements were performed using the colloidal-probe atomic force microscopy (CP-AFM) technique (Figure 3.3a). A modified probe was used with a spherical glass bead ($D = 22 \mu\text{m}$) attached to a cantilever using epoxy resin (Figure S3.5a). The probe was slowly lowered onto the individual immobilised PEG particles to perform the force measurements. Force–deformation curves (Figure 3.3b) were obtained from the raw AFM voltage–displacement data processed using JPK data processing software (v.4.4.29). The Young’s modulus value of PEG particles was evaluated from the linear range (0 to 200 nm) of F – δ curves using Hertz theory (as described in Section 3.3.5). As shown in Figure 3.3c, the Young’s modulus of the PEG particles varied with different PEG building blocks and increased with increasing PEG arm numbers from ~ 1.5 kPa for 3-arm PEG particles to ~ 14.9 kPa for 8-arm PEG particles. As the particles were all constructed with PEG and have similar sizes in aqueous solution, the particle elastic modulus is mainly influenced by material density within the cross-linked structure. 8-Arm PEG particles had the highest Young’s modulus value among the four types of PEG particles, which is consistent with the highest particle density observed in TEM and AFM images in Figure 3.1. All the particles were very soft compared with previously reported polymeric particles and PEG-based particles.^{25, 32}

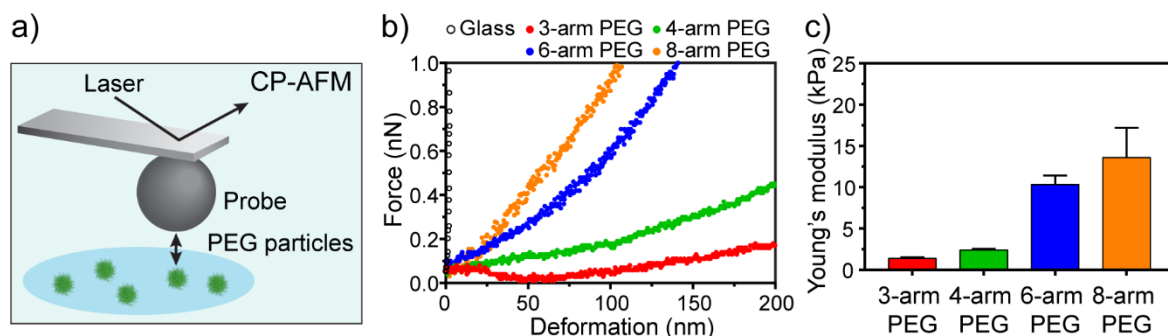


Figure 3.3. (a) Schematic illustration of the elastic modulus measurements between a glass bead probe and PEG particles by CP-AFM. (b) Representative force–deformation (F – δ) curves of 3-arm PEG, 4-arm PEG, 6-arm PEG and 8-arm PEG particles. The F – δ curve of a glass substrate is also shown for calibration. (c) Young's modulus derived from the F – δ analysis for the PEG particles.

3.4.3 Cell Association

To explore the ‘stealth property’ of the PEG particles and the different MS@PEG particles (with mesoporous silica cores intact), their cellular interactions with a commonly studied macrophage cell line RAW 264.7 was examined. The association of MS@PEG particles with RAW 264.7 cells was significantly higher than that of corresponding PEG particles after 12 h of incubation regardless of the PEG building block used (Figure 3.4a). However, negligible differences were detected between the different MS@PEG particles from the statistical analyses shown in Figure S3.6a; all particles displayed >90% cell association. The PEG particles displayed low cell association irrespective of arm number and incubation time (Figure 3.4a and b), which confirms the stealth property of the PEG particles. Increasing the particle number (the particle-to-cell ratio from 100:1 to 1000:1) effectively increased cell association slightly, with 8-arm PEG particles exhibiting the highest binding (Figure S3.6b), probably due to the highest density and stiffness of these particles.^{25, 33} Confocal microscopy analysis was carried out to confirm RAW 264.7 cell association of MS@PEG and PEG particles (Figure 3.5). The images were consistent with flow cytometry results, with the PEG particles associating with the RAW 264.7 cells in low numbers, whereas more than 90% of cells contained MS@PEG particles.

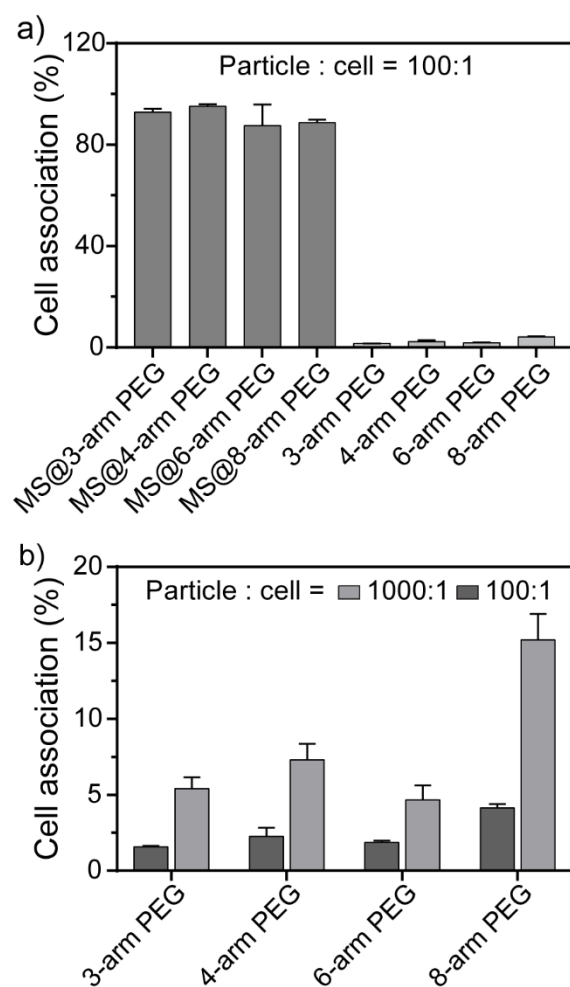


Figure 3.4. RAW 264.7 cell association of (a) MS@PEG and PEG particles after 12 h of incubation at a particle-to-cell ratio of 100:1 and (b) PEG particles after 24 h of incubation at a particle-to-cell ratio of 100:1 and 1000:1. Data are shown as the mean $\pm$ standard error of at least three independent experiments. Statistical data are presented in Figure S3.6.

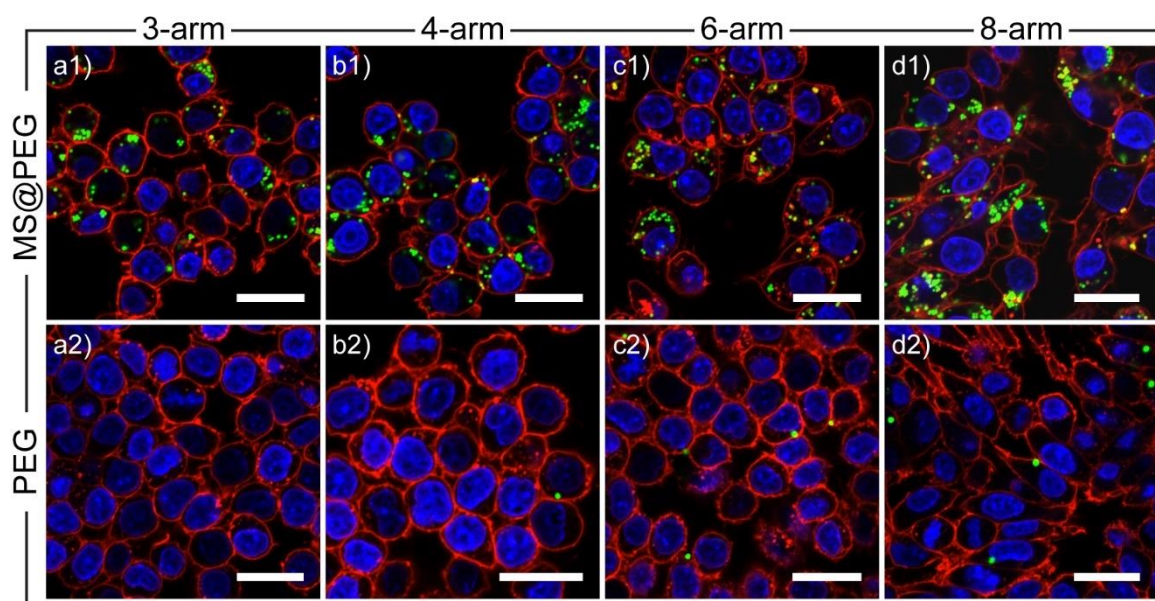


Figure 3.5. Confocal microscopy images of RAW 264.7 cells after incubation with AF488-labelled (a1) MS@3-arm PEG, (b1) MS@4-arm PEG, (c1) MS@6-arm PEG, (d1) MS@8-arm PEG, (a2) 3-arm PEG, (b2) 4-arm PEG, (c2) 6-arm PEG, or (d2) 8-arm PEG particles (green) for 12 h at a particle-to-cell ratio of 100:1. The cell membrane was stained with wheat germ agglutinin Alexa Fluor 594 conjugate (WGA-594, red) and the nuclei were stained with Hoechst 33342 (blue). Scale bars are 20 μm .

Because of the limitation of using a model cell line, wherein cells cultured under non-physiological condition, which is not representative of cells residing in a complex biological environment, human blood was used to predict the behaviour of MS@PEG and PEG particles *in vivo*.³⁴ Many nanostructured materials fail to fulfil their designed function following intravenous administration as they are rapidly recognised and sequestered by the mononuclear phagocyte system in the body, which is responsible for removing dead cells, pathogens and foreign substances from the blood circulation.³⁵ Human whole blood, a highly complex biological environment consisting of red blood cells, immune cells and numerous biomolecules such as plasma proteins and antibodies, is thereby considered to be a more relevant model to study the interactions between particles and biological systems than a mono-cultured cell line.³⁶ Herein, AF647-labelled MS@PEG or PEG particles were incubated in fresh healthy human whole blood at varying particle-to-cell ratios for 1 h at 37 $^{\circ}\text{C}$. The major human immune cells in blood (granulocytes, monocytes, B cells, NK cells, T cells and dendritic cells) were labelled with a fluorescent antibody ‘cocktail’ in titrated concentrations for the gating of cell population.

The cell association measurements were carried out by the flow cytometry and analysed using a previously established gating method (Figure S3.7).

Consistent with the results derived using RAW 264.7 cells, MS@PEG particles resulted in notably higher association with granulocytes, monocytes and B cells compared with the corresponding PEG particles at the same particle-to-cell ratio of 50:1, irrespective of PEG arm numbers (Figure 3.6). The high cell association of MS@PEG particles is likely due to their rigid structure in addition to the net negative surface charge that could lead to the adsorption of biomolecules by electrostatic interactions. At a particle-to-cell ratio of 50:1, cell association for all MS@PEG particles was >90%, which decreased with decreasing particle-to-cell ratios and MS@PEG particles loaded with more-arm PEG had lower association with phagocytes (granulocytes and monocytes) and B cells, likely owing to the thicker and denser PEG protective layer on the surface. At the lower ratios, it was also observed that B cells have a greater association with particles than granulocytes and monocytes for all the MS@PEG particles studied (Figure 3.6a). For instance, cell association with MS@3-arm PEG at a particle-to-cell ratio of 10:1 resulted in 90% of B cells taking up the particles, whereas 65% of granulocytes and 55% of monocytes associated with the particles.

The opposite trend was observed for the PEG particles. B cells associated at a lower extent with PEG particles than granulocytes and monocytes. The association of PEG particles with phagocytic blood cells (granulocytes and monocytes) and B cells increased with increasing arm number and this was more pronounced at higher particle-to-cell ratios (Figure 3.6b). As the particles are made of the same material with a comparable surface charge (~ 0 mV), the differences in cell association are likely due to differences in particle structure, conformation and elasticity, which may lead to different hydration degrees and repulsion forces to the surrounded proteins. The results are opposite to the previous finding that PEG building blocks with the same arm number but higher molecular weight (longer arms) resulted in reduced cell association of assembled PEG particles,²⁶ suggesting that higher molecular weight PEG does not necessarily contribute to lower cell association and better stealth property for PEG replica particles. The architecture of PEG units is an important factor that needs to be considered when studying stealth property.

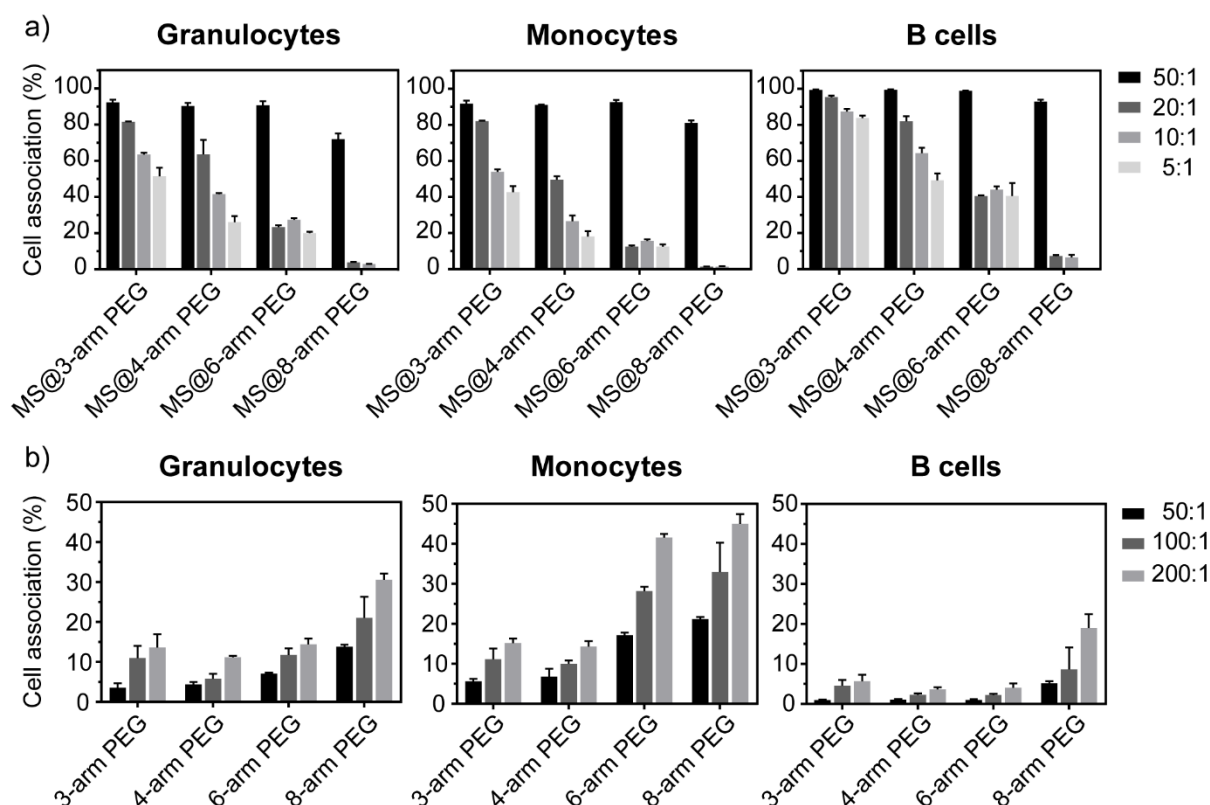


Figure 3.6. Cell association of (a) MS@PEG and (b) PEG particles with granulocytes, monocytes and B cells after incubation in human whole blood for 1 h at varying particle-to-cell ratios. Data are shown as the mean $\pm$ standard error of three independent experiments, with at least 100 000 leukocytes analysed in each individual experiment. Cell associations with the other blood cell populations, i.e. NK cells, T cells and dendritic cells, are shown in Figure S3.8.

To further understand how particle properties affect the formation of a biomolecular corona and subsequent interactions with blood cells, the cell association of MS@PEG and PEG particles incubated under different conditions was examined: (1) human whole blood; (2) human blood plasma followed by washed blood; and (3) human washed blood, where either ‘hard’ and ‘soft’ corona (1 and 2) or ‘no’ corona (3) was formed (Scheme 3.1b). Condition (1) refers to particles treated with whole blood where both biomolecules in plasma and cells are present in the system. Condition (2) refers to particles preincubated in blood plasma to allow the formation of a ‘hard’ and ‘soft’ corona, followed by incubation with cells that had been separated from whole blood via centrifugation (termed washed blood). Condition (3) refers to particles incubated in washed blood directly where only blood cells are present without plasma proteins.

Figure 3.7a shows that the association of MS@PEG particles with cells in washed blood was substantially reduced in contrast to incubation in whole blood, which indicates that the plasma proteins play an essential role in cellular association. This observation is supported by the results of preincubating particles in blood plasma before incubation with washed blood, which recovered cellular association to the high levels. Furthermore, despite the low level of cell association in the absence of protein corona, there were still detectable differences between MS@8-arm PEG particles and the other three types of particles (Figure S3.9), indicating that in addition to the formation of protein corona, the intrinsic properties of a particle directly influence cellular association.

For PEG particles (Figure 3.7b), there was no significant difference between the cell associations with (2) and without (3) protein corona, which suggests that the protein corona has relatively limited influence on low-fouling materials such as PEG. From Figure 3.7b, there was a gradual increase in cellular association with increasing PEG arm numbers, with 8-arm PEG particles exhibiting the highest binding to B cells, granulocytes and monocytes in the presence or absence of a protein corona. This suggests that cellular association for the PEG particles was governed mainly by the synthetic properties of the particles such as structure and elasticity, probably by influencing the mechanism of cell uptake of particles particularly receptor-independent mechanisms.³⁷

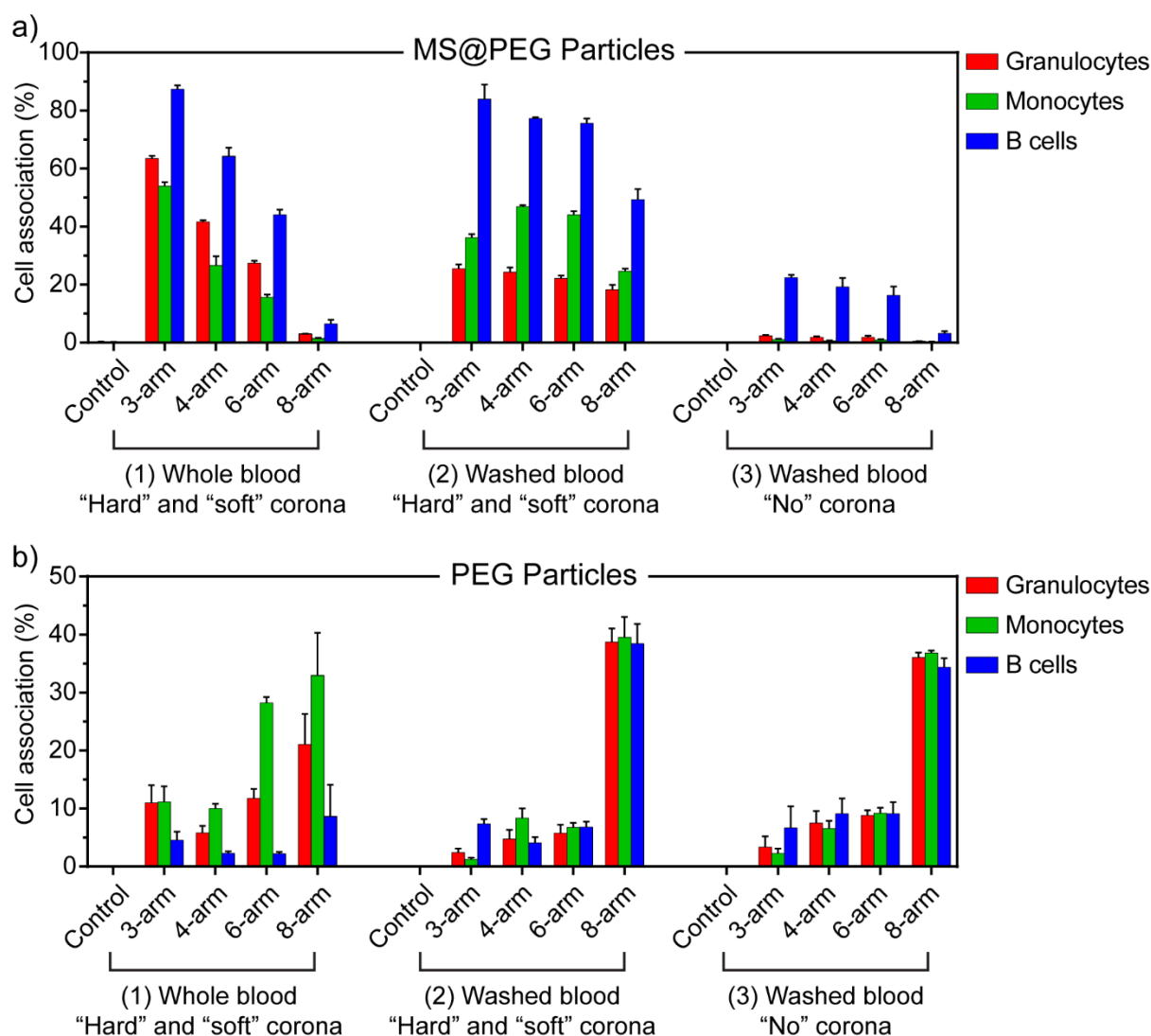


Figure 3.7. Cell association of (a) MS@PEG and (b) PEG particles with granulocytes, monocytes, and B cells after incubation in (1) human whole blood, (2) blood plasma followed by human washed blood or (3) human washed blood. Based on the different incubation conditions, either a 'hard' and 'soft' corona (1 and 2) or 'no' corona (3) was formed on the particles. The particle-to-cell ratio was set at 10:1 for MS@PEG particles and 100:1 for PEG particles. Data are shown as the mean $\pm$ standard error of three independent experiments, with at least 100 000 leukocytes analysed in each individual experiment. Control refers to the cell populations without particle treatment in the incubation medium.

3.4.4 Protein Corona

To confirm the formation of a protein corona on particles and its effect on the particle surface chemistry, the fluorescence intensity (Figure 3.8a) and zeta potential (Figure 3.8b) of

MS@PEG and PEG particles were examined before and after protein corona coating derived from human blood plasma. The fluorescence intensity of both MS@PEG and PEG particles was reduced in the presence of a protein corona (Figure 3.8a). The shift of the MS@PEG particle distribution towards a lower fluorescence intensity was more obvious than that of the PEG particles (Figure S3.10), as a higher surface coverage of proteins led to a greater decrease in particle fluorescence intensity. For both MS@PEG and PEG particles, the increase in fluorescence intensity from 3-arm to 8-arm remained linear after protein corona coating. The adsorption of proteins reduced the zeta potential of MS@3-arm PEG and MS@4-arm PEG particles, whereas no significant changes in the zeta potential of MS@6-arm PEG and MS@8-arm PEG particles were observed (Figure 3.8b). For the PEG particles, except for the 8-arm PEG, the particle zeta potential changed from ~ -2 to ~ -5 mV in the presence of a protein corona, which was likely due to some nonspecific binding of biomolecules on the surface. The negligible change in particle fluorescence intensity and zeta potential for 8-arm PEG particles suggests that few proteins are adsorbed onto particles, which supports the hypothesis that the interaction of 8-arm PEG particles with cells is mainly due to the intrinsic properties of the particles, including higher density and stiffness, whereas the protein corona plays a smaller role. However, it should be noted that the protein corona-coated particles analysed in Figure 3.8 were extensively washed to avoid interference from free proteins, thus the layer of ‘soft’ corona was removed completely. The particles were therefore not the same as those existing in plasma, which have both the ‘hard’ and ‘soft’ corona present. The tightly bound proteins on particles termed as ‘hard’ protein corona were eluted and analysed by SDS-PAGE (Figure S3.12). The results showed a clear band between 64 kDa and 98 kDa which is likely to be histidine-rich glycoprotein for all the MS@PEG particles with smaller bands present for MS@3-arm PEG particles, whereas evidence of protein adsorption on the PEG particles was poor, confirming the low-fouling nature of PEG replica particles.

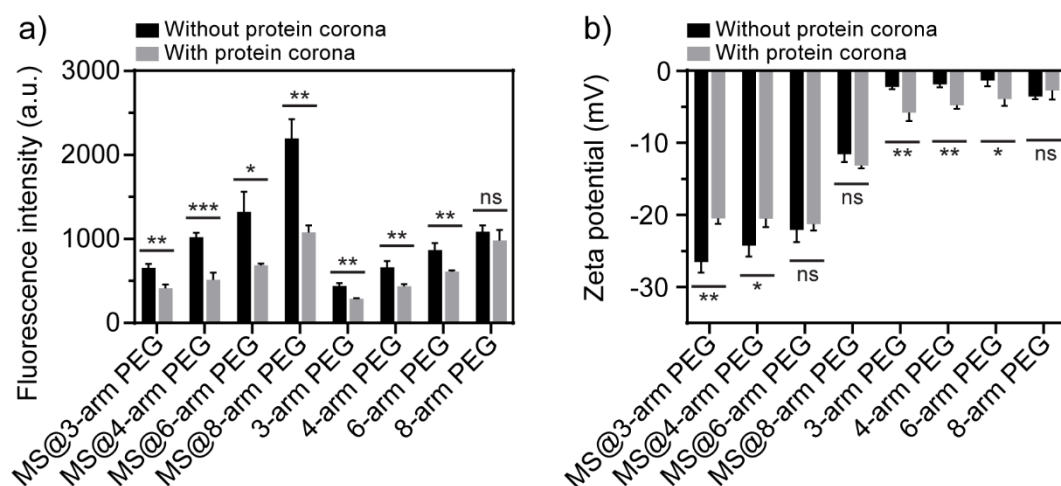


Figure 3.8. (a) Fluorescence intensity and (b) zeta potential of MS@PEG and PEG particles without or with protein corona derived from human blood plasma. Data are shown as the mean $\pm$ standard error of at least three independent experiments (Student *t*-test, ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.5 Conclusions

PEG-based particles were synthesised by a mesoporous silica-templated method. Removal of the silica template resulted in PEG particles. The elastic modulus of the PEG particles was tuned from 1.5 to 14.9 kPa by using PEG building blocks with different arm numbers. For PEGylated mesoporous silica (MS@PEG) particles, increasing the arm numbers of PEG units led to reduced phagocytic blood cell and B cell association, potentially due to a denser PEG coating and reduced negative surface charge at higher PEG arm numbers. However, the opposite trend was observed for the PEG particles, with more-arm PEG building blocks (higher molecular weight) resulting in higher immune cell association, probably due to a denser and stiffer structure. This finding is contrary to previous findings that have revealed that having the same arm number, higher molecular weight PEG (longer arms) contributes to reduced cell association and enhanced stealth property for assembled PEG particles. Thus, this comparison suggests that higher molecular weight PEG does not necessarily contribute to an improved stealth property for PEG replica particles. The present findings suggest that both the intrinsic properties of particles and their interactions with biological components, such as blood proteins, determine the interaction of the particles with immune cells and that the protein corona plays a relatively limited role in low-fouling materials. This work indicates that the building block architecture can influence the mechanical properties of the particles with identical composition,

and subsequently alert their interactions with biological systems, which is an important factor that needs to be carefully considered in the design of functional nanoengineered materials for biomedical applications. It also shows how the synthetic properties of particles can be engineered to control their biological identity to allow modulation of interactions with cells in complex biological environments. PEG-based particles with low-fouling properties are potential candidates for drug delivery to increase retention time in the lungs and can be further engineered to target the epithelial cells underlying the mucus layer. This aspect is interesting and relevant for the scientific community to guide particle design for biomedical applications, including pulmonary delivery.

3.6 Supporting Information

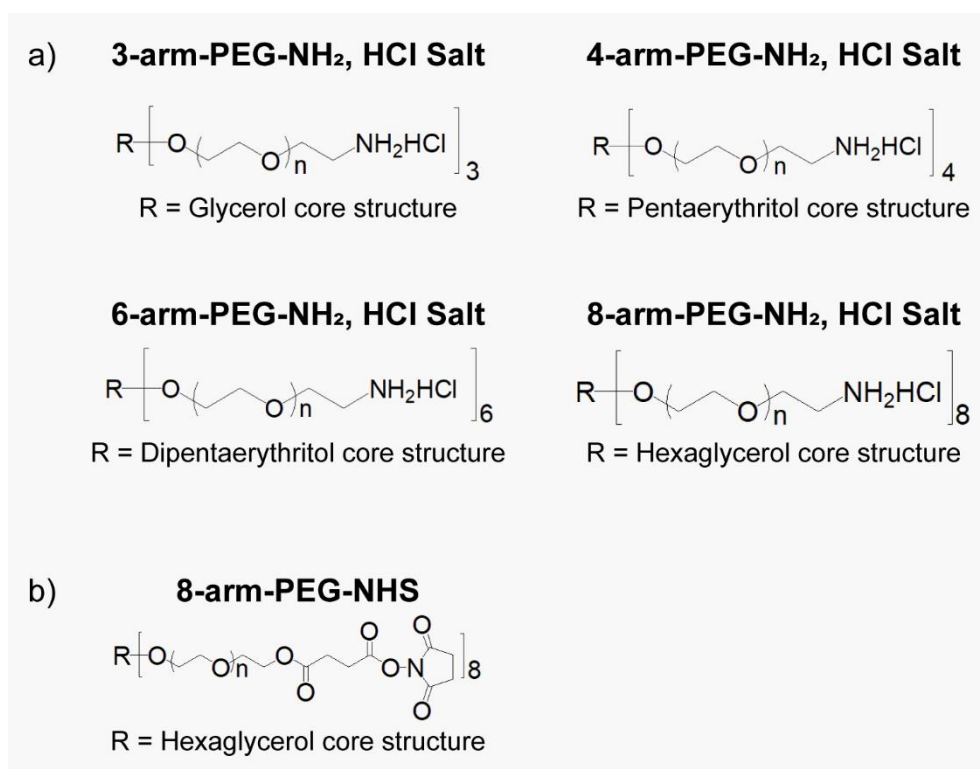


Figure S3.1. Molecular structures of (a) building blocks 3-arm-PEG-NH₂, 4-arm-PEG-NH₂, 6-arm-PEG-NH₂ and 8-arm-PEG-NH₂, and (b) cross-linker 8-arm-PEG-NHS.

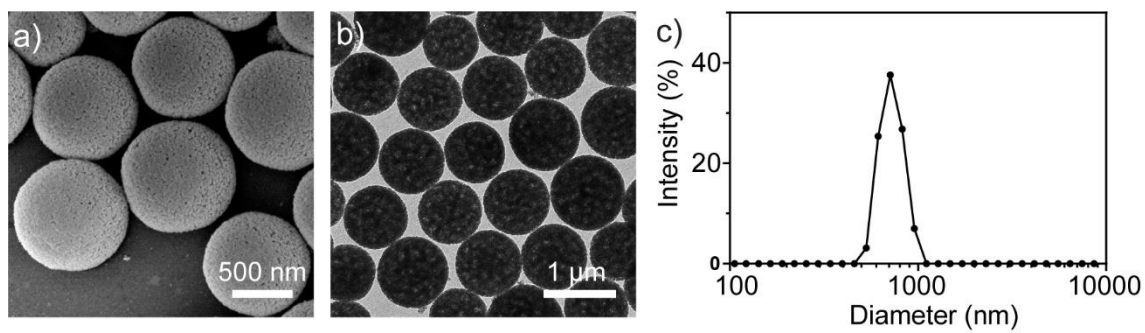


Figure S3.2. (a) SEM and (b) TEM images and (c) size distribution (determined by dynamic light scattering) of MS particles.

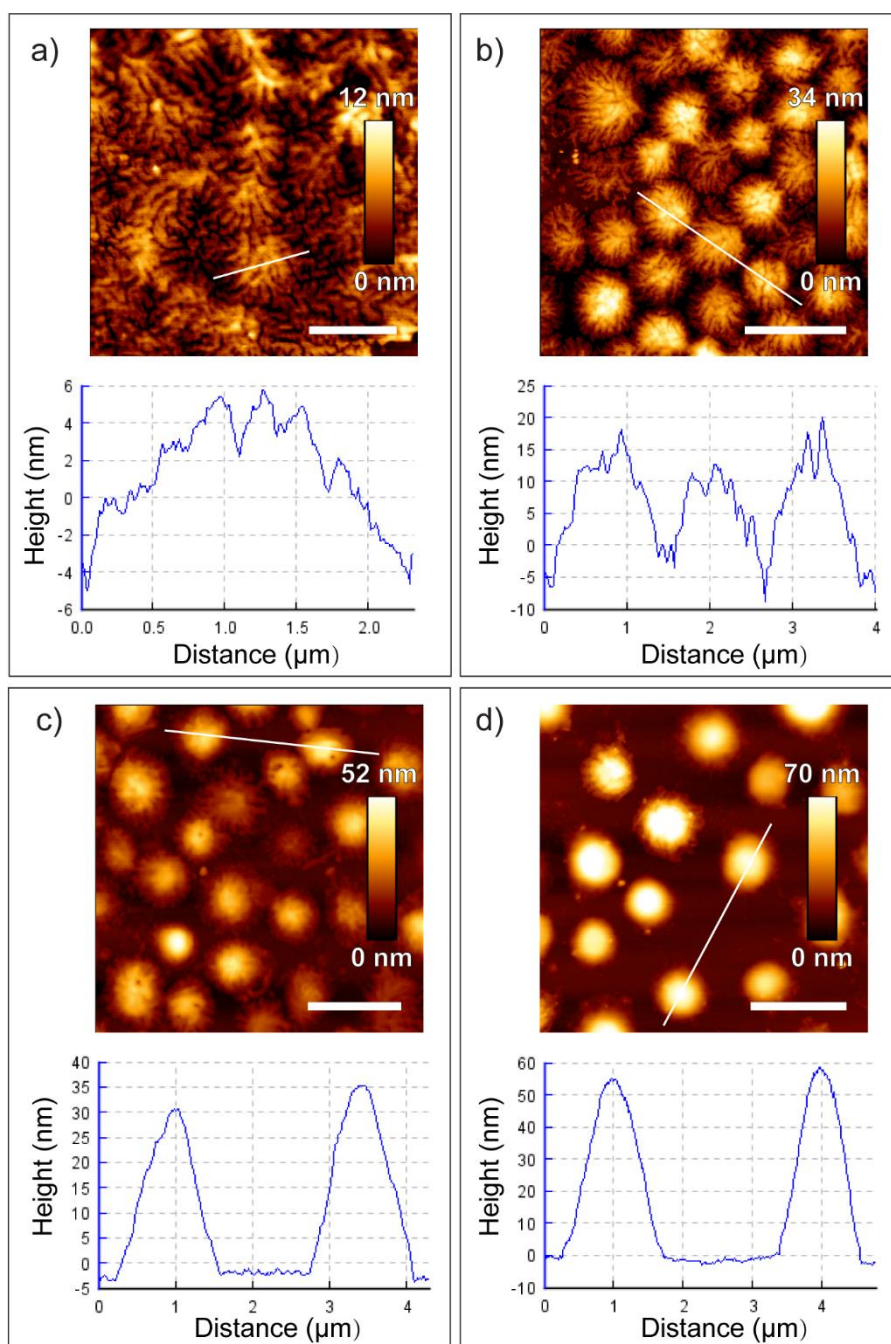


Figure S3.3. Representative AFM images and corresponding height profiles of (a) 3-arm PEG, (b) 4-arm PEG, (c) 6-arm PEG and (d) 8-arm PEG particles. Scale bars are 2 μm .

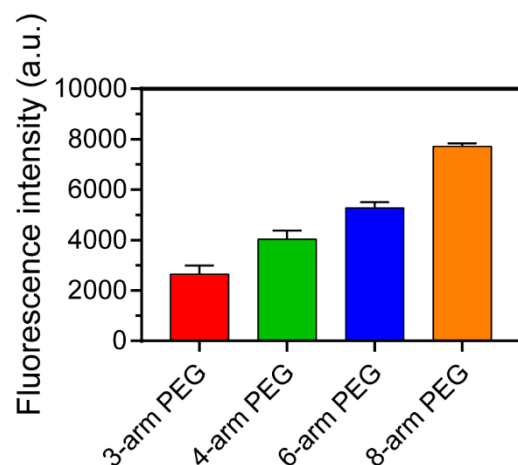


Figure S3.4. Fluorescence intensity of AF488-labelled PEG particles.

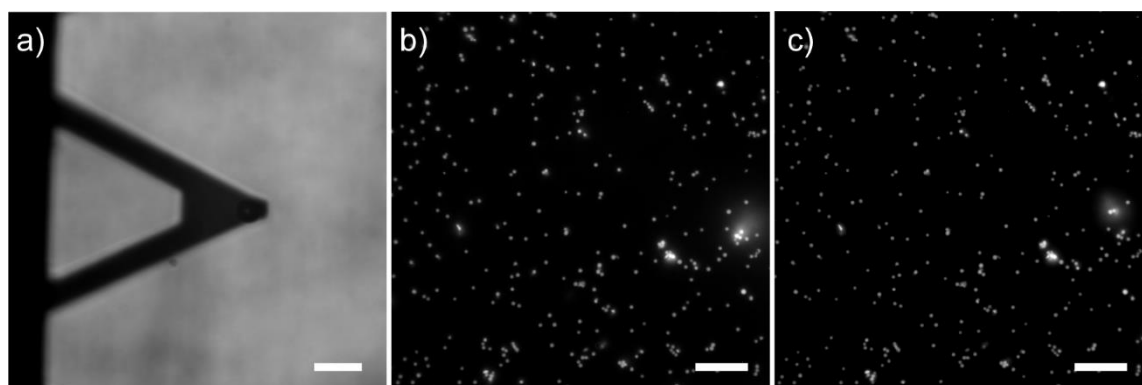


Figure S3.5. (a) Optical microscopy image of the silica colloidal probe used for the CP-AFM force measurements. Fluorescence microscopy images of AF488-labelled 8-arm PEG particles at the base of a FluoroDish (b) before and (c) after the force measurements. Scale bars are 50 μm in (a) and 20 μm in (b) and (c).

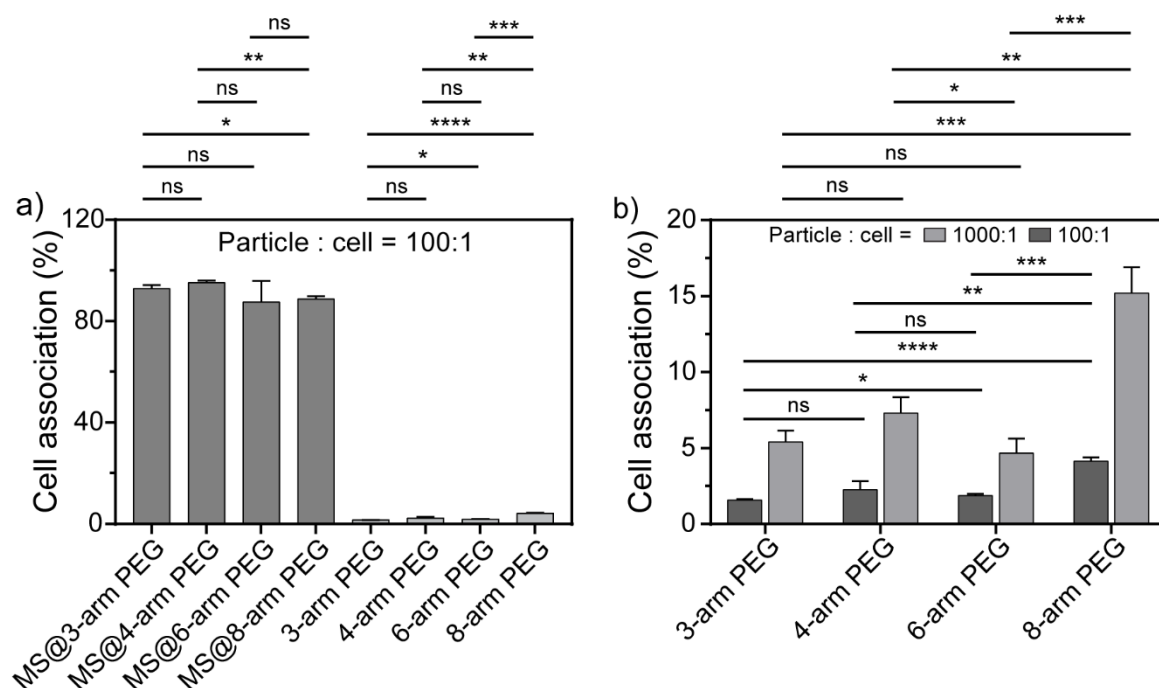


Figure S3.6. RAW 264.7 cell association of (a) MS@PEG and PEG particles after 12 h of incubation at a particle-to-cell ratio of 100:1 and (b) PEG particles after 24 h of incubation at a particle-to-cell ratio of 100:1 or 1000:1. Data are shown as the mean $\pm$ standard error of at least three independent experiments (Student *t*-test, ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

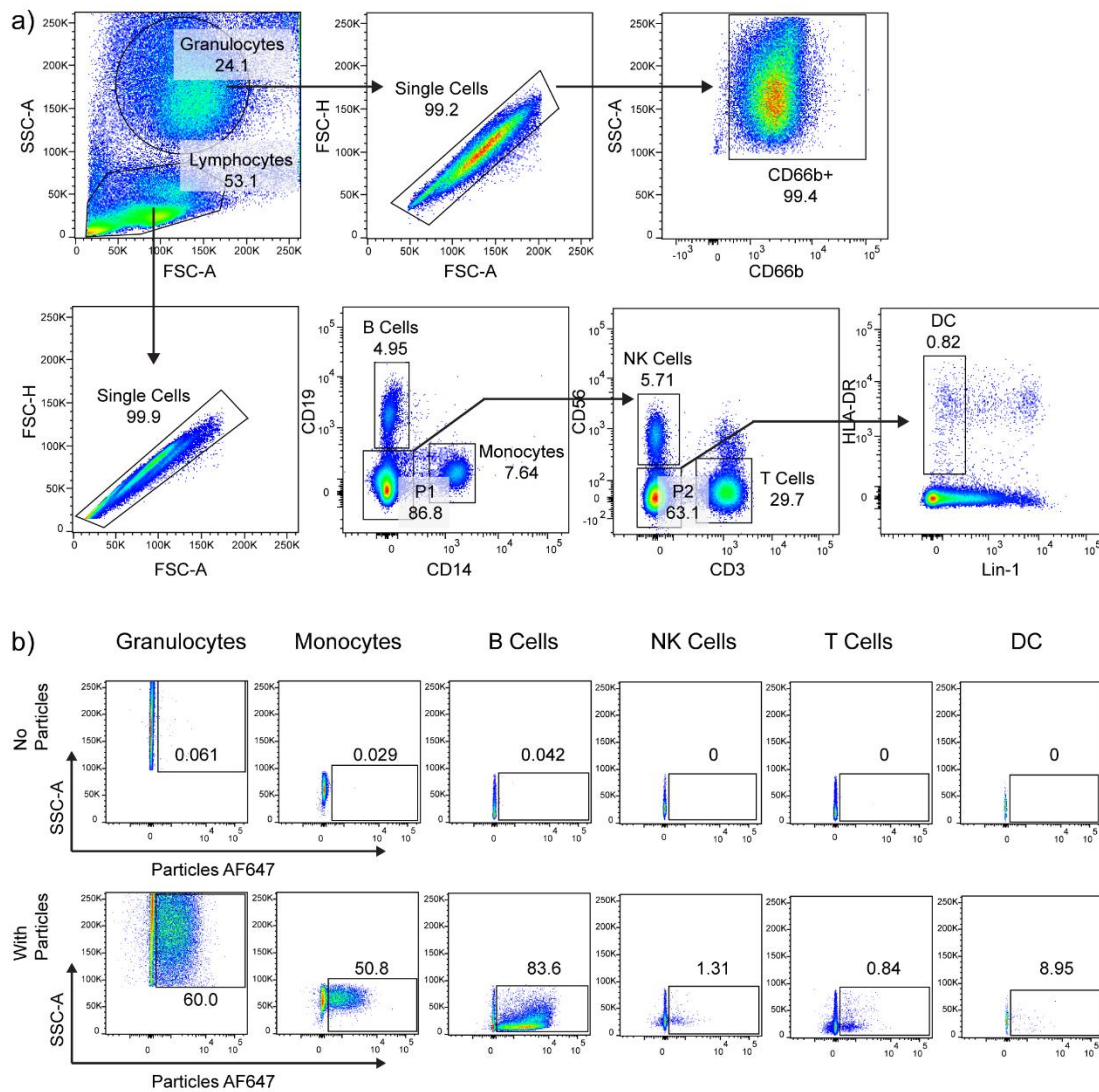


Figure S3.7. (a) Gating method used to identify white blood cell populations. Side and forward scatter were first applied to gate white blood cells before doublets were excluded. The specific cell types were identified according to expression of surface markers: CD66b+ granulocytes; CD14+ monocytes; CD19+ B cells; CD56+ NK cells; CD3+ T cells; and HLA-DR PE-CF594 dendritic cells. (b) Percentage of each cell type positive for the AF647-labelled particles was then measured and an example of the gating and particle association values for one sample is shown.

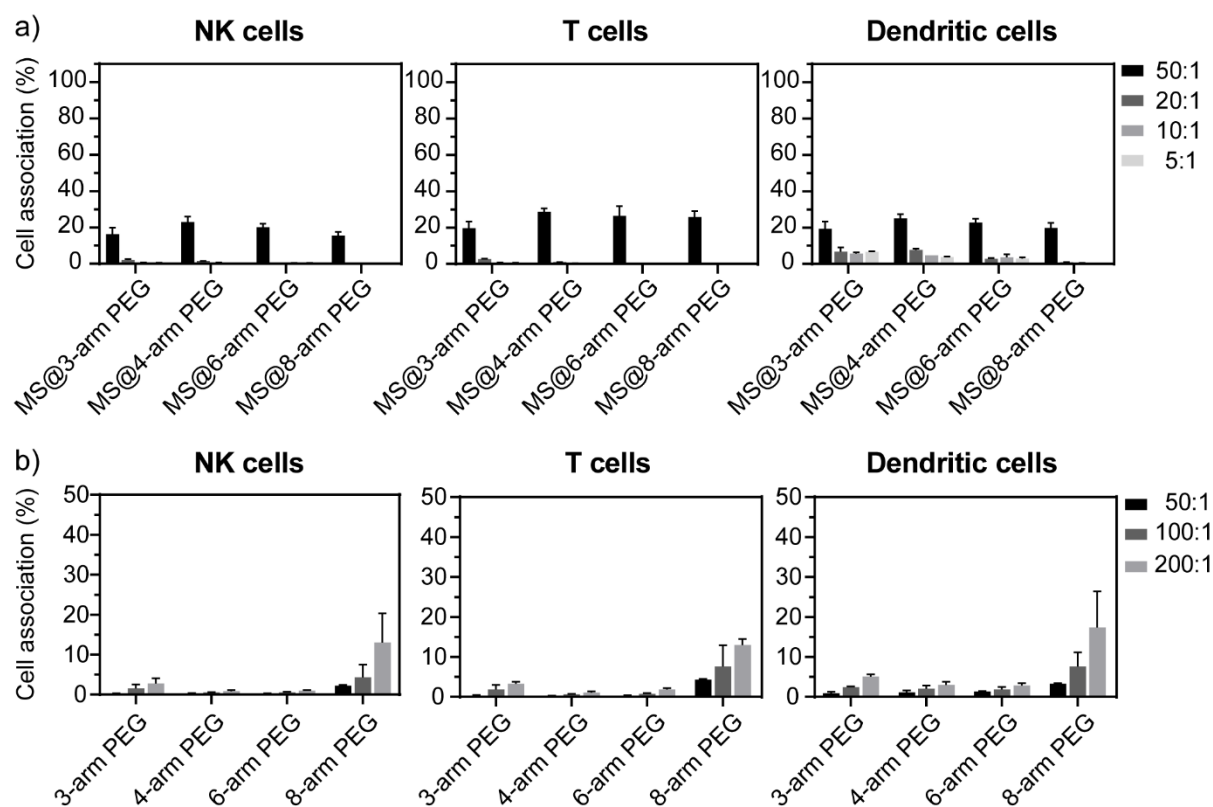


Figure S3.8. Cellular association of (a) MS@PEG and (b) PEG particles with NK cells, T cells and dendritic cells after incubation in human whole blood for 1 h at varying particle-to-cell ratios. Data are shown as the mean $\pm$ standard error of three independent experiments, with at least 100 000 leukocytes analysed in each individual experiment.

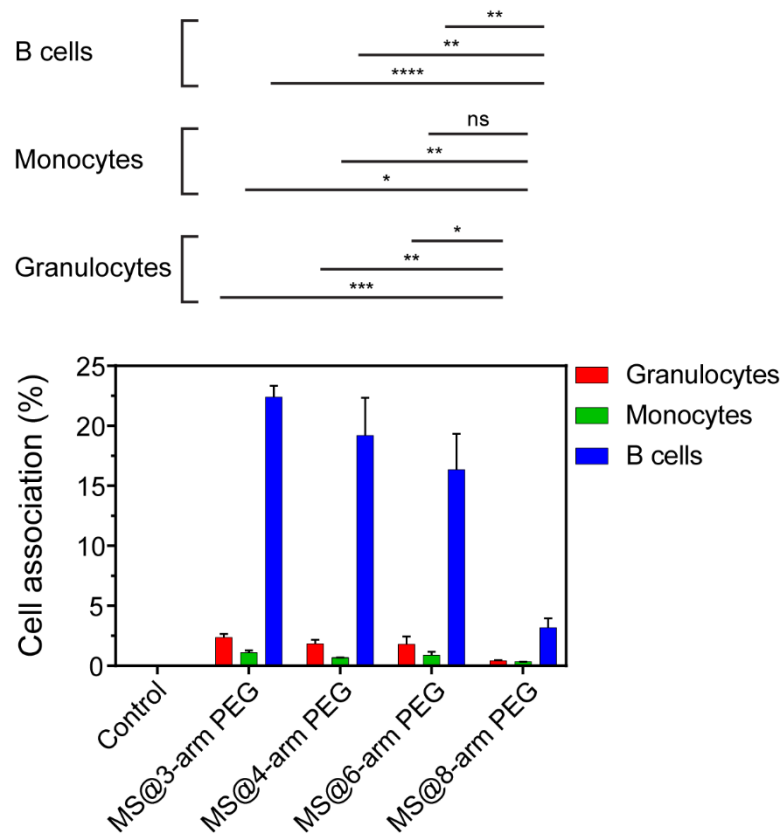


Figure S3.9. Association of MS@PEG particles with granulocytes, monocytes and B cells after incubation in human washed blood at a particle-to-cell ratio of 10:1. Data are shown as the mean $\pm$ standard error of three independent experiments, with at least 100 000 leukocytes analysed in each individual experiment (Student *t*-test, ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

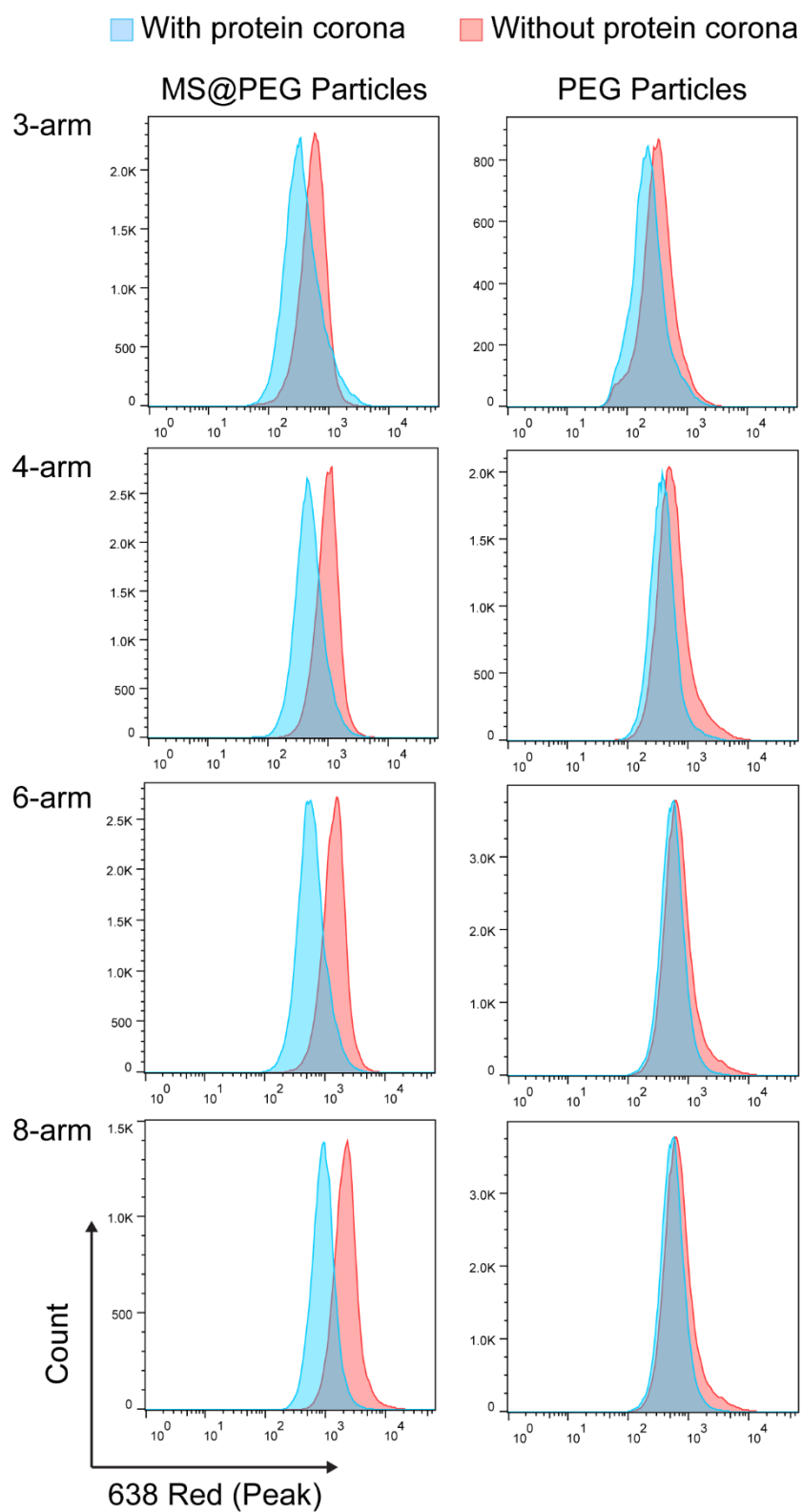


Figure S3.10. Fluorescence intensity distribution of MS@PEG and PEG particles without or with protein corona measured by flow cytometry (Apogee flow).

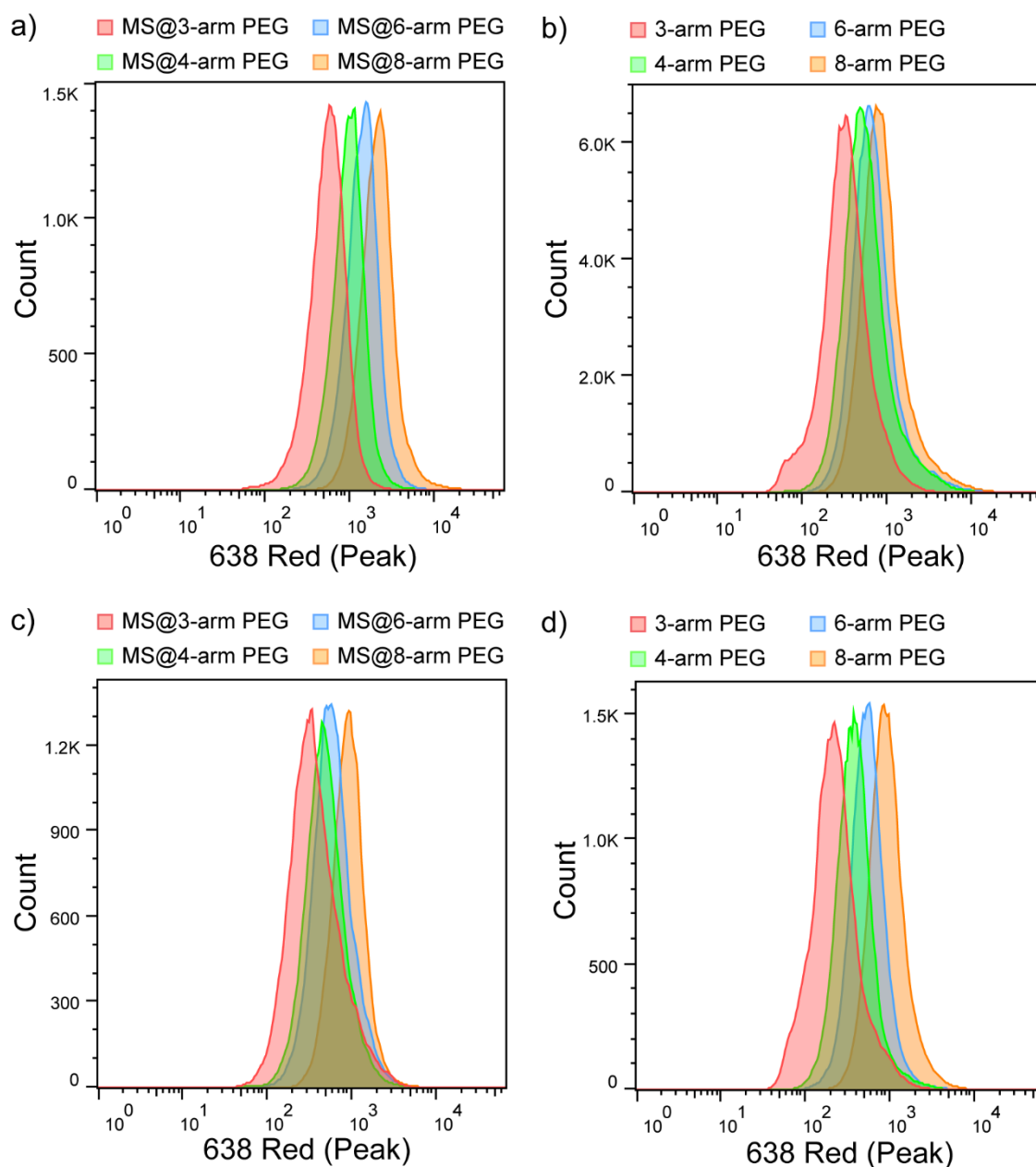


Figure S3.11. Fluorescence intensity distributions of (a) MS@PEG and (b) PEG particles without protein corona, and (c) MS@PEG and (d) PEG particles with protein corona, measured by flow cytometry (Apogee flow).

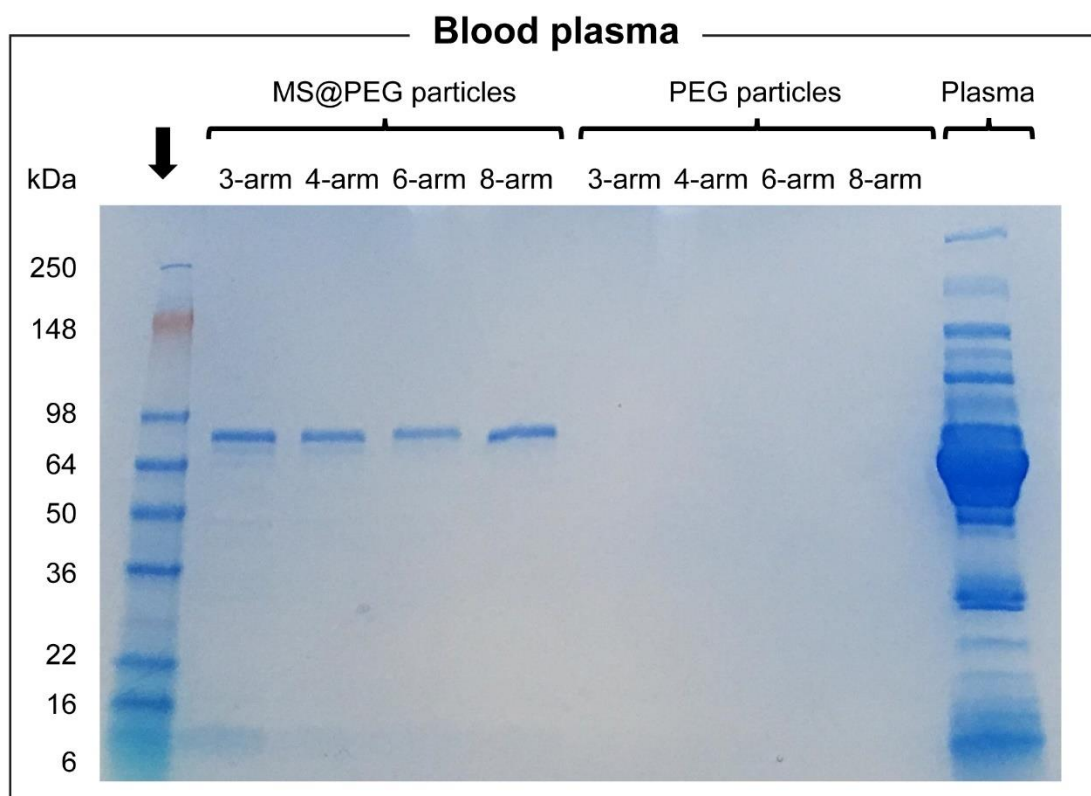


Figure S3.12. SDS-PAGE image of proteins separated from ‘hard’ corona of MS@PEG and PEG particles after 1 h of incubation in human blood plasma at 37 °C. The reference bands related to particular molecular weights are shown on the left in the image as indicated by an arrow. The results of blood plasma are shown on the right.

Table S3.1. Cell count data of human whole blood (before wash) and human washed blood (after wash) for the cell association experiments displayed in Figure 3.6, Figure 3.7 and Figure S3.8

Cell type	Whole blood	Washed blood
WBC [$\times 10^9 \text{ L}^{-1}$]	12.2	11.8
Lymphocytes [%]	20.5	15.4
Monocytes [%]	6.6	6.5
Granulocytes [%]	72.9	78.1
RBC [$\times 10^{12} \text{ L}^{-1}$]	4.76	5.5
Haemoglobin [g L^{-1}]	132	153
Haematocrit [L L^{-1}]	0.396	0.469
Platelets [$\times 10^9 \text{ L}^{-1}$]	285	20
WBC, white blood cells; RBC, red blood cells.		

Table S3.2. Properties of the antibodies used in the human blood assays

Antibody	Colour	Clone	Targeted cells
Lin-1	FITC	Mouse various	Negative dendritic cells
CD66b	BV421	Mouse G10F5, κ	Neutrophils
CD19	BUV395	Mouse SJ25C1, κ	B cells
CD3	AF700	Mouse SP34-2, λ	T cells
CD14	APC-H7	Mouse MΦP9, κ	Monocytes
CD56	PE	Mouse B159, κ	NK cells
HLA-DR	PE-CF594	Mouse G46-6, κ	Dendritic cells

3.7 References

1. Cui, J.; Richardson, J. J.; Bjornmalm, M.; Faria, M.; Caruso, F., Nanoengineered Templated Polymer Particles: Navigating the Biological Realm. *Acc Chem Res* **2016**, *49* (6), 1139-48.
2. Mitragotri, S.; Burke, P. A.; Langer, R., Overcoming the challenges in administering biopharmaceuticals: formulation and delivery strategies. *Nat Rev Drug Discov* **2014**, *13* (9), 655-72.
3. Patra, J. K.; Das, G.; Fraceto, L. F.; Campos, E. V. R.; Rodriguez-Torres, M. D. P.; Acosta-Torres, L. S.; Diaz-Torres, L. A.; Grillo, R.; Swamy, M. K.; Sharma, S.; Habtemariam, S.; Shin, H. S., Nano based drug delivery systems: recent developments and future prospects. *J Nanobiotechnology* **2018**, *16* (1), 71.
4. Zelikin, A. N.; Ehrhardt, C.; Healy, A. M., Materials and methods for delivery of biological drugs. *Nat Chem* **2016**, *8* (11), 997-1007.
5. Tibbitt, M. W.; Dahlman, J. E.; Langer, R., Emerging Frontiers in Drug Delivery. *J Am Chem Soc* **2016**, *138* (3), 704-17.
6. Albanese, A.; Tang, P. S.; Chan, W. C., The effect of nanoparticle size, shape, and surface chemistry on biological systems. *Annu Rev Biomed Eng* **2012**, *14*, 1-16.
7. Walkey, C. D.; Olsen, J. B.; Guo, H.; Emili, A.; Chan, W. C., Nanoparticle size and surface chemistry determine serum protein adsorption and macrophage uptake. *J Am Chem Soc* **2012**, *134* (4), 2139-47.
8. Alexis, F.; Pridgen, E.; Molnar, L. K.; Farokhzad, O. C., Factors affecting the clearance and biodistribution of polymeric nanoparticles. *Mol Pharm* **2008**, *5* (4), 505-15.
9. Jiang, Y.; Huo, S.; Mizuhara, T.; Das, R.; Lee, Y. W.; Hou, S.; Moyano, D. F.; Duncan, B.; Liang, X. J.; Rotello, V. M., The Interplay of Size and Surface Functionality on the Cellular Uptake of Sub-10 nm Gold Nanoparticles. *ACS Nano* **2015**, *9* (10), 9986-93.
10. Song, D.; Cui, J.; Ju, Y.; Faria, M.; Sun, H.; Howard, C. B.; Thurecht, K. J.; Caruso, F., Cellular Targeting of Bispecific Antibody-Functionalized Poly(ethylene glycol) Capsules: Do Shape and Size Matter? *ACS Appl Mater Interfaces* **2019**, *11* (32), 28720-28731.
11. Walkey, C. D.; Chan, W. C., Understanding and controlling the interaction of nanomaterials with proteins in a physiological environment. *Chem Soc Rev* **2012**, *41* (7), 2780-2799.
12. Lazarovits, J.; Chen, Y. Y.; Sykes, E. A.; Chan, W. C., Nanoparticle-blood interactions: the implications on solid tumour targeting. *Chem Commun* **2015**, *51* (14), 2756-67.

13. Ke, P. C.; Lin, S.; Parak, W. J.; Davis, T. P.; Caruso, F., A Decade of the Protein Corona. *ACS Nano* **2017**, *11* (12), 11773-11776.
14. Tenzer, S.; Docter, D.; Kuharev, J.; Musyanovych, A.; Fetz, V.; Hecht, R.; Schlenk, F.; Fischer, D.; Kiouptsi, K.; Reinhardt, C.; Landfester, K.; Schild, H.; Maskos, M.; Knauer, S. K.; Stauber, R. H., Rapid formation of plasma protein corona critically affects nanoparticle pathophysiology. *Nat Nanotechnol* **2013**, *8* (10), 772-81.
15. Jin, Q.; Deng, Y.; Chen, X.; Ji, J., Rational Design of Cancer Nanomedicine for Simultaneous Stealth Surface and Enhanced Cellular Uptake. *ACS Nano* **2019**, *13* (2), 954-977.
16. Pombo Garcia, K.; Zarschler, K.; Barbaro, L.; Barreto, J. A.; O'Malley, W.; Spiccia, L.; Stephan, H.; Graham, B., Zwitterionic-coated 'stealth' nanoparticles for biomedical applications: recent advances in countering biomolecular corona formation and uptake by the mononuclear phagocyte system. *Small* **2014**, *10* (13), 2516-29.
17. Cui, J.; Ju, Y.; Liang, K.; Ejima, H.; Lorcher, S.; Gause, K. T.; Richardson, J. J.; Caruso, F., Nanoscale engineering of low-fouling surfaces through polydopamine immobilisation of zwitterionic peptides. *Soft Matter* **2014**, *10* (15), 2656-63.
18. Suk, J. S.; Xu, Q.; Kim, N.; Hanes, J.; Ensign, L. M., PEGylation as a strategy for improving nanoparticle-based drug and gene delivery. *Adv Drug Deliv Rev* **2016**, *99*, 28-51.
19. Kolate, A.; Baradia, D.; Patil, S.; Vhora, I.; Kore, G.; Misra, A., PEG - a versatile conjugating ligand for drugs and drug delivery systems. *J Control Release* **2014**, *192*, 67-81.
20. Chen, Q.; Yu, S.; Zhang, D.; Zhang, W.; Zhang, H.; Zou, J.; Mao, Z.; Yuan, Y.; Gao, C.; Liu, R., Impact of Antifouling PEG Layer on the Performance of Functional Peptides in Regulating Cell Behaviors. *J Am Chem Soc* **2019**.
21. Perry, J. L.; Reuter, K. G.; Kai, M. P.; Herlihy, K. P.; Jones, S. W.; Luft, J. C.; Napier, M.; Bear, J. E.; DeSimone, J. M., PEGylated PRINT nanoparticles: the impact of PEG density on protein binding, macrophage association, biodistribution, and pharmacokinetics. *Nano Lett* **2012**, *12* (10), 5304-10.
22. Xu, Q.; Ensign, L. M.; Boylan, N. J.; Schon, A.; Gong, X.; Yang, J. C.; Lamb, N. W.; Cai, S.; Yu, T.; Freire, E.; Hanes, J., Impact of Surface Polyethylene Glycol (PEG) Density on Biodegradable Nanoparticle Transport in Mucus ex Vivo and Distribution in Vivo. *ACS Nano* **2015**, *9* (9), 9217-27.
23. Huo, S.; Chen, S.; Gong, N.; Liu, J.; Li, X.; Zhao, Y.; Liang, X. J., Ultrasmall Gold Nanoparticles Behavior in Vivo Modulated by Surface Polyethylene Glycol (PEG) Grafting. *Bioconjug Chem* **2017**, *28* (1), 239-243.

24. Mosqueira, V. C.; Legrand, P.; Morgat, J. L.; Vert, M.; Mysiakine, E.; Gref, R.; Devissaguet, J. P.; Barratt, G., Biodistribution of long-circulating PEG-grafted nanocapsules in mice: effects of PEG chain length and density. *Pharm Res* **2001**, *18* (10), 1411-9.
25. Anselmo, A. C.; Zhang, M.; Kumar, S.; Vogus, D. R.; Menegatti, S.; Helgeson, M. E.; Mitragotri, S., Elasticity of nanoparticles influences their blood circulation, phagocytosis, endocytosis, and targeting. *ACS nano* **2015**, *9* (3), 3169-3177.
26. Cui, J.; De Rose, R.; Alt, K.; Alcantara, S.; Paterson, B. M.; Liang, K.; Hu, M.; Richardson, J. J.; Yan, Y.; Jeffery, C. M.; Price, R. I.; Peter, K.; Hagemeyer, C. E.; Donnelly, P. S.; Kent, S. J.; Caruso, F., Engineering poly(ethylene glycol) particles for improved biodistribution. *ACS Nano* **2015**, *9* (2), 1571-80.
27. Cui, J.; Bjornmalm, M.; Liang, K.; Xu, C.; Best, J. P.; Zhang, X.; Caruso, F., Super-soft hydrogel particles with tunable elasticity in a microfluidic blood capillary model. *Adv Mater* **2014**, *26* (43), 7295-9.
28. Cui, J.; Ju, Y.; Houston, Z. H.; Glass, J. J.; Fletcher, N. L.; Alcantara, S.; Dai, Q.; Howard, C. B.; Mahler, S. M.; Wheatley, A. K., Modulating Targeting of Poly (ethylene glycol) Particles to Tumor Cells Using Bispecific Antibodies. *Adv Healthc Mater* **2019**, 1801607.
29. Cui, J.; Alt, K.; Ju, Y.; Gunawan, S. T.; Braunger, J. A.; Wang, T. Y.; Dai, Y.; Dai, Q.; Richardson, J. J.; Guo, J.; Bjornmalm, M.; Hagemeyer, C. E.; Caruso, F., Ligand-Functionalized Poly(ethylene glycol) Particles for Tumor Targeting and Intracellular Uptake. *Biomacromolecules* **2019**.
30. Hutter, J. L.; Bechhoefer, J., Calibration of atomic-force microscope tips. *Rev Sci Instrum* **1993**, *64* (7), 1868-1873.
31. Hertz, H., Ueber die Berührung fester elastischer Körper. *J Reine Angew Math* **1882**, *1882* (92), 156-171.
32. Best, J. P.; Cui, J.; Mullner, M.; Caruso, F., Tuning the mechanical properties of nanoporous hydrogel particles via polymer cross-linking. *Langmuir* **2013**, *29* (31), 9824-31.
33. Anselmo, A. C.; Mitragotri, S., Impact of particle elasticity on particle-based drug delivery systems. *Adv Drug Deliv Rev* **2017**, *108*, 51-67.
34. Langhans, S. A., Three-Dimensional in Vitro Cell Culture Models in Drug Discovery and Drug Repositioning. *Front Pharmacol* **2018**, *9*, 6.
35. Sathyamoorthy, N.; Dhanaraju, M. D., Shielding Therapeutic Drug Carriers from the Mononuclear Phagocyte System: A Review. *Crit Rev Ther Drug Carrier Syst* **2016**, *33* (6), 489-567.

36. Weiss, A. C. G.; Kelly, H. G.; Faria, M.; Besford, Q. A.; Wheatley, A. K.; Ang, C. S.; Crampin, E. J.; Caruso, F.; Kent, S. J., Link between Low-Fouling and Stealth: A Whole Blood Biomolecular Corona and Cellular Association Analysis on Nanoengineered Particles. *ACS Nano* **2019**, *13* (5), 4980-4991.
37. Banquy, X.; Suarez, F.; Argaw, A.; Rabanel, J.-M.; Grutter, P.; Bouchard, J.-F.; Hildgen, P.; Giasson, S. Effect of mechanical properties of hydrogel nanoparticles on macrophage cell uptake. *Soft Matter* **2009**, *5*, 3984-3991.

Chapter 4

Mucoadhesive vs. Mucus-Penetrating Particles: Nebulisation and Interaction with Mucus

Chapter 4. Mucoadhesive vs. Mucus-Penetrating Particles: Nebulisation and Interaction with Mucus.....	118
4.1 Aim.....	120
4.2 Introduction	120
4.3 Experimental Section	123
4.3.1 Materials	123
4.3.2 Synthesis of Mesoporous Silica (MS) Particles	124
4.3.3 Synthesis of MS@PEG and PEG Particles	124
4.3.4 Poly(methacrylic acid) (PMA) Functionalisation.....	125
4.3.5 Synthesis of MS@PMA _{SH} and PMA _{SH} Particles	125
4.3.6 Particle Characterisation.....	126
4.3.7 Nebulisation of Particles.....	127
4.3.8 Preparation of Artificial Mucus.....	127
4.3.9 Quartz Crystal Microbalance with Dissipation (QCM-D) Analysis	127
4.3.10 Air-Liquid Interface (ALI) Cell Culture.....	128
4.4 Results and Discussions	128
4.4.1 Particle Synthesis and Characterisation.....	128
4.4.2 Nebulisation of Particles.....	130
4.4.3 Particle–Mucus Interactions by QCM-D Analysis.....	132
4.4.4 Particles in ALI Cell Culture	133
4.5 Conclusions	135
4.6 Supporting Information	135
4.7 References	138

4.1 Aim

Mucociliary clearance is the primary clearance mechanism in the airways, which can eliminate foreign particulates to protect the body but also accelerate the clearance of inhaled drug-loaded particles resulting in limited retention time and reduced delivery efficacy. To overcome the mucus barrier, mucoadhesive and mucus-penetrating delivery carriers have been explored to improve drug absorption by increasing the residence time at the targeted mucosal site or allowing penetration of the particles through the mucus layer to interact with the underlying cells, respectively. In this chapter, we aim to study the particle–mucus interactions in the lungs by comparing thiol modified-poly(methacrylic acid) (PMA_{SH}) particles and poly(ethylene glycol) (PEG) particles, which exhibit distinct surface properties using an artificial mucus and air-liquid interface (ALI) cell culture model. The amenability of the particles to the aerosolisation process for inhalation therapy was also investigated.

4.2 Introduction

As a portal entry into the body with features including large surface area suitable for fast absorption, low thickness of the epithelial barrier allowing for penetration, extensive vascularisation and relatively low proteolytic activity in the alveolar region, the lungs represent a promising delivery route of therapeutics either for local lung targeting or systemic circulation.¹⁻³ In contrast to other administration routes such as oral delivery and intravenous administration route, pulmonary delivery can achieve a more rapid onset of drug action, potentially enhanced delivery efficacy and thereby reduced overall drug dosage and less severe side effects.⁴ Particle-based pulmonary delivery, compared to conventional therapeutic formulations, can protect the encapsulated drug against enzymatic and hydrolytic degradation or inactivation, and achieve controlled drug release over prolonged time, overcoming a variety of biological barriers and targeting to specific structures or cells by manipulating the physicochemical properties of the particles.⁵⁻⁷

However, as a major defence organ, the lungs have developed highly efficient clearance mechanisms such as mucociliary clearance, macrophage clearance, and metabolic degradation to protect our body by eliminating foreign substances from entering the systemic circulation, resulting in limited retention time and reduced delivery efficacy of inhaled drug-loaded particles.⁸⁻⁹ Many factors including formulation, size, shape, density, surface chemistry and

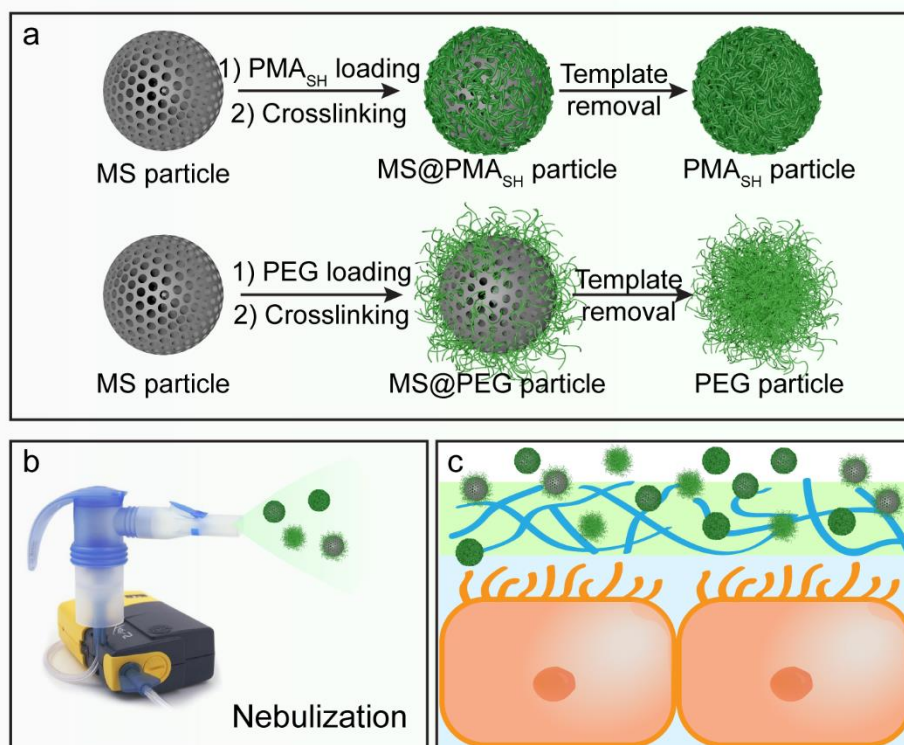
other physicochemical properties affect the deposition of inhaled particles in the lungs.¹⁰ In particular, the aerodynamic diameter of the particles is a critical determinant. Generally, particles larger than 5 μm are mostly exhaled or swallowed while even smaller particles (<1 μm) are likely to remain in the airstream and be exhaled again. Particles with the aerodynamic diameter between 1 μm and 5 μm mainly deposit in the middle and lower regions in the lungs, which is considered desirable for pulmonary delivery.¹¹ Since large particles cannot be effectively taken up by cells, delivery of small particles via aerosolisation is a widely used technique to achieve both high deposition in deep lungs and improved bioavailability for the cells.⁵ Compared with dry powder formulations, nebulisation of particle suspensions can avoid the freeze drying or spray drying processes to form powder and provide finely controlled particle size, allowing the modulation of the bio-nano interactions between the drug-loaded particles and the biological environments where they deposit.

Mucociliary clearance is the primary clearance mechanism in the airways, serving to entrap foreign particulates in the mucus layer and sweep them out of the lungs by the mucociliary escalator.⁸ Airway mucus is a highly hydrated gel-like biological mixture composed of cross-linked mucin glycoproteins, lipids, salts, DNA and other free proteins produced by goblet cells and secretory cells covering the epithelial surface.¹² To overcome the mucus barrier, different strategies have been explored to modulate the surface properties of nanomaterials and subsequent bio-nano interactions.¹³ Endowing particles with a mucoadhesive property (termed mucoadhesive particles) such as modification with chitosan, hyaluronic acid or thiol groups that can establish bonding and interactions with mucin fibres has been commonly applied to improve the drug absorption by increasing the residence time at the targeted mucosal site.¹⁴ Others have developed mucus-penetrating particles with modified inert surface property in order to reduce particle immobilisation in mucus and allow further penetration of the particles through the mucus layer to interact with the underlying cells.¹⁵⁻¹⁸ One of the commonly used techniques is coating the material with a densely packed layer of poly(ethylene glycol) (PEG), a 'stealth' polymer, resulting in a more hydrophilic surface and a large excluded hydrated cloud, which strongly repels the binding of biological components and effectively suppresses the subsequent clearance of particles.¹⁹⁻²¹

In the past decades, polymer particles with distinct properties and functions have been increasingly developed for various biomedical applications including drug delivery.²² Caruso and co-workers have reported a template-mediated assembly for the synthesis of various polymer particles with diverse composition, controllable size and shape, tunable mechanical

property and modifiable surface property.²³⁻²⁵ For example, modified-poly(methacrylic acid) (PMA_{SH}) particles prepared via mesoporous silica (MS) templating were investigated as drug delivery carriers for immobilisation and intracellular delivery of chemotherapeutic compound doxorubicin (DOX), which showed high association with cancer cells and significant cytotoxicity *in vitro*.²⁶ In Chapter 3, the interactions of PEG-based particles with immune cells in human blood were investigated. PEG coating could reduce the immune cell association of particles, and such stealth property of PEGylated particles and PEG replica particles were affected by the architecture of PEG building blocks.

Herein, in this chapter, the study of particle–mucus interactions in artificial lung models was performed by comparing PMA_{SH}-based particles and PEG-based particles (Scheme 4.1a) which exhibit distinct structure and surface properties. The amenability of particles to the nebulisation process were firstly investigated (Scheme 4.1b) and their interactions with artificial mucus and an air-liquid interface (ALI) cell culture model (Scheme 4.1c) were then examined. All the particles were found to be amenable to the nebulisation process maintaining their original structure and morphology without aggregation. PMA_{SH} particles exhibited strong interactions with artificial mucus while PEG particles were found to repel artificial mucus by using quartz crystal microbalance with dissipation (QCM-D) method. PMA_{SH}-loaded MS (MS@PMA_{SH}) particles and PMA_{SH} particles (MS template removed) were found to be entrapped in the mucus layer in ALI cell culture, whereas PEG-loaded MS (MS@PEG) particles and PEG particles (MS template removed) showed negligible adhesion to the mucus layer.



Scheme 4.1. (a) Schematic illustration of the assembly of MS@PMA_{SH} particles (template present), PMA_{SH} particles (template removed), MS@PEG particles (template present) and PEG particles (template removed) via MS particle templating. (b) Schematic illustration of the nebulisation process of particle suspensions. The PARI air-jet nebuliser figure was reproduced from Trek® S Portable Aerosol System, PARI, USA. (c) Schematic illustration of the particle–mucus interactions in ALI cell culture.

4.3 Experimental Section

4.3.1 Materials

Tetraethyl orthosilicate (TEOS), cetyltrimethylammonium bromide (CTAB), poly(acrylic acid) (PAA, M_w ~250 kDa, 35 wt% solution in water), ammonium hydroxide solution (NH₃·H₂O, 28–30%), sodium phosphate dibasic (Na₂HPO₄), hydrofluoric acid (HF, 48 wt%), ammonium fluoride (NH₄F), (3-aminopropyl)-triethoxysilane (APTES), poly(methacrylic acid, sodium salt) (PMA, M_w ~9.5 kDa, 30 wt% solution in water), anhydrous dimethyl sulfoxide (DMSO), N-(3-(dimethylamino)propyl)-N'-ethylcarbodiimide hydrochloride (EDC), N-Chloro-p-toluenesulfonamide sodium salt (chloramine T), dithiothreitol (DTT), 98% sulfuric acid (H₂SO₄), 30% hydrogen peroxide (H₂O₂), 3-(N-morpholino)propanesulfonic acid (MOPS), 2-

(N-Morpholino)ethanesulfonic acid hydrate (MES), Dulbecco phosphate-buffered saline (DPBS), sodium chloride (NaCl), potassium chloride (KCl), mucin from porcine stomach (type III), egg yolk emulsion, diethylenetriaminepentaacetic acid (DTPA), deoxyribonucleic acid from herring sperm and 10% neutral buffered formalin solution were obtained from Sigma-Aldrich (Australia). 8-Arm-poly(ethylene glycol) amine (8-arm-PEG-NH₂, *M_w* 40 kDa) was obtained from JenKem Technology USA Inc. (China). 8-Arm-poly(ethylene glycol) succinimidyl succinate (8-arm-PEG-NHS, hexaglycerol core, *M_w* 10 kDa) was purchased from Creative PEGWorks (USA). Pyridine dithioethylamine hydrochloride (PDA) was obtained from Shanghai Speed Chemical Co. Ltd. (China). Alexa Fluor 488 carboxylic acid succinimidyl ester (AF488-NHS), Alexa Fluor 488 C₅ Maleimide (AF488 Maleimide), RPMI-1640 medium and Hoechst 33342 were purchased from Life Technologies (Australia). B-ALI Bronchial Air-Liquid Interface Medium BulletKit was purchased from Lonza (Australia).

High-purity water used in all experiments with a resistivity greater than 18.2 MΩ cm was generated from a three-stage Millipore Milli-Q plus 185 purification system (Millipore Corporation).

4.3.2 Synthesis of Mesoporous Silica (MS) Particles

MS particles were synthesised following the same protocols described in Chapter 3 (3.3.2).²⁵ Briefly, 1.1 g of CTAB was completely dissolved in 50 mL of water under constant stirring. Then, 4.3 g of PAA solution (35 wt% in water) was added to the above solution. When the solution turned clear after ca. 20 min, 3.5 mL of ammonium hydroxide solution (28–30%) was added under vigorous stirring. Then, 4.46 mL of TEOS was added to the above milky suspension after stirring for 20 min. The suspension was further stirred for 15 min and finally transferred into a Teflon-sealed autoclave allowing 48 h reaction between 80 and 90 °C. After washing with water and ethanol, and subsequent drying at 80 °C, the synthesised MS particles were finally calcined at 550 °C for 30 h for the removal of organic materials.

4.3.3 Synthesis of MS@PEG and PEG Particles

MS@PEG and PEG Particles were fabricated by using the same protocols described in Chapter 3 (3.3.3).²⁵ Briefly, 480 µL of 8-arm-PEG-NH₂ solution (5 mg mL⁻¹ in 100 mM, pH 8.0 phosphate buffer) was added to approximately 6 mg of MS particles for overnight incubation

with constant shaking. 8-Arm-PEG-NH₂ was infiltrated into the pores of the MS particles driven by electrostatic interactions. After removing the excessive polymer by washing with phosphate buffer three times, the particles were subsequently incubated in 400 μ L of 8-arm-PEG-NHS solution (2 mg mL⁻¹ in phosphate buffer) for 2 h to form a cross-linked polymer network by the covalent bonding between 8-arm-PEG-NH₂ and 8-arm-PEG-NHS. MS@PEG particles were labelled with AF488-NHS (5 μ L, 1 mg mL⁻¹ in anhydrous DMSO) during the cross-linking step. The resultant particles were washed three times with water to remove unreacted cross-linker and dye. PEG particles were obtained by dissolving the MS templates with a 2 M HF/8 M NH₄F solution (pH $\sim$ 5). *Caution! HF is highly toxic and extreme care should be taken during handling HF solution.* The resultant PEG particles were washed three times with water and resuspended in water.

4.3.4 Poly(methacrylic acid) (PMA) Functionalisation

Thiol-modified PMA (PMA_{PDA}) was synthesised as described previously via formation of amide bond between the carboxyl groups of PMA and amine groups of PDA mediated by EDC.²⁷ In a typical experiment, 458 mg of PMA solution (30 wt% in water, 1 equiv.) was diluted into 4.875 mL of sodium phosphate buffer (10 mM, pH 7.4). The resulting solution was allowed to react with 97.5 mg of EDC (0.4 equiv.) with stirring for 20 min. Then 70.56 mg of PDA (0.2 equiv.) in 3.538 mL of phosphate buffer was added to the above mixture and the pH was adjusted to 7.6. After incubation with constant shaking overnight, the mixture was transferred into a dialysis membrane (molecular weight cut-off 7000, Thermo) and allowed to dialyse thoroughly against water for three days, and finally, lyophilised for storage until further use. The thiolation degree of as-synthesised thiol-modified PMA (PMA_{PDA}) was quantified as 8% by using a NanoDrop 1000 spectrophotometer. Briefly, PMA_{PDA} was reduced in 0.5 M DTT solution in MOPS buffer (20 mM, pH 8.0) and then diluted in NaOAc buffer (50 mM, pH 4.0) to a final concentration of 0.5 mg mL⁻¹. The thiolation degree was calculated according to the absorbance readings at 343 nm based on a standard curve.

4.3.5 Synthesis of MS@PMA_{SH} and PMA_{SH} Particles

MS@PMA_{SH} and PMA_{SH} particles were prepared according to a previously reported method.²⁸ Firstly, MS particles were modified with amine groups by incubating MS particles in a mixture

of ethanol, ammonia and APTES at a volume ratio of 20 : 1 : 1 overnight followed by thorough washing with ethanol and water. Approximately 3 mg of amine-modified MS particles were incubated in 5 mg mL⁻¹ PMA_{SH} solution in NaOAc buffer (50 mM, pH 4.5) with constant shaking overnight. After washing with NaOAc buffer three times, the particles were exposed to a 10 mM chloramine T solution in MES buffer (50 mM, pH 6) for 2 min to allow cross-linking of the polymer network. After washing with water and sodium phosphate buffer (10 mM, pH 7.4), the resultant MS@PMA_{SH} particles were fluorescently labelled with AF488 maleimide (5 µL, 1 mg mL⁻¹ in DMSO) overnight under gentle shaking. PMA_{SH} particles were obtained by dissolving the silica templates with a 2 M HF/8 M NH₄F solution (pH ~ 5). *Caution! HF is highly toxic and extreme care should be taken when handling the solution.* The resultant fluorescently labelled PMA_{SH} particles were washed three times with sodium phosphate buffer (10 mM, pH 7.4).

4.3.6 Particle Characterisation

Fluorescence microscopy images were taken by using a differential interference contrast (DIC) (U-DICT, Olympus) / inverted fluorescence combined microscope (Olympus IX71) equipped with a 100× oil immersion objective. Transmission electron microscopy (TEM) images were taken with a FEI Tecnai TF20 instrument operating at the voltage of 80 kV for PEG particles and 120 kV for other particles. The TEM samples were prepared by dropping aqueous particle suspension onto plasma-treated formvar-coated copper grids followed by air-drying. Scanning electron microscopy (SEM) images were taken with a Philips XL30 instrument at an operation voltage of 15 kV. The SEM samples were prepared by placing a drop of particle suspension onto clean silicon wafers, followed by sputter-coating with gold. The zeta potential of the particles was measured using a Zetasizer Nano-ZS (Malvern Instruments, Malvern, UK). The size distribution of particles was measured by dynamic light scattering (DLS) (Malvern Instruments, Malvern, UK). The aerosol size distribution was measured by laser diffraction (Spraytec, Malvern, UK). Particle counting was carried out by using flow cytometry (Apogee Flow). Image analysis was conducted using ImageJ software. Nebulisation was performed using a PARI air-jet nebuliser (Trek[®] S Portable Aerosol System, PARI, USA).

4.3.7 Nebulisation of Particles

Aerosol size distribution of nebulised particle suspensions (3×10^5 particles μL^{-1}) using a PARI air-jet nebuliser was measured by laser diffraction (Spraytec, Malvern Instruments) at 25 °C. To compare the particle properties before and after nebulisation, an aliquot of 2 mL of particle suspension was subjected to nebulisation for 5 min. The outlet of the nebuliser was attached to a pipette tip to direct the flow of aerosol into a 1.5 mL Eppendorf tube for the aerosol collection.

4.3.8 Preparation of Artificial Mucus

Artificial mucus was prepared according to a modified method.²⁹ Generally, a mixture of 500 mg of deoxyribonucleic acid (DNA), 250 mg of mucin, 250 mg of NaCl, 110 mg of KCl, 0.295 mg of DTPA, 1 mL of RPMI-1640 media, and 250 μL of egg yolk emulsion were dissolved in water in a final volume of 50 mL. The artificial mucus was allowed to equilibrate at 25 °C for 2 h and used within 24 h.

4.3.9 Quartz Crystal Microbalance with Dissipation (QCM-D) Analysis

QCM-D analysis of interactions between particles and artificial mucus was carried out by using a Q-Sense E4 quartz crystal microbalance from Biolin Scientific (Västra Frölunda, Sweden). Gold-coated quartz crystal microgravimetry sensor crystals purchased from ATA Scientific (Taren Point, Australia) were cleaned using Piranha solution prepared by mixing 98% sulfuric acid (H_2SO_4) and 30% hydrogen peroxide (H_2O_2) at v/v of 3:1. *Caution! Piranha solution is extremely corrosive and reacts violently with organic materials. It should be handled with great care.* The crystals were then rinsed extensively with Milli-Q water followed by ethanol, and dried under a nitrogen stream. Prior to use, the crystals were further cleaned using a UV-ozone cleaner (BioForce Nanosciences, IA) for 30 min. Experiments were conducted at 37 °C at a flow rate of 200 $\mu\text{L min}^{-1}$. The frequency values of the third overtone were recorded for the data analysis, and the fifth, seventh, and ninth overtones all followed similar trends. DPBS was firstly injected into the flow cell to allow a stable baseline. After stabilisation of the signals, $10 \times$ diluted artificial mucus was introduced to the crystal until stabilisation. After rinsing with DPBS three times (approximately 15 min each rinse) to remove unbound components, a stable mucus layer was established. PMA_{SH} or PEG particle suspensions (1×10^6 particles μL^{-1} in water) were introduced to the crystal allowing incubation for approximately 20 min. Finally,

unbound particles were removed by rinsing with DPBS twice (approximately 20 min each rinse).

4.3.10 Air-Liquid Interface (ALI) Cell Culture

ALI cell culture was set up by using a previously reported method.³⁰ Primary human bronchial epithelial cells (HBECS) obtained from Lonza (Waverley, Australia) were cultured using B-ALI Bronchial Air-Liquid Interface Medium BulletKit according to the manufacturer's protocols (50 000 cells per well). The cells were cultured for differentiation for over 21 days at an air-liquid interface on fibrillar collagen-coated 24-well Corning Transwell 0.4 μm pore polyester membrane cell culture inserts (Sigma-Aldrich, MO, USA). Cell differentiation was monitored and confirmed by the measurements of trans-epithelial electrical resistance (TEER) and visualisation of beating cilia. After starvation of the cell culture for 24 h by replacing the complete media with minimal media (Table S4.2), MS@PMA_{SH}, PMA_{SH}, MS@PEG or PEG particles with the size of ~ 400 nm were added onto the apical surface of the cell culture at a particle/cell ratio of 100:1, and then incubated for 24 hours at 37 °C. After incubation, the cell culture was washed with DPBS twice to remove free particles and then fixed with 10% neutral buffered formalin solution. The fixed cell culture was stained with Hoechst 33342 (0.1 mg mL⁻¹) and imaged by confocal microscope.

4.4 Results and Discussions

4.4.1 Particle Synthesis and Characterisation

MS particles with a bimodal pore structure consisting of smaller mesopores in the 2–3 nm range and larger mesopores ranging from 15 nm to 60 nm were synthesised as described above (Figure S4.1).³¹ PMA_{SH} and PEG particles were prepared from PMA_{SH} and PEG building blocks, respectively, via MS particle templating. For the adsorption of PMA_{SH}, the MS particle templates were modified with amine groups to make the surface positively charged (from -24.4 mV to 16.8 mV in pH 7.4 phosphate buffer) for the electrostatic interaction with PMA_{SH} polymer (Figure S4.2b). The amine-modified MS particles (MS-NH₂) maintained monodispersity with similar size to the initial MS particles (Figure S4.2a). PMA_{SH} or PEG-NH₂ was infiltrated into the porous structure of the templates followed by cross-linking, resulting in MS@PMA_{SH} and MS@PEG particles, respectively. After template removal,

PMA_{SH} or PEG particles were obtained. Particles were subsequently labelled with fluorescence dye Alexa Fluor 488 to enable visualisation and imaging.

The characterisation shown in Figure 4.1 and Figure 4.2 reveals the dispersity, morphology and structure of the MS@PMA_{SH}, PMA_{SH}, MS@PEG and PEG particles. The fluorescence microscopy images (Figure 4.1a1–d1) show the monodispersity and homogenous fluorescence of all the particles in aqueous solution. TEM images (Figure 4.2a1–d1) show the detailed morphology and structure of particles in the dry state. MS@PMA_{SH} particles were observed to be more dense (appear darker) compared to MS@PEG particles due to the adsorption of polyelectrolyte. PMA_{SH} particles exhibited a dense, rigid, smooth spherical structure with a thickness of 343.1 ± 24.8 nm from analysis of air-dried particles by AFM (Table 4.1), while PEG particles appeared collapsed after air-drying with a thickness of 55.9 ± 4.1 nm as a result of dehydration and had a relatively loose and rough structure.²⁵⁻²⁶

PMA_{SH}-based and PEG-based particles exhibited different surface properties. MS@PMA_{SH} and PMA_{SH} particles had a zeta potential of ~ -31 mV in phosphate buffer (5 mM, pH 7.4), which was much more negatively charged than MS@PEG particles with ~ -14 mV mainly due to the presence of MS. After MS removal, PEG particles exhibited near neutral charge at pH 7.4 (Table 4.1).

Table 4.1. Zeta potential and thickness of particles.

Particles	Zeta potential in buffer ^a (mV)	Thickness in dry state ^b (nm)
MS@PMA _{SH}	-31.8 ± 0.9	-
PMA _{SH}	-30.7 ± 0.8	343.1 ± 24.8
MS@PEG	-13.9 ± 0.5	-
PEG	-4.2 ± 0.2	55.9 ± 4.1

^a 5 mM phosphate buffer (pH 7.4).

^b The air-dried particle thickness was determined from the distance between the highest point on the particle and the substrate surface using AFM. Twenty particles were analysed for each sample.

4.4.2 Nebulisation of Particles

PMA_{SH} and PEG particles have previously been investigated for the delivery of therapeutics, including vaccines and anti-cancer agents.^{26, 32} To demonstrate their potential for pulmonary delivery, the MS@PMA_{SH}, PMA_{SH}, MS@PEG and PEG particles were aerosolised using an air-jet nebuliser which generates fine inhalable aerosol droplets from a liquid of particle suspension by using compressed air.

To investigate the amenability of particles to the nebulisation process and compare the particle properties before and after nebulisation, the nebulised particles were collected for further characterisation by attaching a pipette tip to the outlet of the nebuliser to direct the flow of aerosol into an Eppendorf tube. The MS@PMA_{SH}, PMA_{SH}, MS@PEG and PEG particles remained well-dispersed in aqueous solution without aggregation after nebulisation (Figure 4.1a2–d2), and there was no significant shift of the particle size distribution following nebulisation (Figure 4.3). The particles maintained intact with the morphology and structure not damaged or altered by nebulisation as observed from the TEM images (Figure 4.2a2–d2), which indicates that all of the particles investigated in our study were able to tolerate the nebulisation process.

Aerosol size distribution of different particle suspensions were measured by laser diffraction as shown in Figure S4.3 and Table S4.1. The droplet volume median diameters (D_v(50)) of water, MS@PMA_{SH}, PMA_{SH}, MS@PEG and PEG particles are all around 5 µm and in the desirable range (1–5 µm) for pulmonary delivery, indicating that the aerodynamic diameter of the liquid aerosol carrying different particles is primarily influenced by the nebuliser properties .

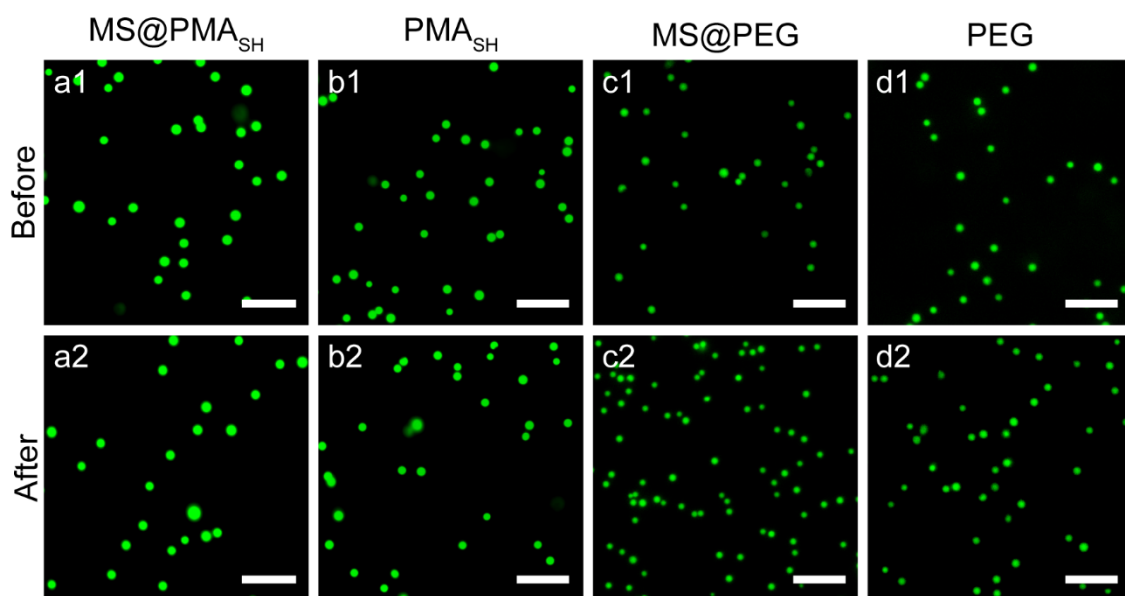


Figure 4.1. Fluorescence microscopy images of AF488-labelled MS@PMA_{SH}, PMA_{SH}, MS@PEG and PEG particles before (a1–d1) and after (a2–d2) nebulisation. The scale bars are 10 μm .

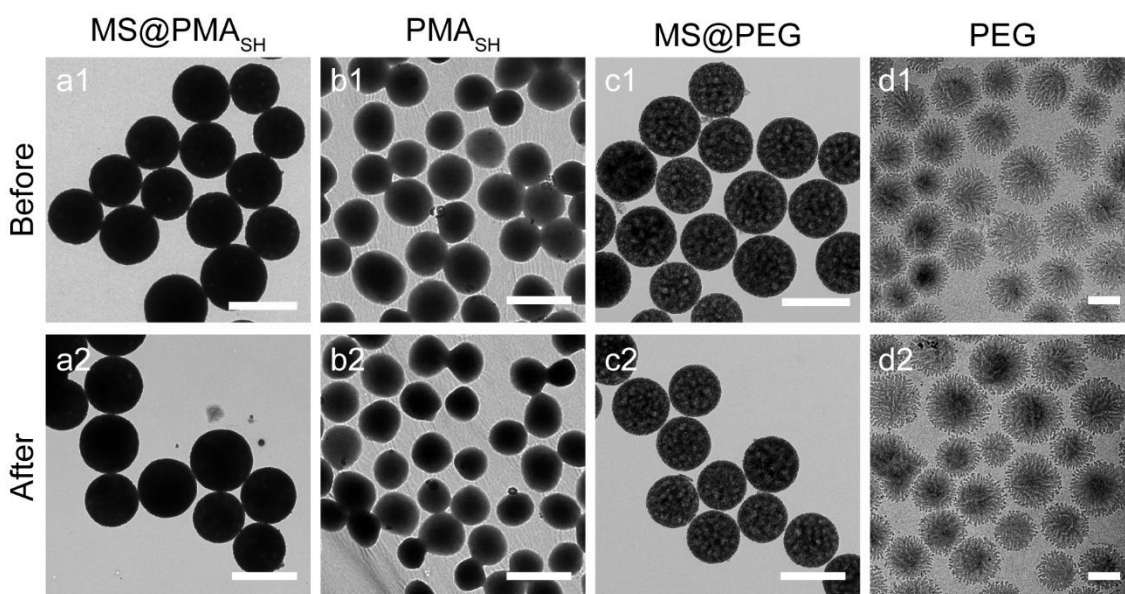


Figure 4.2. TEM images of MS@PMA_{SH}, PMA_{SH}, MS@PEG and PEG particles before (a1–d1) and after (a2–d2) nebulisation. The scale bars are 2 μm .

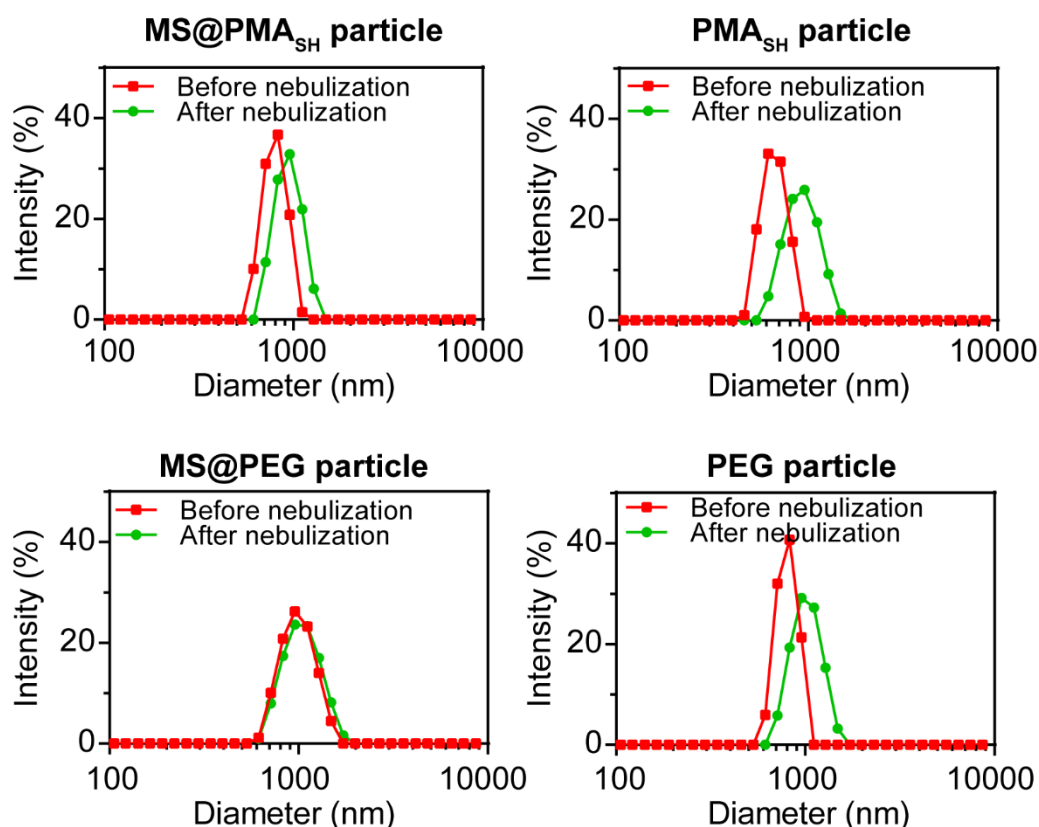


Figure 4.3. Size distribution of MS@PMA_{SH}, PMA_{SH}, MS@PEG and PEG particles before and after nebulisation determined by DLS.

4.4.3 Particle–Mucus Interactions by QCM-D Analysis

To study the interactions between particles and mucus, QCM-D analysis was conducted by measuring the adsorption of PMA_{SH} particles and PEG particles to the artificial mucus-modified sensors (Figure 4.4). QCM-D allows real-time monitoring of adsorption processes as mass is adsorbed leading to the decrease in the frequency of an oscillating piezo-electric crystal. Upon the introduction of mucus to the flow module, the adsorption of mucus components resulted in an immediate decrease in frequency, indicating a mass increase in the system. After rinsing with buffer to remove the unbound mucus, a stable mucus layer was established on the sensor with the decrease in frequency (Δf) of ~ 40 Hz. The subsequent introduction of PMA_{SH} particles to the mucus-modified sensor led to a rapid decrease in frequency (overall $\Delta f \sim 25$ Hz) indicating an increase in mass, which reveals the strong adhesion of PMA_{SH} particles to the mucus layer probably due to the formation of disulfide bonding between the thiol groups of PMA_{SH} particles and the cysteine groups of mucin, and this process appears to occur immediately (within 1 min).^{14, 33} In contrast, PEG particles were not detected to be adsorbed

onto the mucus layer with a negligible Δf after the introduction of PEG particles, confirming the poor mucoadhesive characteristic and low-fouling property of PEG particles resulting from the large excluded hydrated cloud that strongly repels the binding of biological components in mucus.

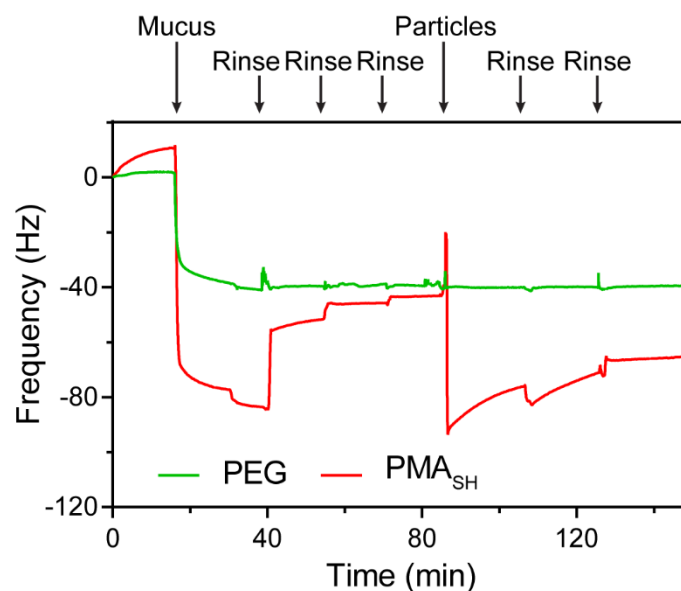


Figure 4.4. Changes in frequency (Δf) with time recorded by QCM-D showing the interactions between artificial mucus with PMA_{SH} particles and PEG particles. The frequency values quoted are for the third overtone only; the fifth, seventh, and ninth overtones all followed similar trends.

4.4.4 Particles in ALI Cell Culture

To investigate the behaviour of MS@PMA_{SH}, PMA_{SH}, MS@PEG and PEG particles in a relatively physiological environment, ALI cell cultures consisting of differentiated human respiratory epithelial cells from primary human bronchial epithelial cells (HBECs) were established. ALI cell cultures as a relevant *in vitro* model provide mimic *in vivo* conditions of the airway epithelium.³⁴⁻³⁶ MS@PMA_{SH}, PMA_{SH}, MS@PEG or PEG particles with diameters ~400 nm were added onto the apical surface of well-differentiated ALI cell cultures (Scheme 4.1c) at a particle/cell ratio of 100:1 for 24 h incubation at 37 °C. The cell culture was then washed with DPBS to remove free particles and fixed for staining and imaging. The mucus distribution on ALI cell culture was determined by Hoechst 33342 staining due to the presence of high content of DNA in mucus. As observed in the confocal microscopy images (Figure 4.5),

MS@PMA_{SH} and PMA_{SH} particles were entrapped in the apical mucus layer potentially due to the interactions between the thiolated particle surface and the biological components in the mucus layer, whereas MS@PEG and PEG particles were not detected in the culture, indicating their negligible adhesion to the mucus. Due to the size filtering of mucin fibres and weak vertical driven forces, MS@PEG and PEG particles did not penetrate through the mucus layer present at the apical site, and were removed during the rinsing steps. The results are in accordance with the results derived from the QCM-D analysis in 4.4.3. As mucins are large molecules forming into cross-linked network with an average mesh diameter from tens of nanometer to hundreds of nanometer³⁷ and only particles smaller than the mesh space have chances to diffuse through the mucus layer, engineering particle size and density in addition to the surface property may help them penetrate through mucus layer.

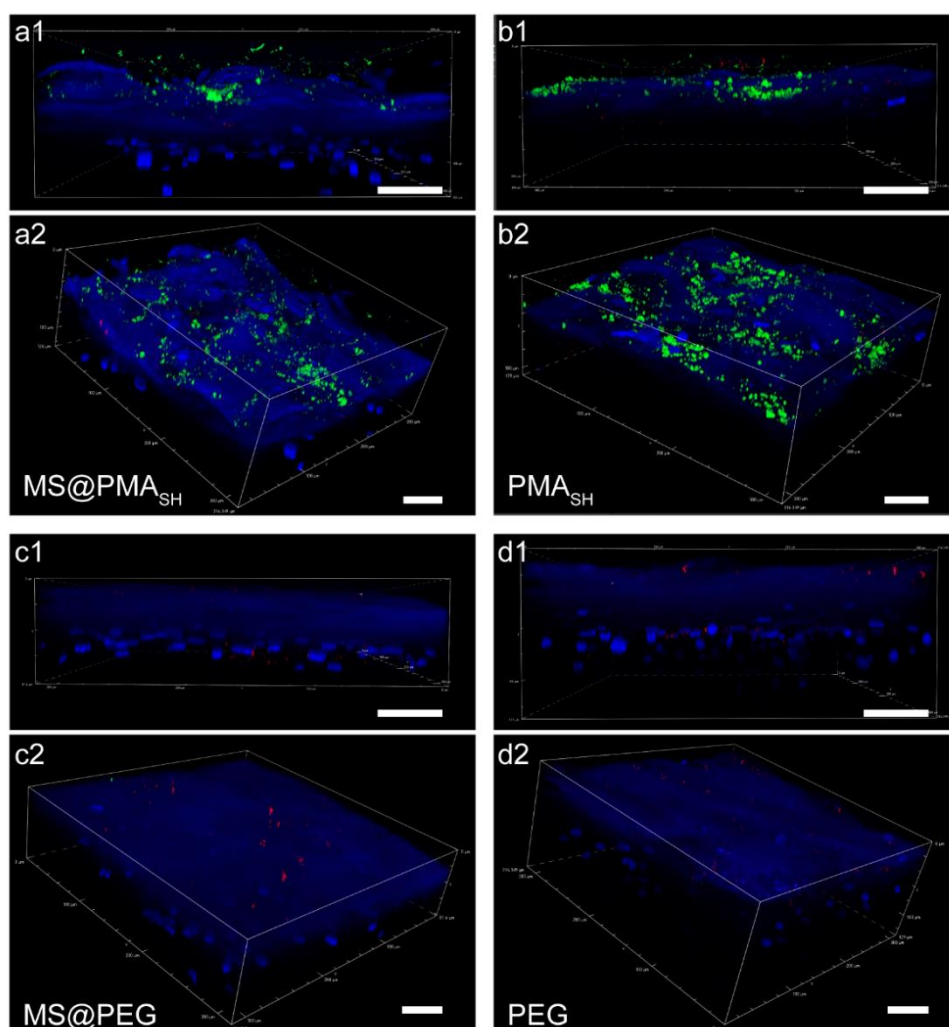


Figure 4.5. Confocal microscopy images of ALI cell culture treated with (a) MS@PMA_{SH}, (b) PMA_{SH}, (c) MS@PEG and (d) PEG particles (green), captured as a volume view. The nuclei and mucus (blue) were stained with Hoechst 33342. The scale bars are 50 μm .

4.5 Conclusions

This chapter investigated the particle–mucus interactions in the lungs by comparing PMA_{SH}-based particles and PEG-based particles which exhibit distinct structure and surface properties by using artificial mucus and an ALI cell culture model. The amenability of particles to the nebulisation process were also investigated. MS@PMA_{SH}, PMA_{SH}, MS@PEG and PEG particles were found to be amenable to the nebulisation process maintaining their original structure and morphology without aggregation, with a droplet size of $\sim 5\ \mu\text{m}$ which is in the desirable size range for pulmonary delivery. PMA_{SH} particles exhibited strong adhesion to the artificial mucus as evaluated by QCM-D, probably due to the interactions between the thiol groups of PMA_{SH} particles and the cysteine groups of mucin. In contrast, PEG particles showed negligible adsorption onto the mucus layer, confirming the poor mucoadhesive characteristic and low-fouling property of PEG particles. The particle behaviours in ALI cell culture were consistent with the results derived from the QCM-D analysis, with MS@PMA_{SH} and PMA_{SH} particles stuck in the mucus layer and no detectable adhesion of MS@PEG and PEG particles to the mucus. Taken together, this study demonstrates the potential of PMA_{SH}-based particles and PEG-based particles which exhibit different structure and surface properties for pulmonary delivery via nebulisation, and provides a preliminary understanding of the interactions of distinct particles with airway mucus and insight on modulating nanoengineered materials for biomedical applications in pulmonary delivery.

4.6 Supporting Information

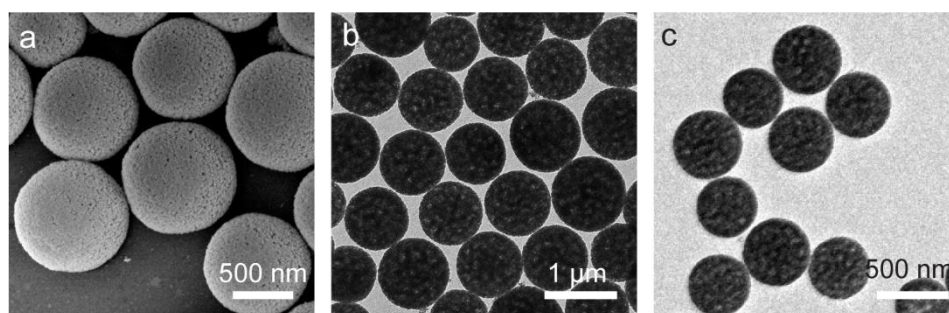


Figure S4.1. (a) SEM and (b) TEM image of 800 nm MS particles. (c) TEM image of 400 nm MS particles.

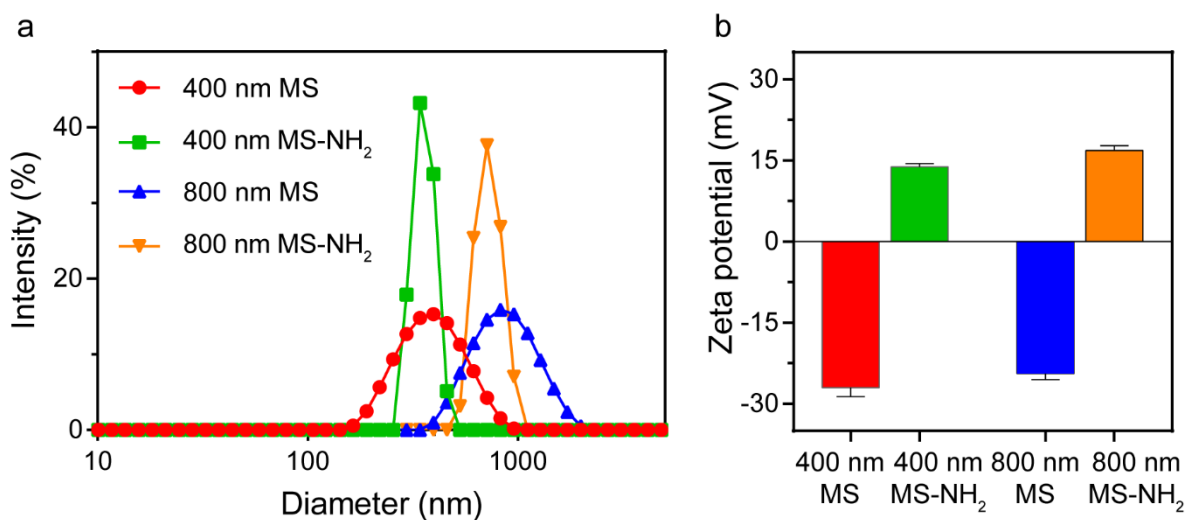


Figure S4.2. (a) Size distribution and (b) zeta potential of 400 nm and 800 nm mesoporous silica particles with or without amine group modification (denoted as MS-NH₂ and MS, respectively).

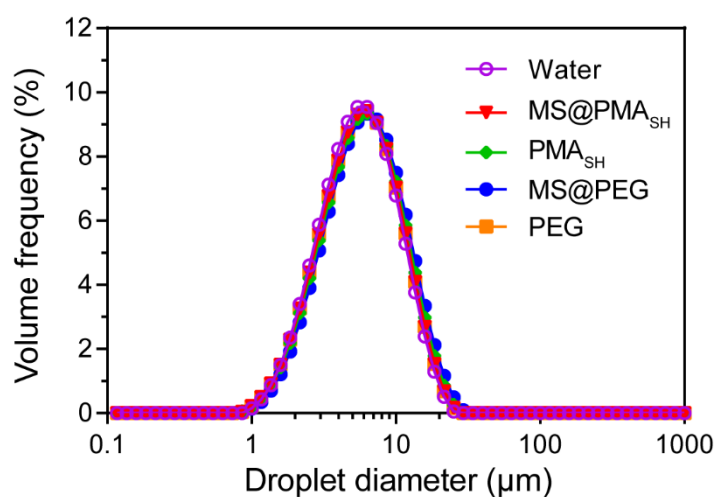


Figure S4.3. Aerosol size distribution of nebulised water, and MS@PMA_{SH}, PMA_{SH}, MS@PEG and PEG particle suspensions as evaluated by laser diffraction.

Table S4.1. Droplet size of different particle suspensions

Particles	Droplet size Dv(50) (μm)
MS@PMA _{SH}	5.419
PMA _{SH}	5.620
MS@PEG	5.654
PEG	5.284
Dv(50): The volume medium diameter	

Table S4.2. Components in complete media vs minimal media for the ALI cell culture

Component	Complete media	Minimal media	Source
BPE (Bovine Pituitary Extract)	52 μg protein contents mL^{-1}	-	SingleQuots
rhEGF	0.5 ng mL^{-1}	-	SingleQuots
Hydrocortisone	0.5 $\mu\text{g mL}^{-1}$ (1.4 μM)	0.035 $\mu\text{g mL}^{-1}$ (0.1 μM)	SingleQuots
Insulin	5 $\mu\text{g mL}^{-1}$ (0.87 μM)	1 $\mu\text{g mL}^{-1}$ (0.18 μM)	SingleQuots
Transferrin	10 $\mu\text{g mL}^{-1}$ (0.125 μM)	As complete media	SingleQuots
Epinephrine	0.5 $\mu\text{g mL}^{-1}$ (2.7 μM)	As complete media	SingleQuots
Triiodothyronine	6.5 ng mL^{-1} (0.01 μM)	As complete media	SingleQuots
Gentamicine	50 $\mu\text{g mL}^{-1}$	As complete media	SingleQuots
Amphotericin B	50 ng mL^{-1}	As complete media	SingleQuots
Retinoic acid	5×10^{-8} M	As complete media	SingleQuots
BSA	3 $\mu\text{g mL}^{-1}$	As complete media	Sigma

4.7 References

1. Patton, J. S.; Byron, P. R., Inhaling medicines: delivering drugs to the body through the lungs. *Nat Rev Drug Discov* **2007**, 6 (1), 67.
2. Muller, L.; Lehmann, A. D.; Johnston, B. D.; Blank, F.; Wick, P.; Fink, A.; Rothen-Rutishauser, B., Inhalation Pathway as a Promising Portal of Entry: What Has to Be Considered in Designing New Nanomaterials for Biomedical Application? *Handbook of Nanotoxicology, Nanomedicine and Stem Cell Use in Toxicology* **2014**, 205-221.
3. Patil, J. S.; Sarasija, S., Pulmonary drug delivery strategies: A concise, systematic review. *Lung India* **2012**, 29 (1), 44-9.
4. Kuzmov, A.; Minko, T., Nanotechnology approaches for inhalation treatment of lung diseases. *J Control Release* **2015**, 219, 500-518.
5. Sung, J. C.; Pulliam, B. L.; Edwards, D. A., Nanoparticles for drug delivery to the lungs. *Trends Biotechnol* **2007**, 25 (12), 563-70.
6. Paranjpe, M.; Muller-Goymann, C. C., Nanoparticle-mediated pulmonary drug delivery: a review. *Int J Mol Sci* **2014**, 15 (4), 5852-73.
7. Chow, A. H.; Tong, H. H.; Chattopadhyay, P.; Shekunov, B. Y., Particle engineering for pulmonary drug delivery. *Pharm Res* **2007**, 24 (3), 411-37.
8. Lippmann, M.; Yeates, D. B.; Albert, R. E., Deposition, retention, and clearance of inhaled particles. *Br J Ind Med* **1980**, 37 (4), 337-62.
9. El-Sherbiny, I. M.; Villanueva, D. G.; Herrera, D.; Smyth, H. D. C., Overcoming Lung Clearance Mechanisms for Controlled Release Drug Delivery. *Adv Del Sci Technol* **2011**, 101-126.
10. Musante, C. J.; Schroeter, J. D.; Rosati, J. A.; Crowder, T. M.; Hickey, A. J.; Martonen, T. B., Factors affecting the deposition of inhaled porous drug particles. *J Pharm Sci* **2002**, 91 (7), 1590-600.
11. Edwards, D. A.; Dunbar, C., Bioengineering of therapeutic aerosols. *Annu Rev Biomed Eng* **2002**, 4, 93-107.
12. García-Díaz, M.; Birch, D.; Wan, F.; Nielsen, H. M., The role of mucus as an invisible cloak to transepithelial drug delivery by nanoparticles. *Adv Drug Deliv Rev* **2017**.
13. Wu, L.; Shan, W.; Zhang, Z.; Huang, Y., Engineering nanomaterials to overcome the mucosal barrier by modulating surface properties. *Adv Drug Deliv Rev* **2018**, 124, 150-163.

14. Sosnik, A.; das Neves, J.; Sarmento, B., Mucoadhesive polymers in the design of nano-drug delivery systems for administration by non-parenteral routes: a review. *Prog Polym Sci* **2014**, 39 (12), 2030-2075.
15. Khutoryanskiy, V. V., Beyond PEGylation: Alternative surface-modification of nanoparticles with mucus-inert biomaterials. *Adv Drug Deliv Rev* **2018**, 124, 140-149.
16. Huckaby, J. T.; Lai, S. K., PEGylation for enhancing nanoparticle diffusion in mucus. *Adv Drug Deliv Rev* **2018**, 124, 125-139.
17. Porsio, B.; Craparo, E. F.; Mauro, N.; Giammona, G.; Cavallaro, G., Mucus and Cell-Penetrating Nanoparticles Embedded in Nano-into-Micro Formulations for Pulmonary Delivery of Ivacaftor in Patients with Cystic Fibrosis. *ACS Appl Mater Interfaces* **2018**, 10 (1), 165-181.
18. Poinard, B.; Kamaluddin, S.; Tan, A. Q. Q.; Neoh, K. G.; Kah, J. C. Y., Polydopamine Coating Enhances Mucopenetration and Cell Uptake of Nanoparticles. *ACS Appl Mater Interfaces* **2019**, 11 (5), 4777-4789.
19. Suk, J. S.; Xu, Q.; Kim, N.; Hanes, J.; Ensign, L. M., PEGylation as a strategy for improving nanoparticle-based drug and gene delivery. *Adv Drug Deliv Rev* **2016**, 99, 28-51.
20. Kolate, A.; Baradia, D.; Patil, S.; Vhora, I.; Kore, G.; Misra, A., PEG - a versatile conjugating ligand for drugs and drug delivery systems. *J Control Release* **2014**, 192, 67-81.
21. Butcher, N. J.; Mortimer, G. M.; Minchin, R. F., Drug delivery: Unravelling the stealth effect. *Nat Nanotechnol* **2016**, 11 (4), 310-1.
22. Tanner, P.; Baumann, P.; Enea, R.; Onaca, O.; Palivan, C.; Meier, W., Polymeric vesicles: from drug carriers to nanoreactors and artificial organelles. *Acc Chem Res* **2011**, 44 (10), 1039-49.
23. Yan, Y.; Lai, Z. W.; Goode, R. J.; Cui, J.; Bacic, T.; Kamphuis, M. M.; Nice, E. C.; Caruso, F., Particles on the move: intracellular trafficking and asymmetric mitotic partitioning of nanoporous polymer particles. *ACS Nano* **2013**, 7 (6), 5558-67.
24. Cui, J.; Richardson, J. J.; Bjornmalm, M.; Faria, M.; Caruso, F., Nanoengineered Templated Polymer Particles: Navigating the Biological Realm. *Acc Chem Res* **2016**, 49 (6), 1139-48.
25. Cui, J.; De Rose, R.; Alt, K.; Alcantara, S.; Paterson, B. M.; Liang, K.; Hu, M.; Richardson, J. J.; Yan, Y.; Jeffery, C. M.; Price, R. I.; Peter, K.; Hagemeyer, C. E.; Donnelly, P. S.; Kent, S. J.; Caruso, F., Engineering poly(ethylene glycol) particles for improved biodistribution. *ACS Nano* **2015**, 9 (2), 1571-80.

26. Cui, J.; Yan, Y.; Wang, Y.; Caruso, F., Templated assembly of pH-labile polymer-drug particles for intracellular drug delivery. *Adv Funct Mater* **2012**, 22 (22), 4718-4723.
27. Dai, Q.; Yan, Y.; Ang, C. S.; Kempe, K.; Kamphuis, M. M.; Dodds, S. J.; Caruso, F., Monoclonal antibody-functionalized multilayered particles: targeting cancer cells in the presence of protein coronas. *ACS Nano* **2015**, 9 (3), 2876-85.
28. Yan, Y.; Gause, K. T.; Kamphuis, M. M.; Ang, C. S.; O'Brien-Simpson, N. M.; Lenzo, J. C.; Reynolds, E. C.; Nice, E. C.; Caruso, F., Differential roles of the protein corona in the cellular uptake of nanoporous polymer particles by monocyte and macrophage cell lines. *ACS Nano* **2013**, 7 (12), 10960-70.
29. Craparo, E. F.; Porsio, B.; Sardo, C.; Giammona, G.; Cavallaro, G., Pegylated Polyaspartamide-Polylactide-Based Nanoparticles Penetrating Cystic Fibrosis Artificial Mucus. *Biomacromolecules* **2016**, 17 (3), 767-77.
30. Xia, Y. C.; Radwan, A.; Keenan, C. R.; Langenbach, S. Y.; Li, M.; Radojicic, D.; Londrigan, S. L.; Gualano, R. C.; Stewart, A. G., Glucocorticoid insensitivity in virally infected airway epithelial cells is dependent on transforming growth factor- β activity. *PLoS pathogens* **2017**, 13 (1), e1006138.
31. Ma, Y.; Bjornmalm, M.; Wise, A. K.; Cortez-Jugo, C.; Revalor, E.; Ju, Y.; Feeney, O. M.; Richardson, R. T.; Hanssen, E.; Shepherd, R. K.; Porter, C. J. H.; Caruso, F., Gel-Mediated Electrospray Assembly of Silica Supraparticles for Sustained Drug Delivery. *ACS Appl Mater Interfaces* **2018**, 10 (37), 31019-31031.
32. Cui, J.; Ju, Y.; Houston, Z. H.; Glass, J. J.; Fletcher, N. L.; Alcantara, S.; Dai, Q.; Howard, C. B.; Mahler, S. M.; Wheatley, A. K., Modulating Targeting of Poly (ethylene glycol) Particles to Tumor Cells Using Bispecific Antibodies. *Adv Healthc Mater* **2019**, 1801607.
33. Wang, Y. Y.; Lai, S. K.; So, C.; Schneider, C.; Cone, R.; Hanes, J., Mucoadhesive Nanoparticles May Disrupt the Protective Human Mucus Barrier by Altering Its Microstructure. *Plos One* **2011**, 6 (6).
34. Lin, H.; Li, H.; Cho, H. J.; Bian, S.; Roh, H. J.; Lee, M. K.; Kim, J. S.; Chung, S. J.; Shim, C. K.; Kim, D. D., Air-liquid interface (ALI) culture of human bronchial epithelial cell monolayers as an in vitro model for airway drug transport studies. *J Pharm Sci* **2007**, 96 (2), 341-50.
35. Wu, N. H.; Yang, W.; Beineke, A.; Dijkman, R.; Matrosovich, M.; Baumgartner, W.; Thiel, V.; Valentin-Weigand, P.; Meng, F.; Herrler, G., The differentiated airway epithelium infected by influenza viruses maintains the barrier function despite a dramatic loss of ciliated cells. *Sci Rep* **2016**, 6, 39668.

36. Bateman, A. C.; Karasin, A. I.; Olsen, C. W., Differentiated swine airway epithelial cell cultures for the investigation of influenza A virus infection and replication. *Influenza Other Respir Viruses* **2013**, 7 (2), 139-50.
37. Groo, A. C.; Lagarce, F., Mucus models to evaluate nanomedicines for diffusion. *Drug Discov Today* **2014**, 19 (8), 1097-108.

Chapter 5

The Interplay of Pulmonary Corona and Plasma Protein Corona on Macrophage Uptake of Polymer Particles

Chapter 5. The Interplay of Pulmonary Corona and Plasma Protein

Corona on Macrophage Uptake of Polymer Particles.....142

5.1 Aim.....	144
5.2 Introduction	144
5.3 Experimental Section	147
5.3.1 Materials	147
5.3.2 Synthesis of Mesoporous Silica (MS) Particles	148
5.3.3 Synthesis of MS@PEG and PEG Particles	148
5.3.4 Poly(methacrylic acid) (PMA) Functionalisation.....	149
5.3.5 Synthesis of MS@PMA _{SH} and PMA _{SH} Particles	149
5.3.6 Particle Characterisation.....	150
5.3.7 Collection of Mouse Bronchoalveolar Lavage Fluid (BALF).....	150
5.3.8 Formation of Protein Corona-Coated Particles	151
5.3.9 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).....	151
5.3.10 Proteomics Analysis by Mass Spectrometry	151
5.3.11 Cell Culture.....	153
5.3.12 Cell Association by Flow Cytometry	153
5.3.13 Cell Association by Confocal Microscopy	153
5.4 Results and Discussions	154
5.4.1 Particle Preparation and Characterisation.....	154
5.4.2 Protein Corona Effects on Particle Surface Charge.....	155
5.4.3 Protein Corona Effects on Cell Association.....	156
5.4.4 Protein Corona Analysis by SDS-PAGE and Mass Spectrometry	161
5.5 Conclusions	166
5.6 Supporting Information	167
5.7 References	172

5.1 Aim

Nanoengineered particles have been found to adsorb biomolecules, especially proteins, when they enter a biological environment, which results in the formation of a ‘protein corona’ around particles. This theory has primarily considered the interactions of particles with components in blood plasma. Pulmonary surfactant is a complex biological aqueous mixture in deep lungs, composed of high contents of surfactant specific proteins, lipids, DNA and other components. The surface association of these components results in a complex corona, which is likely to be different from the one formed in blood plasma. Therapeutics for systemic circulation via pulmonary delivery go through the lung surfactant layer before entering the blood circulation system, while injected therapeutics targeting the lung regions contact blood plasma first before they cross the lung environment. This chapter aims to study the role of the protein corona in the modulation of particle–cell interactions, focusing on the interplay of pulmonary corona and plasma protein corona on the macrophage clearance of polymer particles to mimic different administration routes.

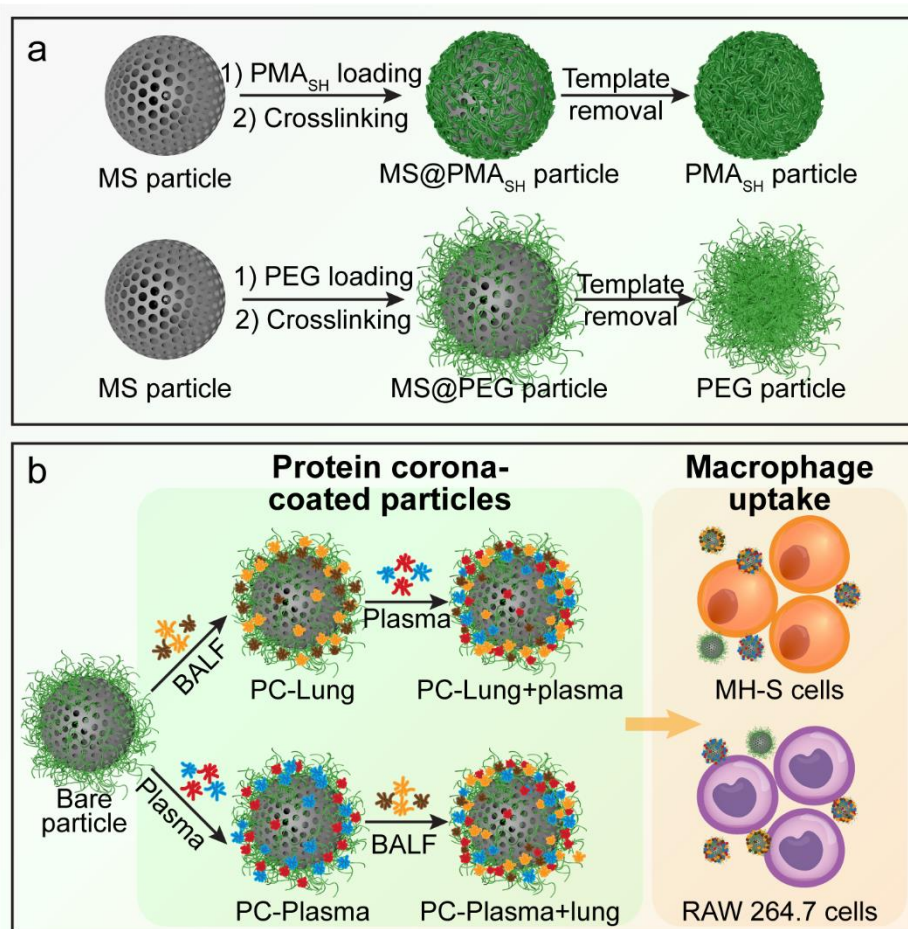
5.2 Introduction

The interactions of nanoengineered particles with biological fluids upon entering a physiological environment, such as the adsorption of biomacromolecules and the resultant formation of a biomolecular corona on particle surface, endow the particles with a ‘biological identity’ that might be different from their original ‘synthetic identity’, which could in turn alter their interactions with cells and the eventual fate in the biological system.¹⁻³ The biomolecular corona is generally composed of two parts with an inner tightly bound near-monolayer termed ‘hard corona’ and an outer layer termed ‘soft corona’ in which components are associated more loosely by lower affinity with a higher protein exchange rate.⁴ The formation of a biomolecular corona is influenced by both the particle physicochemical properties including size, shape, surface chemistry and elasticity, and the distinct composition of the surrounding physiological environments.⁵ As the interactions between particles and biological fluids are highly dynamic processes with continuous adsorption and desorption of components on the particle surface, the components associated with particles are likely to be replaced leading to an altered composition of the biomolecular corona during the transportation of particles from one biological environment to another.⁶⁻⁷

The pulmonary surfactant layer covering the epithelial cells in the deep lungs is a complex biological aqueous mixture consisting of high proportions of surfactant specific proteins, lipids, DNA and other components, constituting the important line of body defence at the air-blood barrier.⁸ Therapeutics or particles for systemic circulation via pulmonary delivery will encounter the lung surfactant environment in the lungs before entering the blood circulation system.⁹ The surface association of the components in pulmonary surfactant will result in a complex mixed corona around particles, which is likely to be different from the conventionally studied protein corona formed in blood plasma, and can potentially affect the downstream biological interactions in blood. On the other hand, therapeutics targeted for the lung regions via the traditional administration route of injection will contact blood first, followed by the lung environment. Despite the numerous investigations on the interaction of particles with blood plasma,¹⁰⁻¹⁴ less attention has been paid to the formation and composition of the pulmonary corona around inhaled particles and its influence on subsequent biological responses, such as macrophage clearance which results in the poor efficacy of nanomaterial delivery to the target cells or tissues and bioavailability of nanomaterials in the body. Recently, some studies have reported the proteomic and lipidomic analysis of the pulmonary corona,⁸ there is little known about the interplay of the pulmonary corona and plasma protein corona on particle–cell interactions during the delivery of particles via different administration routes.

In this chapter, the role of the pulmonary corona and plasma protein corona in the cellular associations of particles with different physicochemical properties was studied. The specific aim was to explore the interplay of pulmonary corona and plasma protein corona on the macrophage uptake of polymer particles to mimic different administration routes. Modified-poly(methacrylic acid) (PMA_{SH})-based particles and poly(ethylene glycol) (PEG)-based particles developed in Chapter 4 (Scheme 5.1a) were incubated in (i) mouse plasma, (ii) mouse bronchoalveolar lavage fluid (BALF), (iii) mouse plasma followed by BALF, or (iv) mouse BALF followed by plasma, to form (i) plasma protein corona-coated particles, (ii) lung protein corona-coated particles, (iii) plasma plus lung protein corona-coated particles, or (iv) lung plus plasma protein corona-coated particles, respectively (Scheme 5.1b). The composition of the protein coronas derived via different ways was analysed by mass spectrometry. The influence of various protein coronas on particle clearance by macrophages were investigated by using mouse alveolar macrophage MH-S cells and murine monocyte/macrophage-like RAW 264.7 cells (Scheme 5.1b). Pulmonary corona, plasma protein corona and the mixed coronas altered the surface properties and cell association of engineered particles differently. The study reveals

that the protein corona initially formed from biological environment A can be partially retained when the particles are subsequently transferred into another biological environment B, and this effect is influenced by the sequence, which means that the resulting protein corona on particles transported from environment B then A is not necessarily equal to the protein corona formed from environment A then B. The interplay of both protein coronas determines the composition of the mixed protein corona, and influences particle properties and subsequent particle–cell interactions.



Scheme 5.1 (a) Schematic illustration of the assembly of MS@PMA_{SH} particles (template present), PMA_{SH} particles (template removed), MS@PEG particles (template present) and PEG particles (template removed) via mesoporous silica (MS) particle templating. Adapted from Scheme 4.1. (b) Schematic illustration of the experimental setup: MS@PMA_{SH}, PMA_{SH}, MS@PEG, and PEG particles were incubated in (i) plasma, (ii) BALF, (iii) plasma followed by BALF, or (iv) BALF followed by plasma, to form (i) plasma protein corona-coated particles (denoted as PC-Plasma), (ii) lung protein corona-coated particles (denoted as PC-Lung), (iii)

plasma plus lung protein corona-coated particles (denoted as PC-Plasma+lung), or (iv) lung plus plasma protein corona-coated particles (denoted as PC-Lung+plasma), respectively, followed by incubating with mouse alveolar macrophage MH-S cells or murine monocyte/macrophage-like RAW 264.7 cells.

5.3 Experimental Section

5.3.1 Materials

Tetraethyl orthosilicate (TEOS), cetyltrimethylammonium bromide (CTAB), poly(acrylic acid) (PAA, M_w ~250 kDa, 35 wt% solution in water), ammonium hydroxide solution ($\text{NH}_3 \cdot \text{H}_2\text{O}$, 28–30%), sodium phosphate dibasic (Na_2HPO_4), hydrofluoric acid (HF, 48 wt%), ammonium fluoride (NH_4F), (3-aminopropyl)-triethoxysilane (APTES), poly(methacrylic acid, sodium salt) (PMA, M_w ~15 kDa, 30 wt% solution in water), N-(3-(dimethylamino)propyl)-N'-ethylcarbodiimide hydrochloride (EDC), N-Chloro-p-toluenesulfonamide sodium salt (chloramine T), DL-dithiothreitol (DTT), 3-(N-morpholino)propanesulfonic acid (MOPS), 2-(N-Morpholino)ethanesulfonic acid hydrate (MES), Dulbecco phosphate-buffered saline (DPBS), paraformaldehyde (PFA), mouse plasma, acetonitrile (CH_3CN), tetraethylammonium bromide (TEAB), tris(2-carboxyethyl)phosphine hydrochloride (TCEP), trifluoroacetic acid (TFA), trifluoroethanol (TFE), iodoacetamide (IAM), trypsin from porcine pancreas (proteomics grade), and formic acid were obtained from Sigma-Aldrich (Australia). 8-arm-poly(ethylene glycol) amine (8-arm-PEG- NH_2 , M_w 40 kDa) was obtained from JenKem Technology USA Inc. (China). 8-Arm-poly(ethylene glycol) succinimidyl succinate (8-arm-PEG-NHS, hexaglycerol core, M_w 10 kDa) was purchased from Creative PEGWorks (USA). Pyridine dithioethylamine hydrochloride (PDA) was obtained from Shanghai Speed Chemical Co. Ltd. (China). Alexa Fluor 488 carboxylic acid succinimidyl ester (AF488-NHS), Alexa Fluor 488 C₅ Maleimide (AF488 Maleimide), RPMI-1640 medium, fetal bovine serum (FBS), NuPAGE LDS sample buffer, NuPAGE sample reducing agent, SeeBlue Plus2 pre-stained protein standard, and SimplyBlue safe stain, Dulbecco's Modified Eagle's Medium (DMEM), wheat germ agglutinin Alexa Fluor 594 conjugate (WGA-594), Hoechst 33342 were purchased from Life Technologies (Australia). BioRad 4–20% Mini-PROTEAN TGX gel, and Tris/glycine/sodium dodecyl sulfate running buffer were purchased from BioRad (CA, USA).

High-purity water used in all experiments with a resistivity greater than 18.2 M Ω cm was generated from a three-stage Millipore Milli-Q plus 185 purification system (Millipore Corporation).

5.3.2 Synthesis of Mesoporous Silica (MS) Particles

MS particles were synthesised following the same protocol described in Chapter 3 (3.3.2).¹⁵ Briefly, 1.1 g of CTAB was completely dissolved in 50 mL of water under constant stirring. Then, 4.3 g of PAA solution (35 wt% in water) was added to the above solution. When the solution turned clear after ca. 20 min, 3.5 mL of ammonium hydroxide solution (28–30%) was added under vigorous stirring. Then, 4.46 mL of TEOS was added to the above milky suspension after stirring for 20 min. The suspension was further stirred for 15 min and finally transferred into a Teflon-sealed autoclave allowing 48 h reaction between 80 and 90 °C. After washing with water and ethanol, and subsequent drying at 80 °C, the synthesised MS particles were finally calcined at 550 °C for 30 h for the removal of organic materials.

5.3.3 Synthesis of MS@PEG and PEG Particles

MS@PEG and PEG particles were fabricated by using a modified published method as described in Chapter 3 (3.3.3).¹⁵ Briefly, 480 μ L of 8-arm-PEG-NH₂ solution (5 mg mL⁻¹ in 100 mM, pH 8.0 phosphate buffer) was added to approximately 6 mg of MS particles for overnight incubation with constant shaking. 8-Arm-PEG-NH₂ was infiltrated into the pores of the MS particles driven by electrostatic interactions. After removing the excessive polymer by washing with phosphate buffer three times, the particles were subsequently incubated in 400 μ L of 8-arm-PEG-NHS solution (2 mg mL⁻¹ in phosphate buffer) for 2 h to form a cross-linked polymer network by the covalent bonding between 8-arm-PEG-NH₂ and 8-arm-PEG-NHS. MS@PEG particles were labelled with AF488-NHS (5 μ L, 1 mg mL⁻¹ in anhydrous DMSO) during the cross-linking step. The resultant particles were washed three times with water to remove unreacted cross-linker and dye. PEG particles were obtained by dissolving the MS templates with a 2 M HF/8 M NH₄F solution (pH ~5). *Caution! HF is highly toxic and extreme care should be taken during handling HF solution.* The resultant PEG particles were washed three times with water and resuspended in water.

5.3.4 Poly(methacrylic acid) (PMA) Functionalisation

Thiol-modified PMA (PMA_{PDA}) was synthesised as described previously via formation of amide bond between the carboxyl groups of PMA and amine groups of PDA mediated by EDC.¹¹ In a typical experiment, 458 mg of PMA solution (30 wt% in water, 1 equiv.) was diluted into 4.875 mL of sodium phosphate buffer (10 mM, pH 7.4). The resulting solution was allowed to react with 97.5 mg of EDC (0.4 equiv.) with stirring for 20 min. Then 70.56 mg of PDA (0.2 equiv.) in 3.538 mL of phosphate buffer was added to the above mixture and the pH was adjusted to 7.6. After incubation with constant shaking overnight, the mixture was transferred into a dialysis membrane (molecular weight cut-off 7000, Thermo) and allowed to dialyse thoroughly against water for three days, and finally, lyophilised for storage until further use. The thiolation degree of as-synthesised thiol-modified PMA (PMA_{PDA}) was quantified as 8% by using a NanoDrop 1000 spectrophotometer. Briefly, PMA_{PDA} was reduced in 0.5 M DTT solution in MOPS buffer (20 mM, pH 8.0) and then diluted in NaOAc buffer (50 mM, pH 4.0) to a final concentration of 0.5 mg mL⁻¹. The thiolation degree was calculated according to the absorbance readings at 343 nm based on a standard curve.

5.3.5 Synthesis of MS@PMA_{SH} and PMA_{SH} Particles

MS@PMA_{SH} and PMA_{SH} particles were prepared according to a previously reported method as described in Chapter 4 (4.3.5).¹⁰ Firstly, MS particles were modified with amine groups by incubating MS particles in a mixture of ethanol, ammonia and APTES at a volume ratio of 20 : 1 : 1 overnight followed by thorough washing with ethanol and water. Approximately 3 mg of amine-modified MS particles were incubated in 5 mg mL⁻¹ PMA_{SH} solution in NaOAc buffer (50 mM, pH 4.5) with constant shaking overnight. After washing with NaOAc buffer three times, the particles were exposed to a 10 mM chloramine T solution in MES buffer (50 mM, pH 6) for 2 min to allow cross-linking of the polymer network. After washing with water and sodium phosphate buffer (10 mM, pH 7.4), the resultant MS@PMA_{SH} particles were fluorescently labelled with AF488 maleimide (5 μL , 1 mg mL⁻¹ in DMSO) overnight under gentle shaking. PMA_{SH} particles were obtained by dissolving the silica templates with a 2 M HF/8 M NH₄F solution (pH $\sim$ 5). *Caution! HF is highly toxic and extreme care should be taken when handling the solution.* The resultant fluorescently labelled PMA_{SH} particles were washed three times with sodium phosphate buffer (10 mM, pH 7.4).

5.3.6 Particle Characterisation

Fluorescence microscopy images were taken by using a differential interference contrast (DIC) (U-DICT, Olympus) / inverted fluorescence combined microscope (Olympus IX71) equipped with a 100× oil immersion objective. Transmission electron microscopy (TEM) images were taken with a FEI Tecnai TF20 instrument operating at the voltage of 80 kV for PEG particles and 120 kV for other particles. The TEM samples were prepared by dropping aqueous particle suspension onto plasma-treated formvar-coated copper grids followed by air-drying. Scanning electron microscopy (SEM) images were taken with a Philips XL30 instrument at an operation voltage of 15 kV. The SEM samples were prepared by placing a drop of particle suspension onto clean silicon wafers, followed by sputter-coating with gold. The zeta potential of particles was measured by a Zetasizer Nano-ZS (Malvern Instruments, Malvern, UK). The size distribution of particles was measured by dynamic light scattering (DLS) (Zetasizer Nano-ZS, Malvern Instruments, Malvern, UK). Particle counting was carried out by using flow cytometry (Apogee Flow). Image analysis was conducted using ImageJ software.

5.3.7 Collection of Mouse Bronchoalveolar Lavage Fluid (BALF)

Lavage procedure of the mouse lungs was carried out by using a reported method.¹⁶ All procedures were conducted in accordance with ethical guidelines from the University of Melbourne Animal Ethics Committee (AEEC#1513736.1). The trachea of lethally anesthetized C57Bl/6 mice (20–25 g) was surgically exposed and intubated with a syringe catheter. The lungs were lavaged with DPBS four times (300 μ L each time) and a total of $\sim$ 1.2 mL of BALF was obtained from each mouse. After removal of the cells in BALF by centrifugation (2500 *g*, 5 min) at 4 °C, the supernatant was stored at –80 °C until further use. Protein concentration in BALF was determined by using a Micro BCA Protein Assay Reagent (Fisher Thermo Scientific) according to the manufacturer's protocol. Absorbance of samples at 562 nm was converted to the protein concentration based on a standard curve generated with serial dilutions of bovine serum albumin (BSA) (0–40.0 μ g mL^{–1}).

5.3.8 Formation of Protein Corona-Coated Particles

MS@PEG particles, PEG particles, MS@PMA_{SH} or PMA_{SH} particles (2×10^8 dispersed in DPBS) were incubated in 100 μ L of 10 mg mL⁻¹ mouse plasma or BALF for 1 h at 37 °C with constant shaking (600 rpm), and then washed with DPBS thoroughly resulting in plasma protein corona-coated particles or lung protein corona-coated particles, respectively. The as-obtained protein corona-coated particles were further incubated in 100 μ L of as-obtained BALF or 10 mg mL⁻¹ mouse plasma for 1 h at 37 °C with shaking, followed by extensive washing with DPBS, to form (i) plasma plus lung protein corona-coated particles or (ii) lung plus plasma protein corona-coated particles. These protein corona-coated particles were used directly for particle characterisation, cell association and protein corona analysis.

5.3.9 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The plasma protein corona-coated particles, lung protein corona-coated particles, plasma plus lung protein corona-coated particles, or lung plus plasma protein corona-coated particles, were obtained using the above described method (5.3.8). The adsorbed proteins were eluted from the particles by incubating particles with NuPAGE LDS sample buffer and NuPAGE sample reducing agent, followed heating at 70 °C for 10 min. After removal of particles by centrifugation, the supernatant was loaded on the 4–20% Mini-PROTEAN TGX gel (BioRad) running at 120 V for 40 min. Diluted mouse plasma and BALF were loaded as controls. After washing with RO water, the gel was stained with SimplyBlue overnight, and rinsed with RO water three times prior to imaging.

5.3.10 Proteomics Analysis by Mass Spectrometry

The protein corona-coated particles were obtained using the above described method (5.3.8). Samples for mass spectrometry analysis were prepared following a standard protocol. Briefly, the protein corona-coated particles were incubated in a mixture of 25 μ L of 100 mM TEAB, 25 μ L of TFE and 2.5 μ L of 200 mM TCEP at 60 °C for 45 min for protein denaturation. 10 μ L of 200 mM IAM (in 50 mM TEAB) was then added to the mixture, allowing 45 min-incubation at room temperature in the dark. Then, 2.5 μ L of 200 mM TCEP was added to the mixture for 30 min-incubation at room temperature in the dark, to destroy the excess IAM. After that, the mixture was diluted by adding 300 μ L of water and the pH was raised to between

7.5 and 8.0 by adding 100 μ L of 100 mM TEAB. The eluted proteins were digested by incubating with 0.2 μ g of trypsin overnight at 37 °C. The trypsin activity was stopped by adding 2 μ L of formic acid to lower the pH. The resultant mixture was centrifuge at 15000 *g* for 10 min to remove any pellets. The collected supernatant was freeze dried followed by resuspending in 20 μ L of 2% CH₃CN/0.05% TFA.

Three replicates of each samples were analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a QExactive plus Orbitrap mass spectrometer (Thermo Scientific) with a nanoESI interface in conjunction with an Ultimate 3000 RSLC nanoHPLC (Dionex Ultimate 3000). The LC system was equipped with an Acclaim Pepmap nano-trap column (Dionex-C18, 100 Å, 75 μ m $\times$ 2 cm) and an Acclaim Pepmap RSLC analytical column (Dionex-C18, 100 Å, 75 μ m $\times$ 50 cm). The tryptic peptides were injected to the enrichment column at an isocratic flow of 5 μ L min⁻¹ of 2% v/v CH₃CN containing 0.1% v/v formic acid for 5 min applied before the enrichment column was switched in-line with the analytical column. The eluents were 0.1% v/v formic acid (solvent A) and 100% v/v CH₃CN in 0.1% v/v formic acid (solvent B). The flow gradient was as follows: (i) 0–6min at 3% B, (ii) 6–40 min, 3–25% B, (iii) 40–48 min, 25–45% B, (iv) 48–50 min, 40–80% B, (v) 50–53 min, 85–85% B, (vi) 53–54 min, 85–3% and equilibrated at 3% B for 10 minutes before the next sample injection. The QExactive plus mass spectrometer was operated in the data-dependent mode, whereby full MS1 spectra were acquired in positive mode, 70 000 resolution, AGC target of 3e⁶ and maximum IT time of 50 ms. Fifteen of the most intense peptide ions with charge states ≥ 2 and intensity threshold of 1.7e⁴ were isolated for MS/MS. The isolation window was set at 1.2m/z and precursors fragmented using normalised collision energy of 30 at 17 500 resolution, AGC target of 1e⁵ and maximum IT time of 100 ms. Dynamic exclusion was set to be 30 s.

Raw files were processed using the MaxQuant platform (version 1.6.10.43) and searched against UniProt human database (January 2019) using default settings for Orbitrap instrument.¹⁷ Trypsin was selected as the enzyme with the number of maximum missed cleavages set to 2. Fixed modification was carbamidomethyl of cysteine while variable modifications included acetyl (protein N-terminus) and oxidation of methionine. False discovery rate (FDR) was set to 1% on both protein and peptide level. Label free quantitation required a minimum of two peptides with at least one razor or unique peptide. Protein groups identified in MaxQuant were imported into Perseus for further analysis.¹⁸

5.3.11 Cell Culture

Mouse alveolar macrophage MH-S cells obtained from American Type Culture Collection (ATCC, USA) were maintained in complete RPMI-1640 media containing 10% FBS and 0.05 mM 2-mercaptoethanol at 37 °C in a 5% CO₂ humidified atmosphere. Mouse RAW 264.7 cells (ATCC, USA) were cultured in complete DMEM containing 10% FBS and 2 mM glutamine at 37 °C in a 5% CO₂ humidified atmosphere.

5.3.12 Cell Association by Flow Cytometry

MH-S cells or RAW 264.7 cells were treated with AF488-labelled MS@PMA_{SH}, PMA_{SH}, MS@PEG or PEG particles in the absence or presence of various protein coronas (bare particles, plasma protein corona-coated particles, lung protein corona-coated particles, plasma plus lung protein corona-coated particles, and lung plus plasma protein corona-coated particles) in serum free medium at a particle-to-cell ratio of 100:1 (8×10^4 cells per well, 24-well plate). After 2 h incubation at 37 °C, the cells were detached from the plate using trypsin and subsequently washed with DPBS three times by centrifugation at 100 *g*, 10 min for MH-S cells, and 300 *g*, 5 min for RAW 264.7 cells. The resultant cell pellets were resuspended in DPBS and analysed by flow cytometry (Accuri Flow).

5.3.13 Cell Association by Confocal Microscopy

MH-S cells or RAW 264.7 cells were treated with AF488-labelled MS@PMA_{SH}, PMA_{SH}, MS@PEG or PEG particles in the absence or presence of various protein coronas (bare particles, plasma protein corona-coated particles, lung protein corona-coated particles, plasma plus lung protein corona-coated particles, and lung plus plasma protein corona-coated particles) in serum free medium at a particle-to-cell ratio of 100:1 (5×10^4 cells per well, 8-well Lab-Tek chambered coverglass slides) at 37 °C for 2 h. After incubation, the cells were gently washed with DPBS and then fixed with paraformaldehyde (4% in DPBS) for 15 min at room temperature. The fixed cells were then stained with WGA-594 ($5 \mu\text{g mL}^{-1}$) for 5 min and Hoechst 33342 (0.1 mg mL^{-1}) for 5 min at room temperature.

Cell association imaging was carried out using a laser scanning confocal microscope (Nikon Corporation, Japan) equipped with a Plan Apo λ 60 $\times$ 1.4 NA oil immersion objective, and 405, 488, 561, and 640 nm lasers. Images were analysed by ImageJ software.

5.4 Results and Discussions

5.4.1 Particle Preparation and Characterisation

MS particles with an average diameter of 891 ± 83 nm were synthesised as described above (Figure S5.1). PMA_{SH} particles which showed high cell association as reported in previous study,¹⁹ and PEG particles which exhibited ‘stealth property’ in bloodstream,¹⁵ were used in this study for comparison. PMA_{SH} and PEG particles were prepared from PMA_{SH} and PEG building blocks, respectively, via MS particle templating. For the adsorption of PMA_{SH}, the MS particle templates were modified with amine groups to make the surface positively charged (from -24.4 mV to 16.8 mV in pH 7.4 phosphate buffer) for the electrostatic interaction with PMA_{SH} polymer. The amine-modified MS particles (MS-NH₂) maintained monodispersity with similar size to the initial MS particles (Figure S5.2). PMA_{SH} or PEG-NH₂ was infiltrated into the porous structure of the templates followed by crosslinking, resulting in MS@PMA_{SH} or MS@PEG particles. After template removal by HF, PMA_{SH} or PEG particles were obtained. Particles were subsequently labelled with fluorescence dye Alexa Fluor 488 to enable visualisation and imaging.

The characterisation shown in Figure 5.1 reveals the dispersity, morphology and structure of the MS@PMA_{SH}, PMA_{SH}, MS@PEG and PEG particles. The fluorescence microscopy images (Figure 5.1a1–d1) show the monodispersity and homogenous fluorescence of all the particles in aqueous solution. TEM images (Figure 5.1a2–d2) show the detailed morphology and structure of particles in the dry state. MS@PMA_{SH} particles were observed to be more electron dense compared to MS@PEG particles due to the adsorption of PMA_{SH}. PMA_{SH} particles exhibited a dense, rigid, smooth spherical structure with a thickness of 343.1 ± 24.8 nm from analysis of air-dried particles by AFM, while PEG particles appeared collapsed after air-drying with a thickness of 55.9 ± 4.1 nm as a result of dehydration and had a relatively loose and rough structure.

MS@PMA_{SH}, PMA_{SH}, MS@PEG, and PEG particles were incubated in (i) plasma, (ii) BALF, (iii) plasma followed by BALF, or (iv) BALF followed by plasma, to form (i) plasma protein

corona-coated particles, (ii) lung protein corona-coated particles, (iii) plasma plus lung protein corona-coated particles, or (iv) lung plus plasma protein corona-coated particles, respectively, following the methods described in 5.3.8.

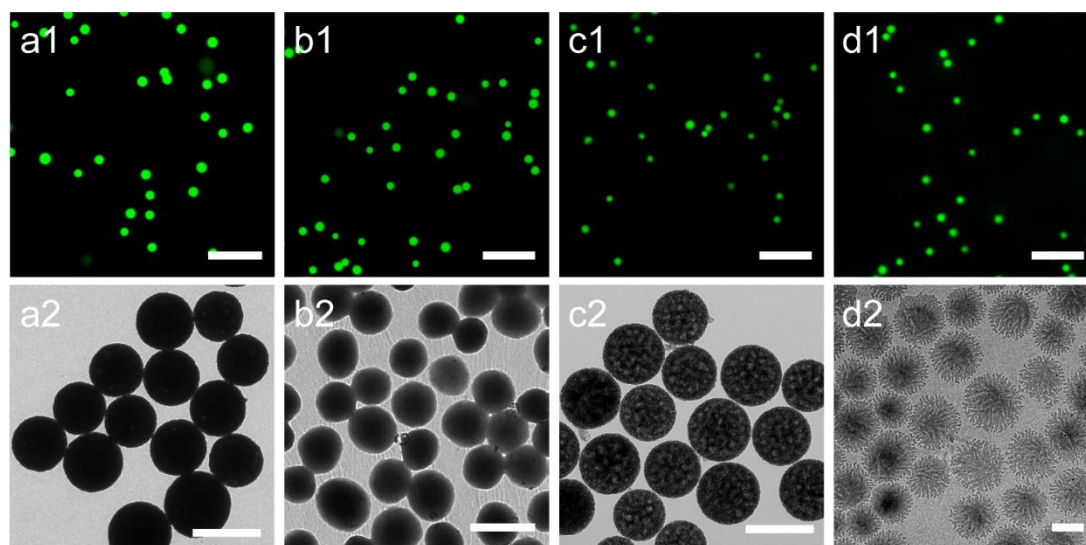


Figure 5.1. Fluorescence microscopy images of AF488 fluorescently labelled (a1) MS@PMA_{SH}, (b1) PMA_{SH}, (c1) MS@PEG and (d1) PEG particles. TEM images of (a2) MS@PMA_{SH}, (b2) PMA_{SH}, (c2) MS@PEG and (d2) PEG particles. Scale bars are 10 μm in (a1–d1), and 1 μm in (a2–d2). Modified from Figure 4.1 and 4.2.

5.4.2 Protein Corona Effects on Particle Surface Charge

To evaluate the effect of protein corona on particle surface charge, the zeta potential of the particles with or without protein corona was measured using microelectrophoresis (Figure 5.2a). The bare PMA_{SH}-based and PEG-based particles without protein corona exhibited different surface properties. MS@PMA_{SH} and PMA_{SH} particles both had a zeta potential of ~ -31 mV in phosphate buffer (5 mM, pH 7.4), which was much more negatively charged than MS@PEG particles with zeta potential of ~ -14 mV, due to the different surface chemistry. PEG particles exhibited near neutral charge at pH 7.4. In general, the adsorption of proteins from BALF and/or plasma resulted in neutralisation of the particle surface charge for MS@PMA_{SH} and PMA_{SH} particles, whereas this charge neutralisation effect was negligible for MS@PEG and PEG particles probably due to the repulsion force from the hydrated PEG surface. The heatmap of the statistical analysis (Figure 5.2b) showed the significant differences between different protein corona-coated particles. Notably, plasma protein corona, plasma plus lung protein corona, and lung plus plasma protein corona resulted in similar alteration of particle surface

charge, suggesting that the plasma protein corona played a dominant role in the eventual mixed protein corona no matter with the sequence of protein corona formation. The lung protein corona had less influence on the particle surface charge probably due to the more diluted protein concentration ($\sim 180 \mu\text{g mL}^{-1}$) in BALF, and resultant lower amount of adsorbed proteins and different protein composition of the lung corona on particles (Figure 5.6).

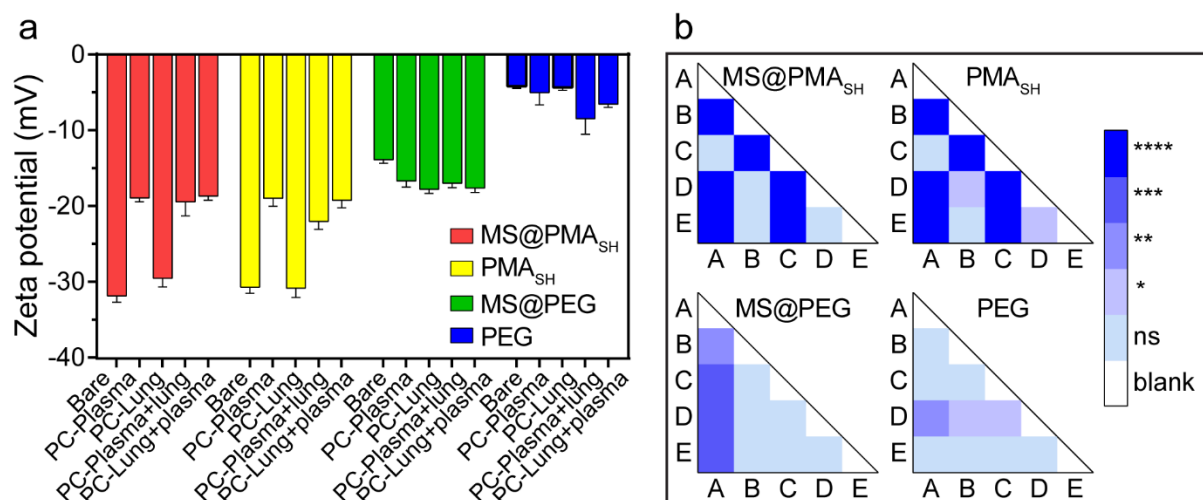


Figure 5.2. (a) Zeta potential and (b) corresponding statistic analysis of MS@PMA_{SH}, PMA_{SH}, MS@PEG or PEG particles without a protein corona (PC) (A: bare) or with different protein coronas (B: PC-Plasma; C: PC-Lung; D: PC-Plasma+lung; and E: PC-Lung+plasma). Data are shown as the mean $\pm$ standard error of at least three independent experiments. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (one-way ANOVA with Tukey's multiple comparisons test).

5.4.3 Protein Corona Effects on Cell Association

As a protein corona endows particles with a 'biological identity' that might be different from their original 'synthetic identity', we then investigated the effects of different protein coronas on the cell association of MS@PMA_{SH}, PMA_{SH}, MS@PEG and PEG particles using mouse alveolar macrophage MH-S cells and murine monocyte/macrophage-like RAW 264.7 cells. MS@PMA_{SH}, PMA_{SH}, MS@PEG and PEG particles were fluorescently labelled with AF488, and counted using flow cytometry before and after the formation of protein corona. MH-S cells and RAW 264.7 cells were treated with MS@PMA_{SH}, PMA_{SH}, MS@PEG and PEG particles in the absence or presence of various protein coronas (plasma, lung, plasma plus lung, and lung

plus plasma protein coronas) for 2 h at 37 °C. The cells associated with particles were then evaluated quantitatively and qualitatively by flow cytometry (Figure 5.3) and confocal microscopy (Figure 5.4 and 5.5), respectively.

In general, the formation of protein corona led to reduced cell association of MS@PMA_{SH} and PMA_{SH} particles, with stronger reduction for PMA_{SH} particles than MS@PMA_{SH} particles, which is consistent with a previous study of PMA_{SH} capsules and PMA_{SH} core-shell particles.¹² This is probably due to the difference of rigidity and deformability between MS@PMA_{SH} and PMA_{SH} particles. The protein corona, however, resulted in increased macrophage uptake of MS@PEG particles, but had negligible effect on PEG particles which retained a low level of cell association, for both MH-S cells and RAW 264.7 cells. This indicates that the effect of protein corona on particle–cell interactions was dependent on the particle properties (Figure 5.3). The inhibition effect of protein corona on cell association of MS@PMA_{SH} and PMA_{SH} particles were likely due to the reduced free energy of the particle surface in the presence of protein corona, and a consequently decreased adhesion to the cell membrane of protein corona-coated particles compared to bare particles.^{20–21} Furthermore, the adsorption of proteins could reduce the exposure of thiol groups present on MS@PMA_{SH} and PMA_{SH} particle surface, which have been found to enhance cellular association and internalisation via interactions with exofacial thiols on the cell surface,²² and thereby reduced the cell uptake. In contrast, protein corona promoted macrophage uptake for MS@PEG particles. In the absence of proteins corona, MS@PEG particles had little (~13%) association with cells due to limited interactions between the highly hydrated PEG surface and the cell surface.²³ The presence of adsorbed proteins on particle surface enhanced the cell association to >40% for both MH-S cells and RAW 264.7 cells. This could probably result from the protein–receptor interactions between specific components in the protein corona and cell recognition receptors on cell membranes as suggested in a previous study.²⁴ The PEG particles retained a low level of cell association mainly due to the small amount of proteins adsorbed on the particles (Figure 5.6).

Furthermore, it was found that different protein coronas played different roles in cell association of particles (Figure 5.3). Although the lung protein corona had limited influence on particle surface charge as shown in Figure 5.2, it altered their association with macrophages, especially for PMA_{SH} and MS@PEG particles. As shown in Figure 5.3a, the association of bare PMA_{SH} particles with MH-S cells was 94%, while in the presence of lung protein corona, the association was reduced to 82%. And the lung protein corona led to significantly increased cell association of MS@PEG particles (44%) compared to bare MS@PEG particles (13%).

Furthermore, although the particles exhibited similar zeta potential after coating with plasma, plasma+lung or lung+plasma protein corona (Figure 5.2), the cell associations of these particles were significantly different (Figure 5.3). For example, the plasma+lung protein corona resulted in 28% of PMA_{SH} particles associated with MH-S cells, which was significantly lower than that of plasma protein corona (66%) and lung+plasma protein corona (67%). For MS@PEG particles, the cell association in the presence of plasma+lung protein corona was 72%, which was higher than that of plasma protein corona (63%) and lung+plasma protein corona (38%). Similar results were found for RAW 264.7 cells. These findings revealed that the protein corona altered the particle surface properties especially their biological identity in addition to the particle surface charge. Notably, the plasma+lung protein corona-coated particles and the lung+plasma protein corona-coated particles exhibited different levels of cell association under the same conditions (e.g., MH-S cells: difference ~39% for PMA_{SH} particles and ~34% for MS@PEG particles), suggesting that even though plasma+lung protein corona and lung+plasma protein corona were generated from the same biological environments (plasma and BALF), their composition might be different due to the different corona formation sequence, leading to subsequently different particle–cell interactions.

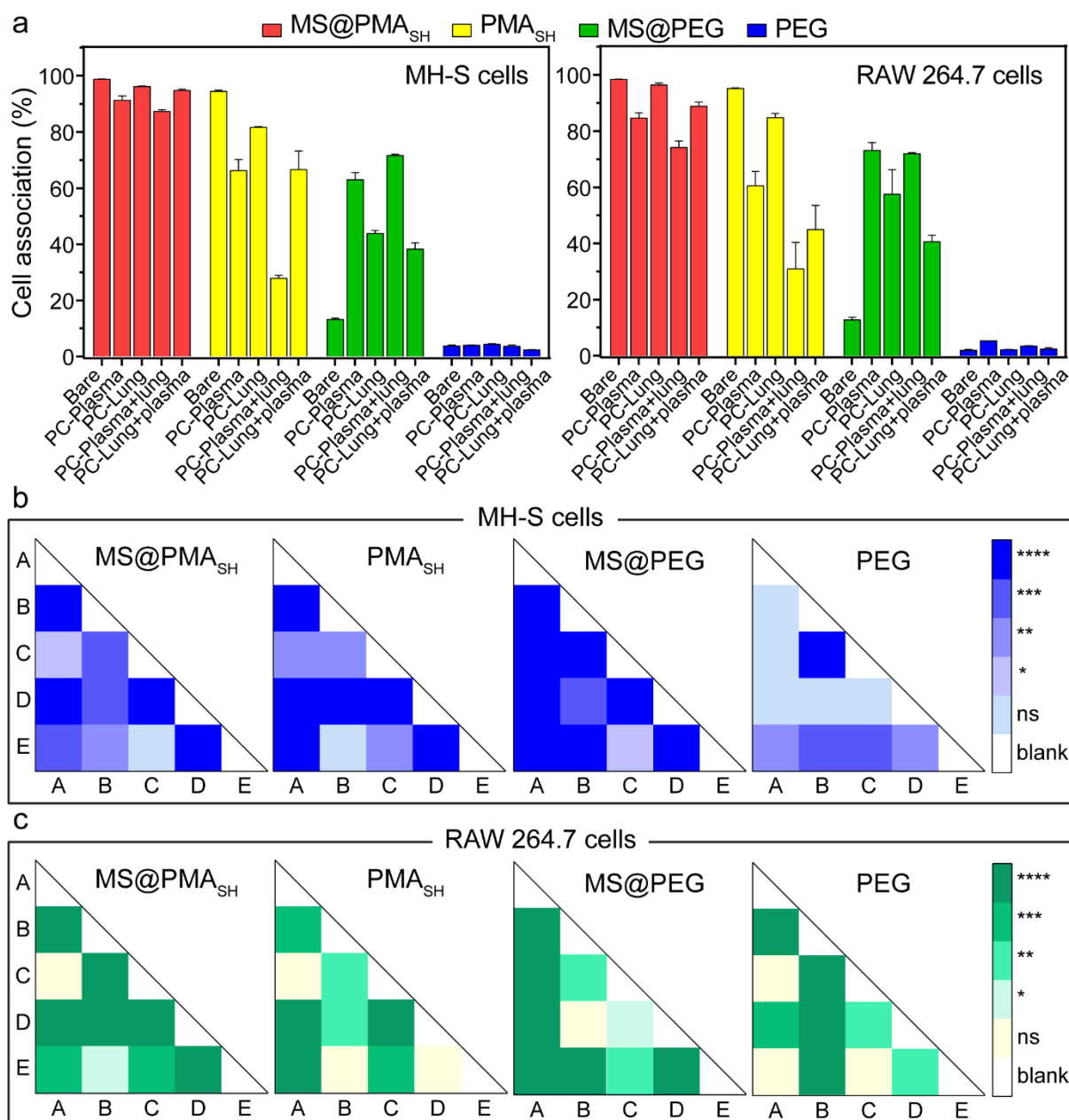


Figure 5.3. (a) Cell association and (b, c) corresponding statistical analysis of MS@PMA_{SH}, PMA_{SH}, MS@PEG or PEG particles without a protein corona (PC) (bare, A) or with different protein coronas (B: PC-Plasma; C: PC-Lung; D: PC-Plasma+lung; and E: PC-Lung+plasma) with MH-S cells and RAW 264.7 cells after 2 h incubation at a particle-to-cell ratio of 100:1. Data are shown as the mean $\pm$ standard error of at least three independent experiments. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (one-way ANOVA with Tukey's multiple comparisons test).

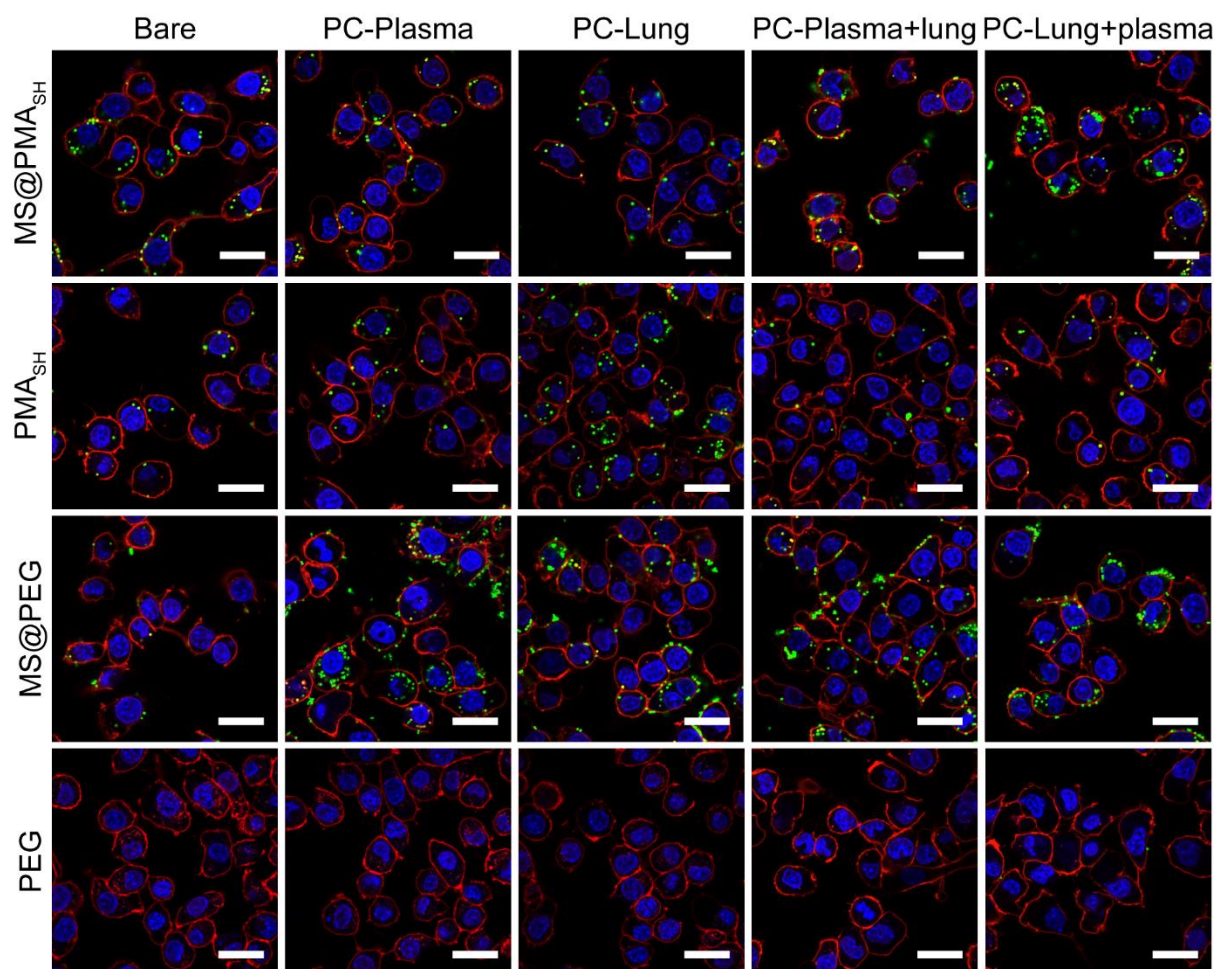


Figure 5.4. Confocal microscopy images of MH-S cells after incubation with AF488-labelled MS@PMA_{SH}, PMA_{SH}, MS@PEG or PEG particles without a protein corona (PC) (bare) or with different protein coronas (PCs) (PC-Plasma, PC-Lung, PC-Plasma+lung, and PC-Lung+plasma) (green) for 2 h at a particle-to-cell ratio of 100:1. The cell membranes were stained with Alexa Fluor 594-conjugated wheat germ agglutinin (WGA-594, red), and the nuclei were stained with Hoechst 33342 (blue). Scale bars are 20 μ m.

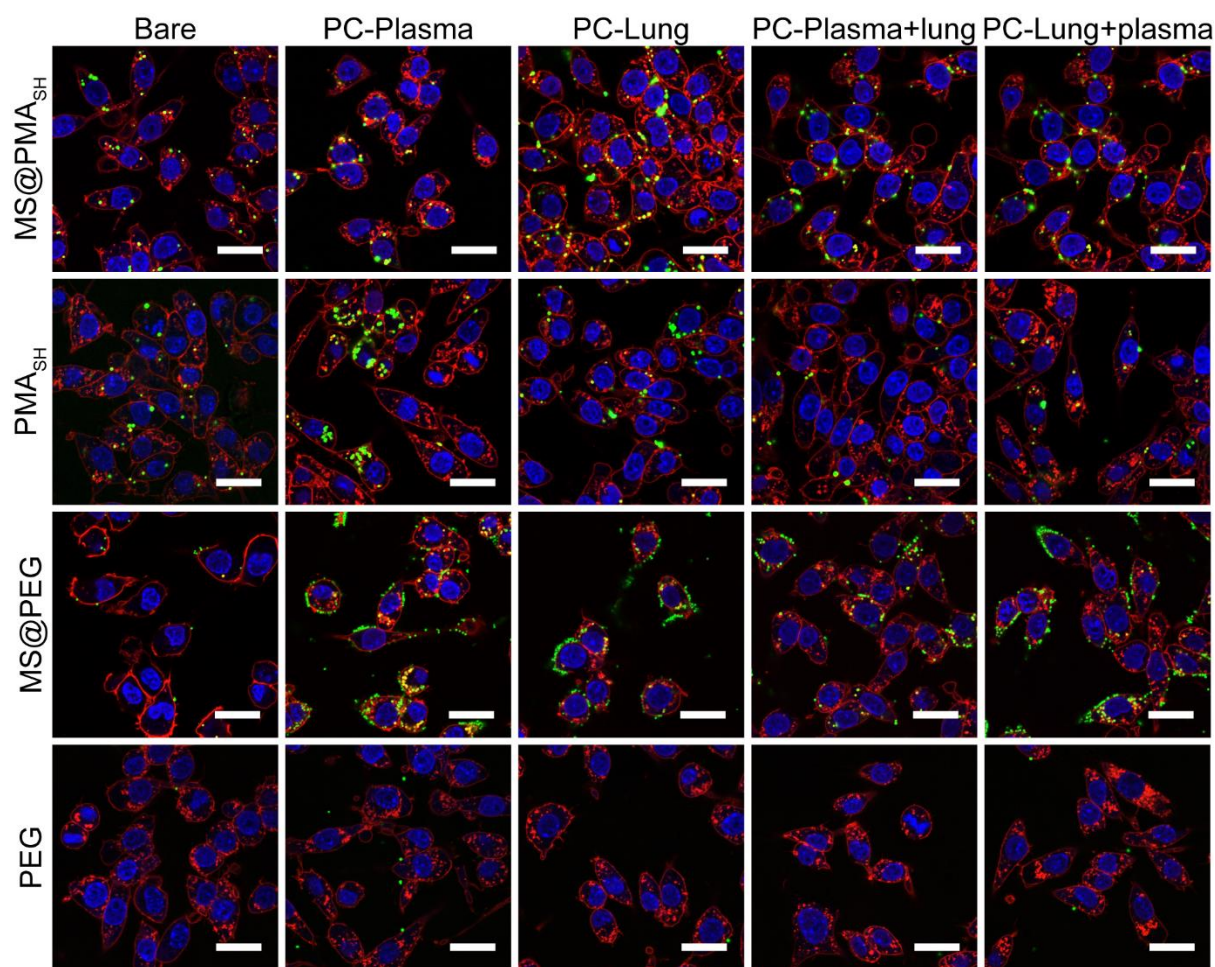


Figure 5.5. Confocal microscopy images of RAW 264.7 cells after incubation with AF488-labelled MS@PMA_{SH}, PMA_{SH}, MS@PEG or PEG particles without a protein corona (PC) (bare) or with different protein coronas (PCs) (PC-Plasma, PC-Lung, PC-Plasma+lung, and PC-Lung+plasma) (green) for 2 h at a particle-to-cell ratio of 100:1. The cell membranes were stained with Alexa Fluor 594-conjugated wheat germ agglutinin (WGA-594, red), and the nuclei were stained with Hoechst 33342 (blue). Scale bars are 20 μ m.

5.4.4 Protein Corona Analysis by SDS-PAGE and Mass Spectrometry

To analyse the composition of the protein corona, proteins eluted from various protein corona-coated particles were separated by SDS-PAGE, and then stained with SimplyBlue for observation. The present protein bands were slightly different varying from different protein coronas and particles, indicating that the composition of protein corona was dependent on both biological environments and particle properties (Figure 5.6). Due to the low profile of SDS-PAGE, proteomics analysis was carried out by mass spectrometry (LC-MS/MS) to obtain

detailed protein information. Three replicates of each sample were analysed and only proteins that were identified in at least two replicates were evaluated. The percentage of the top 20 most abundant proteins from the total adsorbed proteins derived from the plasma+lung protein corona and lung+plasma protein corona on MS@PMA_{SH}, PMA_{SH}, MS@PEG and PEG particles as shown in Figure 5.7 and 5.8. The values were calculated from the intensity based absolute quantification (iBAQ) value, by dividing a certain protein's iBAQ value to the sum iBAQ value of all identified proteins, generating a relative iBAQ (riBAQ) value for each protein. The results show the differences in protein composition of the plasma+lung protein corona and lung+plasma protein corona, which could serve as evidence to explain their different influence on particle–cell interaction as discussed in 5.4.3. In general, for a certain particle system, even though these two types of mixed protein corona were derived from the same biological environments (plasma and BALF), the protein content was not necessarily the same especially for the relatively more abundant proteins, due to the different corona formation sequence. For example, with MS@PMA_{SH} particles (Figure 5.7a), the most abundant protein in plasma+lung protein corona was HBA1 (hemoglobin subunit alpha) at 16.5%, while it was only 8.7% in the lung+plasma protein corona and much lower than the most abundant FGB (fibrinogen beta chain) (17.9%). Similar results were found in PMA_{SH}, MS@PEG and PEG particles. Notably, such differences in protein composition between plasma+lung protein corona and lung+plasma protein corona were more obvious for MS@PMA_{SH} and PMA_{SH} particles than MS@PEG and PEG particles. For MS@PEG and PEG particles, except for the most abundant HBA1(hemoglobin subunit alpha) protein, the proportions of the other 19 proteins were at a similar level in both types of protein corona.

The signal intensity of proteins identified from the different protein coronas is presented through heatmaps (Figure S5.5, S5.6 and S5.7). Some proteins (e.g., apolipoprotein B-100 in Figure S5.6) detected in plasma protein corona but undetected in lung protein corona were found in plasma+lung and lung+plasma protein corona. Similarly, some proteins (e.g., apolipoprotein C-III in FigureS5.6) undetected in plasma protein corona but detected in lung protein corona were found in plasma+lung and lung+plasma protein corona. There were also some proteins detected in both plasma protein corona and lung protein corona but undetected in plasma+lung protein corona or lung+plasma protein corona. These findings suggested that previously adsorbed proteins may be replaced by the proteins from the new environment when the particles are transferred from one biological environment into another, though the protein corona derived from the former environment can be partially retained. Figure S5.7 shows the

signal intensity of proteins in plasma+lung protein corona and lung+plasma protein corona on MS@PMA_{SH}, PMA_{SH}, MS@PEG or PEG particles by heatmap. Many differences were observed either between different particles or between different protein coronas within a certain particle system, revealing that the features of protein corona are dependent on both biological environments and the particle properties. Furthermore, the addition of protein corona B to protein corona A is not necessarily equal to the addition of protein corona A to protein corona B. The interplay of both protein coronas determines the composition of the mixed protein corona (Figure 5.7, S5.5, S5.6, S5.7), and the resultant influence on particle properties (Figure 5.2) and subsequent particle–cell interactions (Figure 5.3).

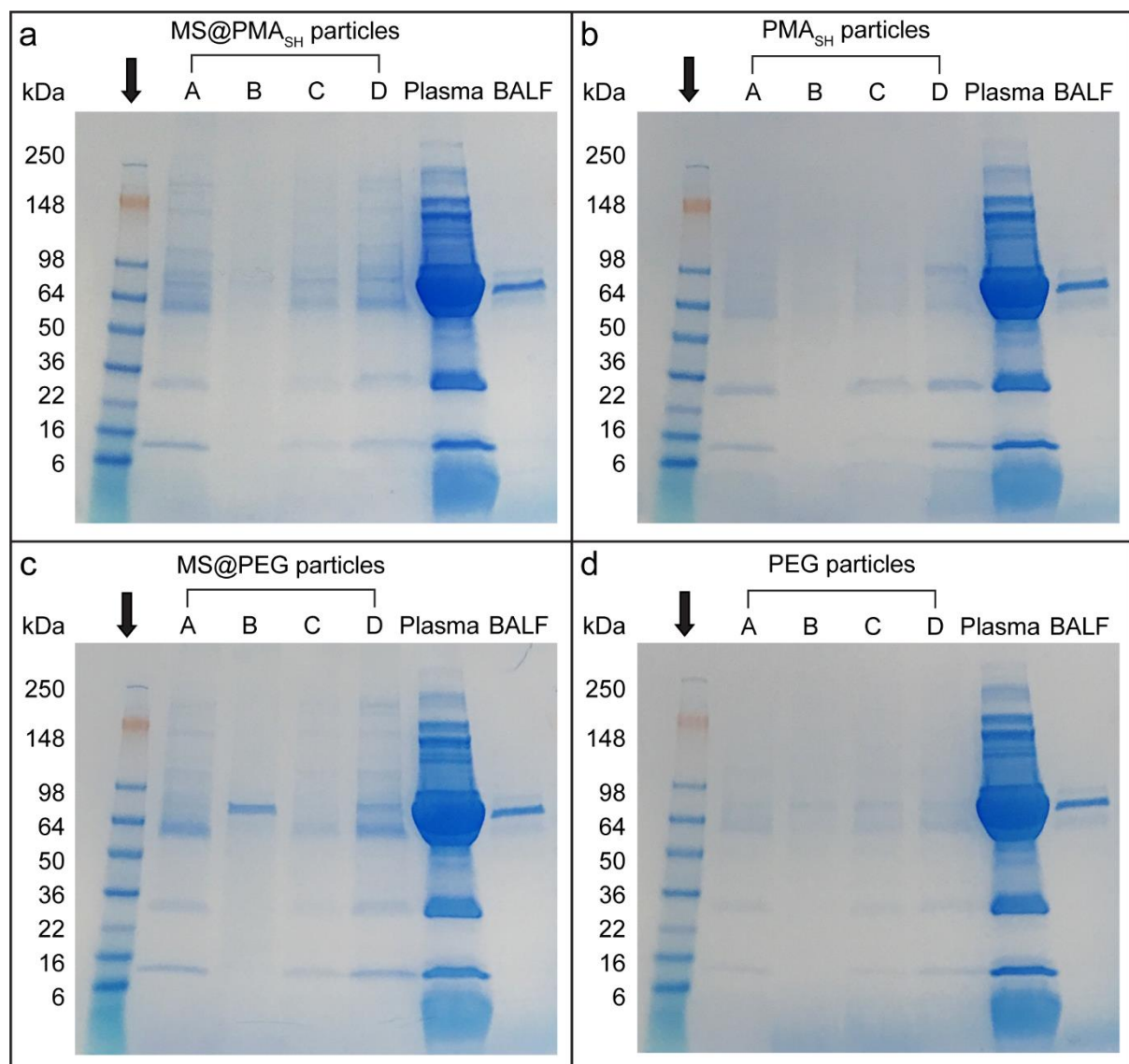


Figure 5.6. SDS-PAGE images of separated corona proteins derived from (a) MS@PMA_{SH}, (b) PMA_{SH}, (c) MS@PEG and (d) PEG particles. A = PC-Plasma, B = PC-Lung, C = PC-Lung+plasma, and D = PC-Plasma+lung.

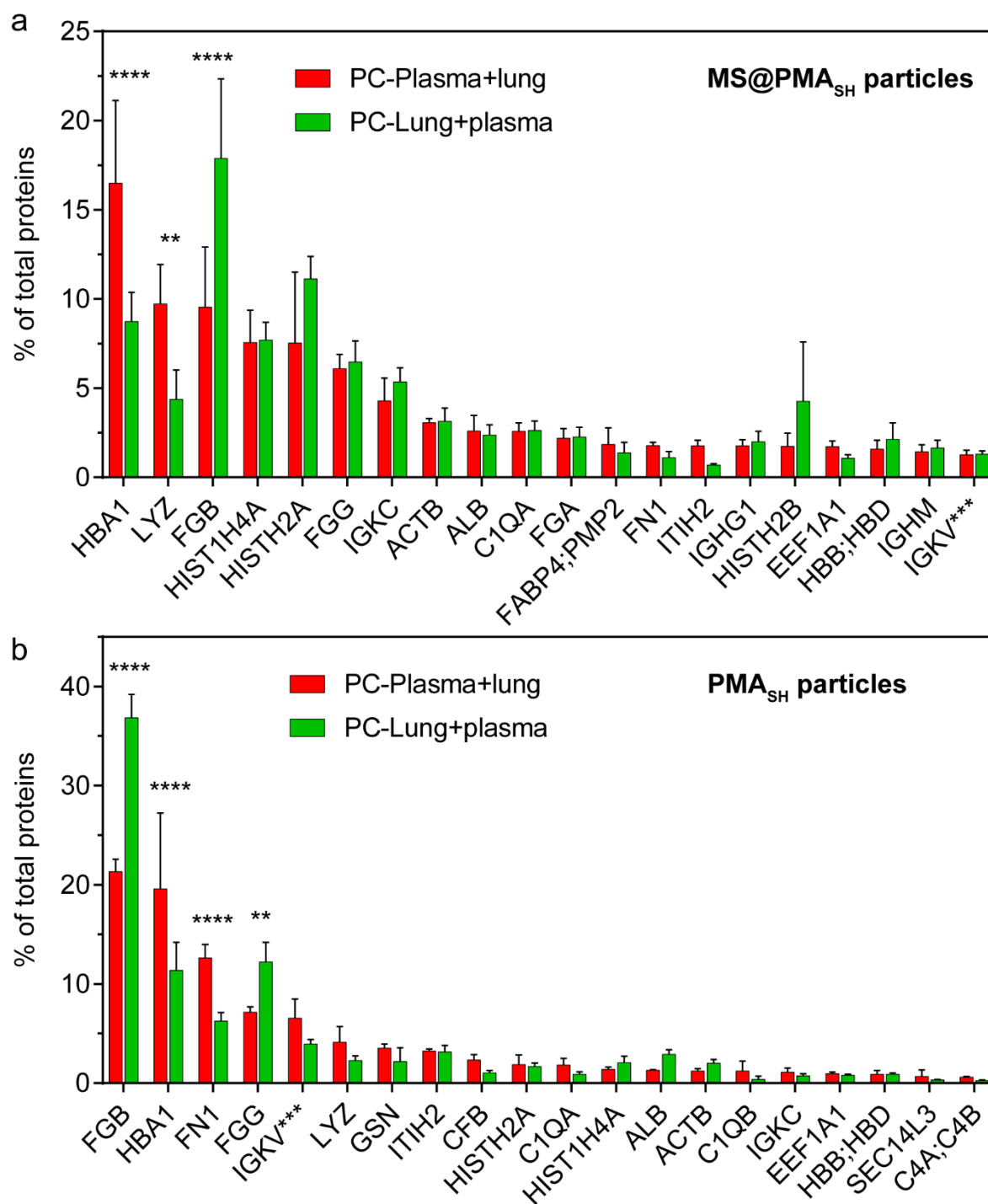


Figure 5.7. The top 20 most abundant proteins found in plasma+lung protein corona and lung+plasma protein corona on (a) MS@PMA_{SH} particles and (b) PMA_{SH} particles. Data represent the mean value $\pm$ standard deviation ($n = 3$). ** $p < 0.01$, **** $p < 0.0001$ (two-way ANOVA with Tukey's multiple comparisons test).

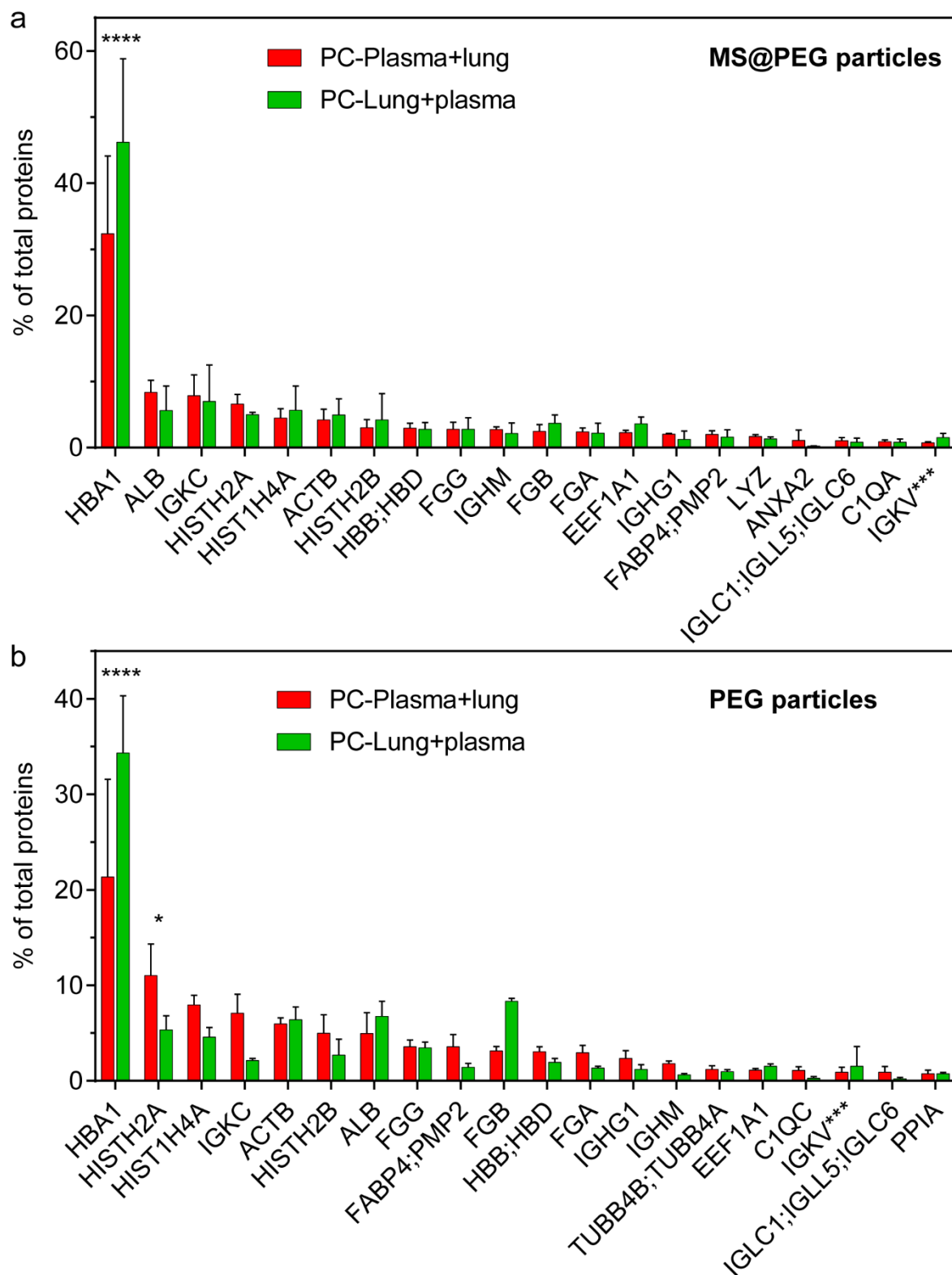


Figure 5.8. The top 20 most abundant proteins found in plasma+lung protein corona and lung+plasma protein corona on (a) MS@PEG particles and (b) PEG particles. Data represent the mean value $\pm$ standard deviation ($n = 3$). * $p < 0.05$, **** $p < 0.0001$ (two-way ANOVA with Tukey's multiple comparisons test).

5.5 Conclusions

This chapter demonstrated the influence of pulmonary corona and plasma protein corona on particle properties, and the interplay of both protein coronas on the composition of the mixed protein corona obtained when particles were transported from the lung environment to blood and vice versa. The influence of the protein corona on macrophage uptake of the particles was also studied. Protein coronas formed from plasma or lung environment alone and the mixed coronas had different effects on the particle properties (e.g., zeta potential) and the subsequent particle–cell interactions. Overall, the presence of a protein corona led to reduced macrophage uptake of MS@PMA_{SH} and PMA_{SH} particles, but increased macrophage uptake of MS@PEG particles, and negligible effect on the PEG particles. The plasma+lung protein corona-coated particles and the lung+plasma protein corona-coated particles exhibited different levels of cell association under the same condition, due to the different composition and proportions of protein in the coronas as evaluated by mass spectrometry. The relative abundance of specific functional proteins (e.g., immunoglobulins) may play an important role in regulating the particle–cell interactions. The study reveals that even though the previously adsorbed proteins may be replaced by the proteins from the new environment when the particles are transferred from one biological environment into another, the protein corona derived from the former environment can be partially retained. The features of the protein corona including the protein composition and proportions are dependent on both biological environments and the particle properties. The addition of protein corona B to protein corona A is not necessarily equal to the addition of protein corona A to protein corona B, in terms of both the features of the mixed protein corona and its influence on subsequent particle-cell interactions. The interplay of both protein coronas determines the composition of the mixed protein corona, and the resultant influence on particle properties and their interactions with biological systems. This study provides improved understanding of the role of mixed protein coronas from two different environment in particle–cell interactions, which is helpful for the design of particle systems to overcome the biological barriers present in multiple environments and achieve promising biological outcomes.

5.6 Supporting Information

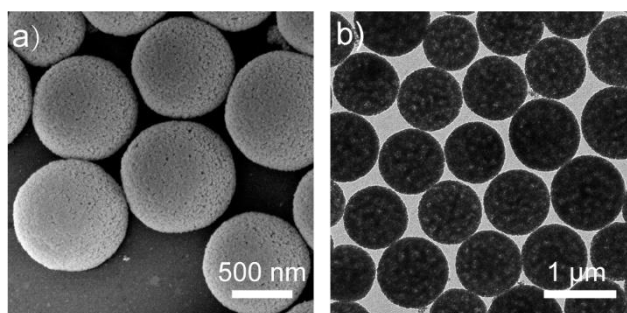


Figure S5.1. (a) SEM and (b) TEM images of MS particles.

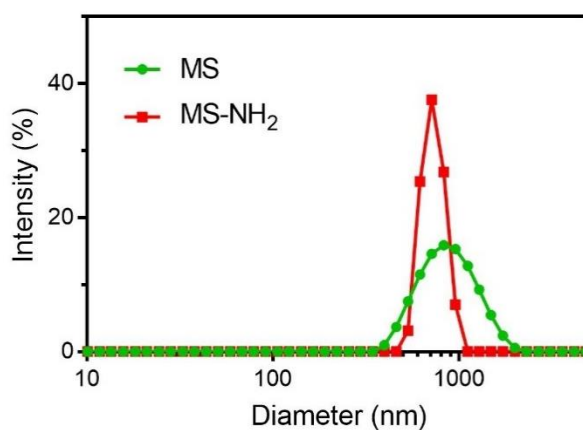


Figure S5.2. Size distribution of MS particles with or without amine group modification (denoted as MS-NH₂ and MS, respectively) measured by dynamic light scattering (DLS).

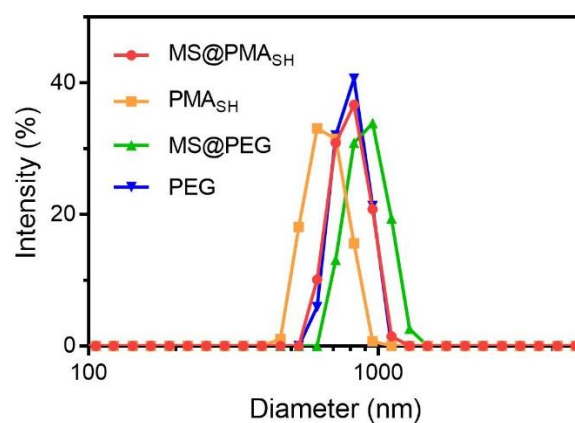


Figure S5.3. Size distribution of MS@PMA_{SH}, PMA_{SH}, MS@PEG and PEG particles measured by dynamic light scattering (DLS).

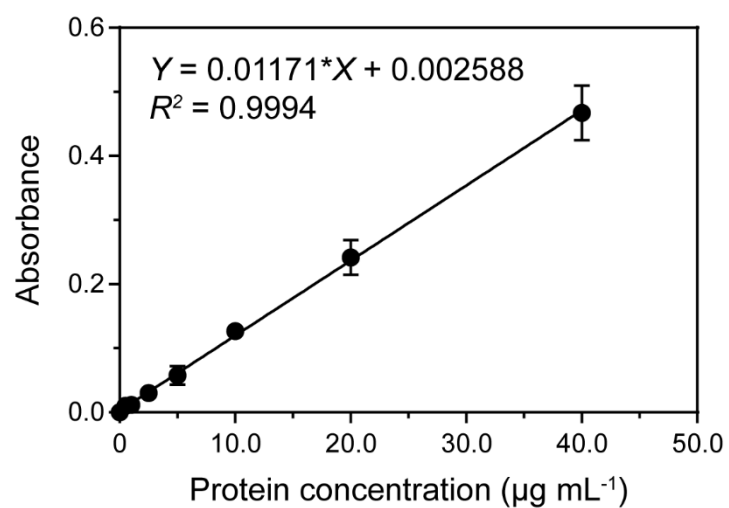


Figure S5.4. Bovine serum albumin (BSA) standard curve produced using a Micro BCA protein assay. Data represent the mean value $\pm$ standard deviation ($n = 3$).

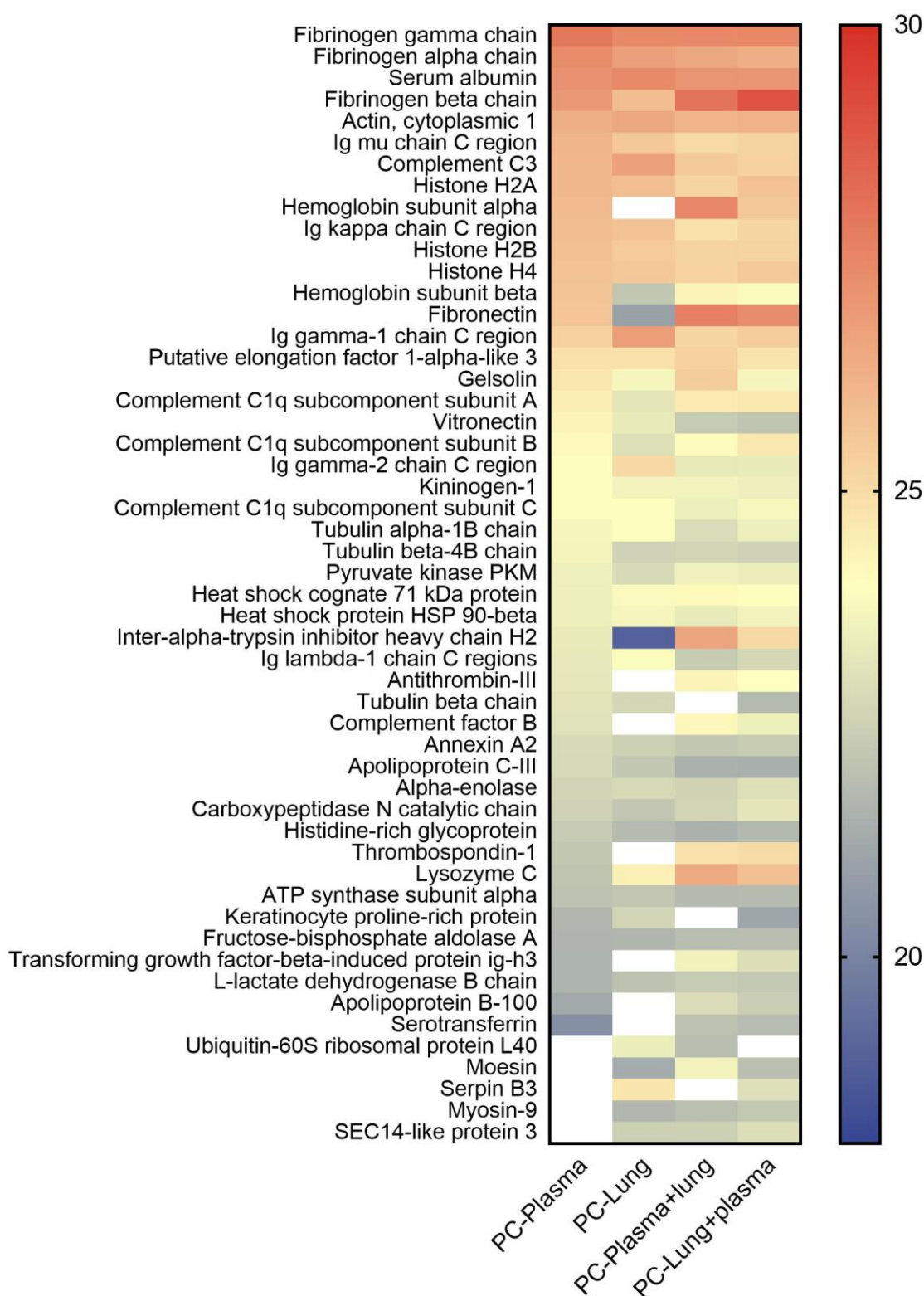


Figure S5.5. Heatmap of identified proteins from plasma+lung protein corona or lung+plasma protein corona on MS@PMA_{SH} particles. Data represent the mean value from at least two replicates. Blank cells refer to absence of proteins.

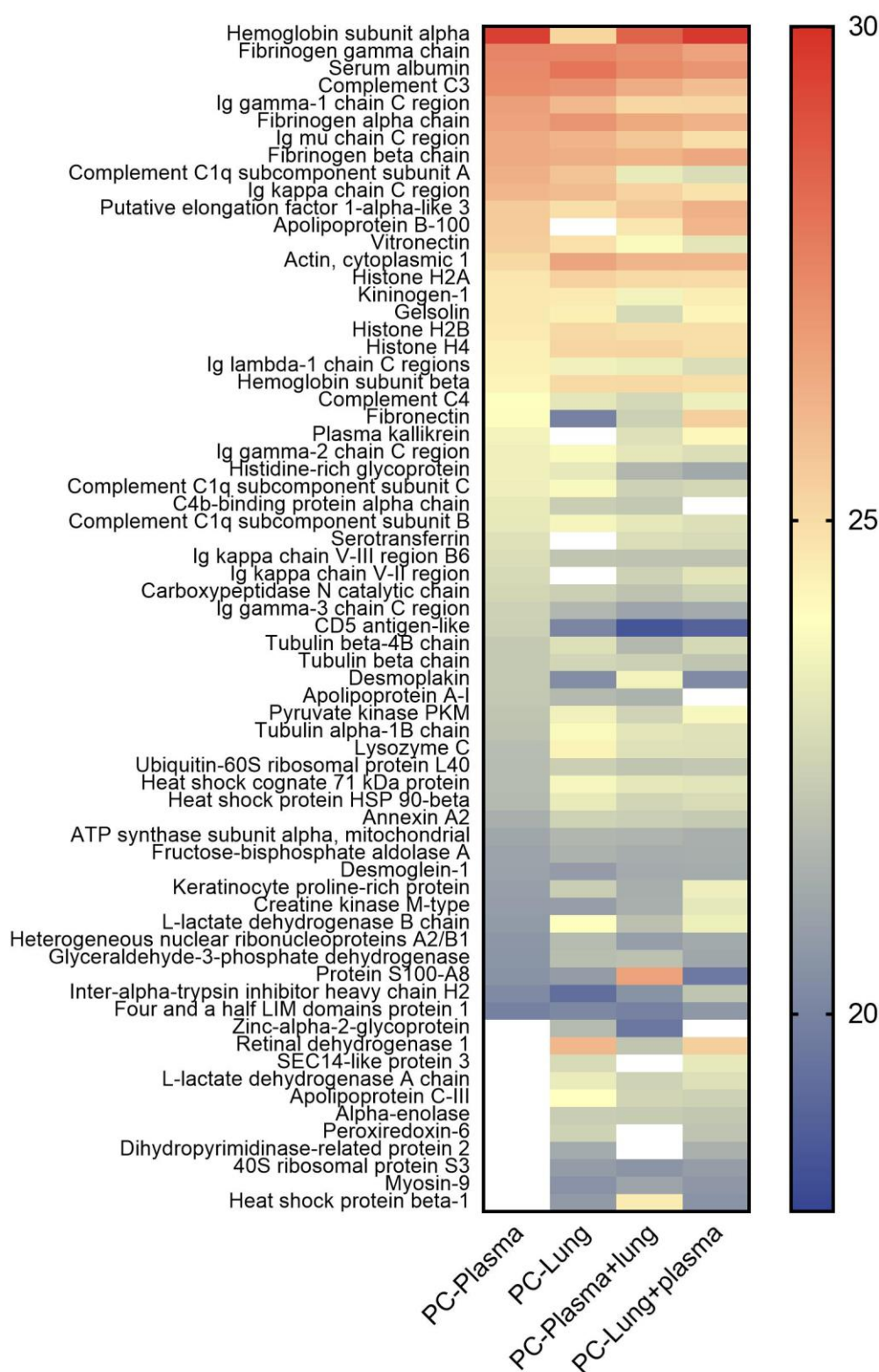


Figure S5.6. Heatmap of identified proteins from plasma+lung protein corona or lung+plasma protein corona on MS@PEG particles. Data represent the mean value from at least two replicates. Blank cells refer to absence of proteins.

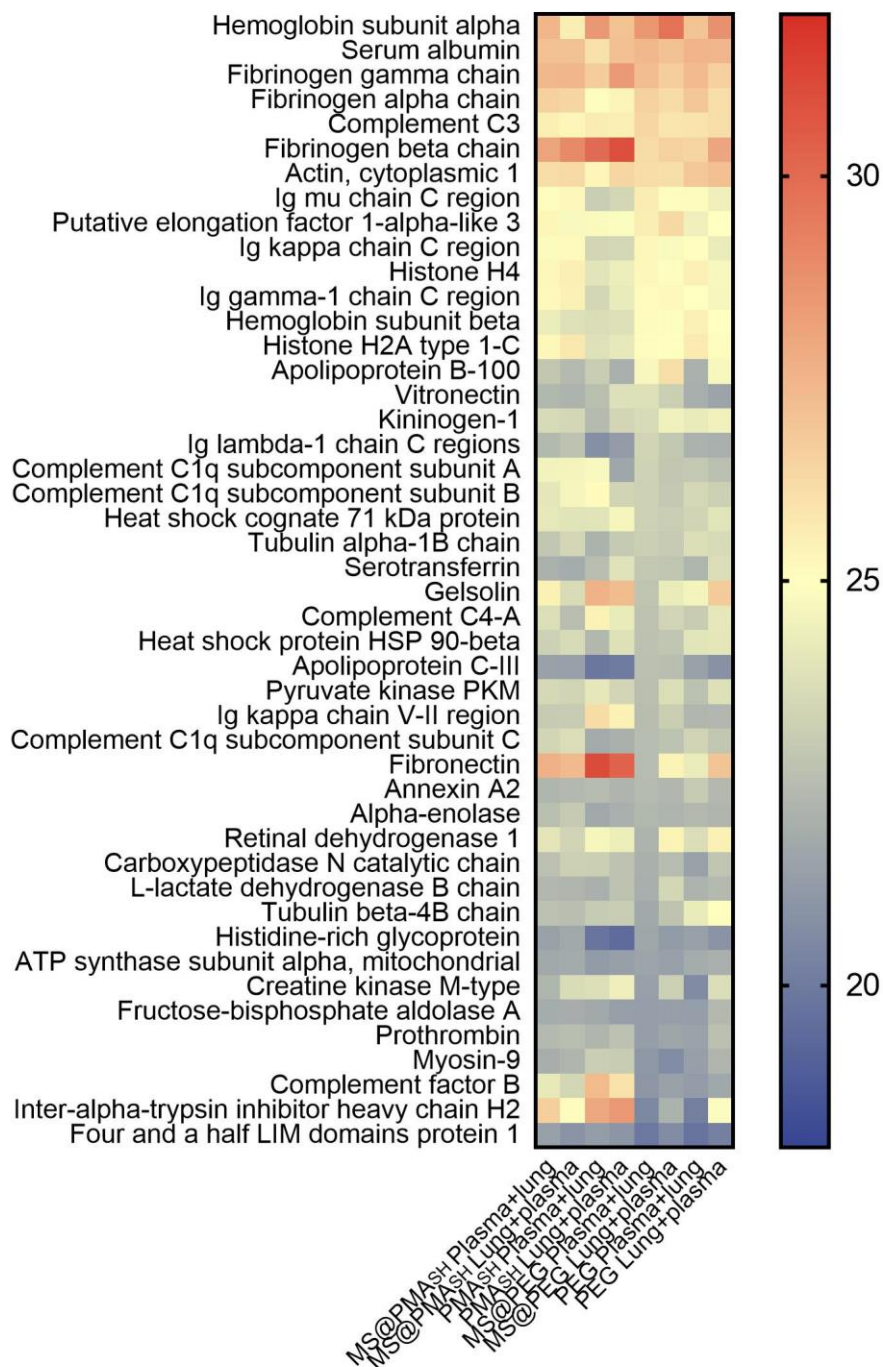


Figure S5.7. Heatmap of identified proteins from plasma+lung protein corona or lung+plasma protein corona on MS@PMA_{SH}, PMA_{SH}, MS@PEG or PEG particles. Data represent the mean value from at least two replicates.

5.7 References

1. Walkey, C. D.; Chan, W. C., Understanding and controlling the interaction of nanomaterials with proteins in a physiological environment. *Chem Soc Rev* **2012**, *41* (7), 2780-2799.
2. Ke, P. C.; Lin, S.; Parak, W. J.; Davis, T. P.; Caruso, F., A Decade of the Protein Corona. *ACS Nano* **2017**, *11* (12), 11773-11776.
3. Digiacomo, L.; Pozzi, D.; Palchetti, S.; Zingoni, A.; Caracciolo, G., Impact of the protein corona on nanomaterial immune response and targeting ability. *Wiley Interdiscip Rev Nanomed Nanobiotechnol* **2020**, e1615.
4. Monopoli, M. P.; Aberg, C.; Salvati, A.; Dawson, K. A., Biomolecular coronas provide the biological identity of nanosized materials. *Nat Nanotechnol* **2012**, *7* (12), 779-86.
5. Jiang, Y.; Huo, S.; Mizuhara, T.; Das, R.; Lee, Y. W.; Hou, S.; Moyano, D. F.; Duncan, B.; Liang, X. J.; Rotello, V. M., The Interplay of Size and Surface Functionality on the Cellular Uptake of Sub-10 nm Gold Nanoparticles. *ACS Nano* **2015**, *9* (10), 9986-93.
6. Casals, E.; Pfaller, T.; Duschl, A.; Oostingh, G. J.; Puentes, V., Time evolution of the nanoparticle protein corona. *ACS Nano* **2010**, *4* (7), 3623-32.
7. Lundqvist, M.; Stigler, J.; Cedervall, T.; Berggard, T.; Flanagan, M. B.; Lynch, I.; Elia, G.; Dawson, K., The evolution of the protein corona around nanoparticles: a test study. *ACS Nano* **2011**, *5* (9), 7503-9.
8. Raesch, S. S.; Tenzer, S.; Storck, W.; Rurainski, A.; Selzer, D.; Ruge, C. A.; Perez-Gil, J.; Schaefer, U. F.; Lehr, C. M., Proteomic and Lipidomic Analysis of Nanoparticle Corona upon Contact with Lung Surfactant Reveals Differences in Protein, but Not Lipid Composition. *ACS Nano* **2015**, *9* (12), 11872-85.
9. Hidalgo, A.; Cruz, A.; Perez-Gil, J., Pulmonary surfactant and nanocarriers: Toxicity versus combined nanomedical applications. *Biochim Biophys Acta Biomembr* **2017**, *1859* (9 Pt B), 1740-1748.
10. Yan, Y.; Gause, K. T.; Kamphuis, M. M.; Ang, C. S.; O'Brien-Simpson, N. M.; Lenzo, J. C.; Reynolds, E. C.; Nice, E. C.; Caruso, F., Differential roles of the protein corona in the cellular uptake of nanoporous polymer particles by monocyte and macrophage cell lines. *ACS Nano* **2013**, *7* (12), 10960-70.
11. Dai, Q.; Yan, Y.; Ang, C. S.; Kempe, K.; Kamphuis, M. M.; Dodds, S. J.; Caruso, F., Monoclonal antibody-functionalized multilayered particles: targeting cancer cells in the presence of protein coronas. *ACS Nano* **2015**, *9* (3), 2876-85.

12. Dai, Q.; Guo, J.; Yan, Y.; Ang, C. S.; Bertleff-Zieschang, N.; Caruso, F., Cell-Conditioned Protein Coronas on Engineered Particles Influence Immune Responses. *Biomacromolecules* **2017**, *18* (2), 431-439.
13. Ju, Y.; Dai, Q.; Cui, J.; Dai, Y.; Suma, T.; Richardson, J. J.; Caruso, F., Improving Targeting of Metal-Phenolic Capsules by the Presence of Protein Coronas. *ACS Appl Mater Interfaces* **2016**, *8* (35), 22914-22.
14. Weiss, A. C. G.; Kelly, H. G.; Faria, M.; Besford, Q. A.; Wheatley, A. K.; Ang, C. S.; Crampin, E. J.; Caruso, F.; Kent, S. J., Link between Low-Fouling and Stealth: A Whole Blood Biomolecular Corona and Cellular Association Analysis on Nanoengineered Particles. *ACS Nano* **2019**, *13* (5), 4980-4991.
15. Cui, J.; De Rose, R.; Alt, K.; Alcantara, S.; Paterson, B. M.; Liang, K.; Hu, M.; Richardson, J. J.; Yan, Y.; Jeffery, C. M.; Price, R. I.; Peter, K.; Hagemeyer, C. E.; Donnelly, P. S.; Kent, S. J.; Caruso, F., Engineering poly(ethylene glycol) particles for improved biodistribution. *ACS Nano* **2015**, *9* (2), 1571-80.
16. Sun, F.; Xiao, G.; Qu, Z., Murine Bronchoalveolar Lavage. *Bio Protoc* **2017**, *7* (10).
17. Tyanova, S.; Temu, T.; Cox, J., The MaxQuant computational platform for mass spectrometry-based shotgun proteomics. *Nat Protoc* **2016**, *11* (12), 2301-2319.
18. Tyanova, S.; Temu, T.; Sinitcyn, P.; Carlson, A.; Hein, M. Y.; Geiger, T.; Mann, M.; Cox, J., The Perseus computational platform for comprehensive analysis of (prote)omics data. *Nat Methods* **2016**, *13* (9), 731-40.
19. Cui, J.; Yan, Y.; Wang, Y.; Caruso, F., Templated assembly of pH-labile polymer-drug particles for intracellular drug delivery. *Adv Funct Mater* **2012**, *22* (22), 4718-4723.
20. Lesniak, A.; Fenaroli, F.; Monopoli, M. P.; Aberg, C.; Dawson, K. A.; Salvati, A., Effects of the presence or absence of a protein corona on silica nanoparticle uptake and impact on cells. *ACS Nano* **2012**, *6* (7), 5845-57.
21. Lesniak, A.; Salvati, A.; Santos-Martinez, M. J.; Radomski, M. W.; Dawson, K. A.; Aberg, C., Nanoparticle adhesion to the cell membrane and its effect on nanoparticle uptake efficiency. *J Am Chem Soc* **2013**, *135* (4), 1438-44.
22. Torres, A. G.; Gait, M. J., Exploiting cell surface thiols to enhance cellular uptake. *Trends Biotechnol* **2012**, *30* (4), 185-90.
23. Kolate, A.; Baradia, D.; Patil, S.; Vhora, I.; Kore, G.; Misra, A., PEG - a versatile conjugating ligand for drugs and drug delivery systems. *J Control Release* **2014**, *192*, 67-81.

24. Walkey, C. D.; Olsen, J. B.; Guo, H.; Emili, A.; Chan, W. C., Nanoparticle size and surface chemistry determine serum protein adsorption and macrophage uptake. *J Am Chem Soc* **2012**, *134* (4), 2139-47.

Chapter 6

Immobilisation and Intracellular Delivery of Structurally Nanoengineered Antimicrobial Peptide Polymers Using Polyphenol-Based Capsules

Chapter 6. Immobilisation and Intracellular Delivery of Structurally Nanoengineered Antimicrobial Peptide Polymers Using Polyphenol-Based Capsules	175
6.1 Aim.....	178
6.2 Introduction	178
6.3 Experimental Section	180
6.3.1 Materials	180
6.3.2 Synthesis of Structurally Nanoengineered Antimicrobial Peptide Polymers (SNAPPs)	181
6.3.2.1 Synthesis of D,L-Valine N-Carboxyanhydrides (Val NCA) and (Z)-L-Lysine N-Carboxyanhydrides (Lys NCA)	181
6.3.2.2 Synthesis of Poly(Z-L-Lysine- <i>r</i> -D,L-Valine) _{arm} PAMAM-(NH ₂) _{m,core} Star Peptide Polymers	182
6.3.2.3 Deprotection of Star Peptide Polymers.....	182
6.3.2.4 FITC Labelling of SNAPP	183
6.3.3 Synthesis of Calcium Carbonate Particles.....	183
6.3.4 Preparation of SNAPP-Tannic Acid (SNAPP-TA) Capsules.....	183
6.3.5 Preparation of SNAPP-loaded MPN (SNAPP-Fe ^{III} -TA) Capsules	184
6.3.6 Capsule Characterisation	184
6.3.7 Nebulisation of Capsules	185
6.3.8 SNAPP Loading Capacity of SNAPP-TA and SNAPP-Fe ^{III} -TA Capsules.....	185
6.3.9 <i>In Vitro</i> SNAPP Release.....	186
6.3.10 Capsule Permeability Measurements.....	186
6.3.11 Cell Culture.....	186
6.3.12 Cell Association Analysis by Flow Cytometry	187
6.3.13 Cell Association Imaging by Confocal Microscopy.....	187
6.3.14 Cell Internalisation Analysis by Imaging Flow Cytometry.....	187
6.3.15 Cell Viability Analysis by XTT Assay	188
6.3.16 Co-localisation by Confocal Laser Scanning Microscopy	188
6.3.17 <i>In Vitro</i> Antimicrobial Activity	189
6.4 Results and Discussions	189
6.4.1 Preparation and Characterisation of SNAPP-TA and SNAPP-Fe ^{III} -TA Capsules	189
6.4.2 SNAPP Loading and <i>In Vitro</i> Release.....	192

6.4.3 <i>In Vitro</i> Cell Interactions Study	193
6.4.4. Intracellular Delivery	195
6.4.5. <i>In Vitro</i> Antimicrobial Activity	196
6.5 Conclusions	198
6.6 Supporting Information	199
6.7 References	208

6.1 Aim

Polyphenols (e.g., tannic acid) are ubiquitous and naturally occurring compounds capable of forming networks or conjugates with metal ions via coordination bonding and various biomacromolecules, including DNA and proteins, via complex interactions. Polyphenol-based drug delivery systems have developed as promising delivery carriers for biomedical applications due to their distinct properties including pH responsiveness, high bioavailability and biocompatibility. This chapter aims to explore drug encapsulation and intracellular delivery by using promising carriers for the potential applications in pulmonary delivery. Specifically, structurally nanoengineered antimicrobial peptide polymers termed SNAPPs, a new class of synthetic antimicrobial peptide mimics that exhibit excellent antimicrobial properties with negligible toxicity to mammalian cells and no bacterial resistance are investigated. Their immobilisation, *in vitro* time-dependent release, cellular delivery and antimicrobial activity were examined by loading into polyphenol-based delivery systems, with implications for the delivery of therapeutics for lung diseases caused by bacterial infection (e.g., tuberculosis).

6.2 Introduction

With the development of nanoengineering technology, a variety of particle systems with unique properties and functions, including liposomes,¹ polymersomes,² protein–drug conjugates,³ micelles,⁴ dendrimers,⁵ polymer particles⁶ and polymer capsules,⁷ have been developed for drug delivery with the aim of improving therapeutic outcomes.⁸ Compared to conventional delivery of therapeutic formulations, particle-based delivery can protect the loaded drug against enzymatic and hydrolytic degradation or inactivation and achieve controlled drug release over prolonged time, and thereby overcoming a variety of biological barriers and achieving targeting to specific structures or cells by manipulating the physicochemical properties of the particles such as via functionalisation with targeting ligands.⁹⁻¹⁰

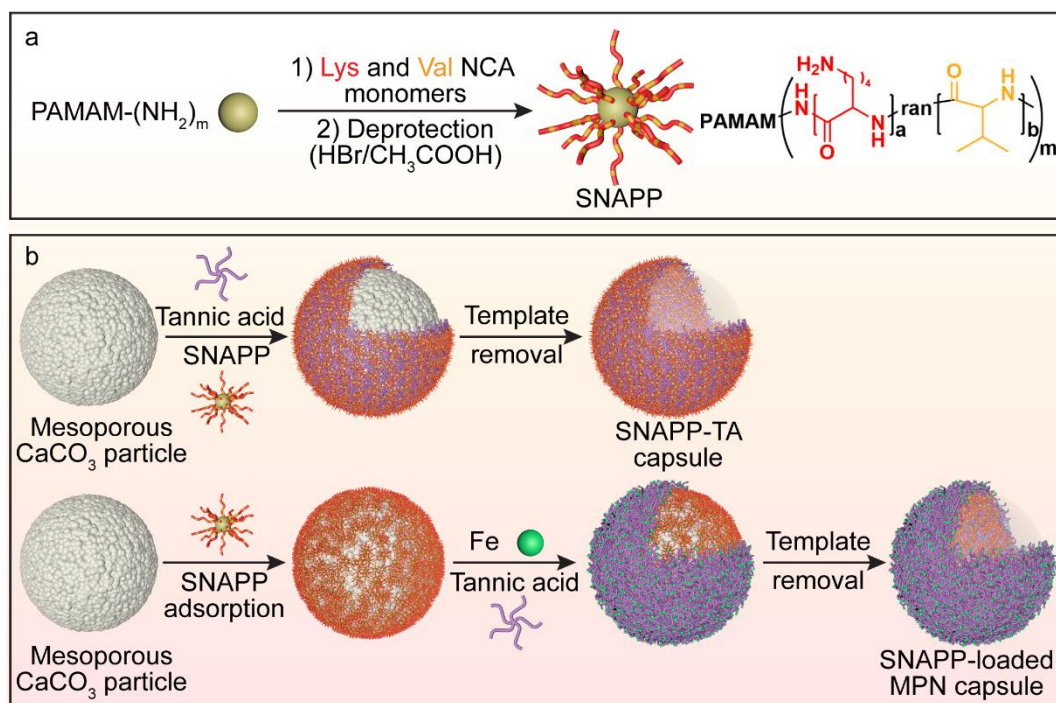
Polyphenols (e.g., tannic acid) are ubiquitous and naturally occurring compounds widely found in plants and capable of forming networks or conjugates with metal ions via coordination bonding and various biomacromolecules, such as DNA and proteins, via complex interactions, including electrostatic interactions, hydrogen bonding and hydrophobic interactions.¹¹⁻¹⁴ Recently, polyphenols have been reported for their application as a multifunctional coating

molecule for various types of materials of different shapes and composition via the formation of metal–phenolic networks (termed MPNs) or protein–phenolic assemblies.^{11, 13, 15} MPN capsules prepared via templated assembly have emerged as a promising drug delivery carrier owing to their distinct properties, including high biocompatibility, pH-dependent degradability and cargo release, and controllable intracellular delivery.¹⁶ In a previous study by Caruso and co-workers, MPN capsules were investigated as pulmonary delivery carriers by assessing the airway distribution of the nebulised capsules using an *in vitro* lung model, capsule biodistribution at both single cell and organ levels, cellular interactions, capsule biocompatibility and resulting inflammatory in the lungs.¹⁷ MPN capsules could be delivered to the deeper regions of the lungs with a priority of association with alveolar macrophages and neutrophils, which are major phagocytic cell subsets present in the lungs, without leading to severe lung inflammation or toxicity,¹⁷ thus demonstrating the potential of the MPN capsules as a highly attractive candidate for pulmonary delivery.

Bacterial infection is a common cause of lung diseases in the lower respiratory regions.¹⁸ Antibiotics are administered to the deep lungs for the therapy of bacterial infections.¹⁹ However, there is likely to be increasing incidence of antibiotic resistance among bacteria with the increase of antibiotic administration, which leads to reduced efficacy.²⁰ Recently, Lam et al. reported a new type of synthetic antimicrobial peptides (AMP) mimics termed structurally nanoengineered antimicrobial peptide polymers (SNAPPs) via the formation of star-shaped polypeptide nanoparticles containing cationic lysine and hydrophobic valine amino acid residues (Scheme 6.1a).²¹ The SNAPPs exhibited excellent antimicrobial properties with low toxicity toward mammalian cells and no bacterial resistance and thus considered to be a promising therapeutic for lung diseases caused by bacterial infection (e.g., tuberculosis).^{21–22}

Herein, two effective methods of SNAPP immobilisation and encapsulation were reported from the assembly of SNAPP-TA and SNAPP-loaded MPN (SNAPP-Fe^{III}-TA) capsules using a sacrificial calcium carbonate particle templating method (Scheme 6.1b). Maximum loading amounts of 0.8 pg capsule⁻¹ and 4.4 pg capsule⁻¹ were achieved for SNAPP-TA capsules and SNAPP-Fe^{III}-TA capsules, respectively. *In vitro* time-dependent SNAPP release at different pH, cellular interactions, intracellular delivery and antimicrobial activity of SNAPP-TA and SNAPP-Fe^{III}-TA capsules were investigated. Both SNAPP-TA and SNAPP-Fe^{III}-TA capsules showed controlled SNAPP release at physiology pH and were effectively taken up by mouse alveolar macrophages with negligible cytotoxicity. The released SNAPP retained its high antimicrobial activity, as evaluated using an *Escherichia coli* (*E. coli*) model. The present study

provides important insights and promising prospects for the delivery of therapeutics for lung diseases caused by bacterial infection (e.g., tuberculosis) by using polyphenol-based capsules.



Scheme 6.1. (a) Schematic of the synthesis of a SNAPP via ring-opening polymerisation (ROP), resulting in the formation of star-shaped polypeptide nanoparticles containing cationic lysine and hydrophobic valine amino acid residues. (b) Schematic depicting the immobilisation and encapsulation of SNAPPs by the assembly of SNAPP-TA and SNAPP-loaded MPN (SNAPP- Fe^{III} -TA) capsules on sacrificial calcium carbonate particle templates.

6.3 Experimental Section

6.3.1 Materials

Poly(sodium 4-styrenesulfonate) (PSS, 70 kDa), calcium nitrate tetrahydrate ($\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$), sodium carbonate (Na_2CO_3), iron(III) chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), tannic acid (TA), 3-(N-morpholino)propanesulfonic acid (MOPS), ethylenediaminetetraacetic acid (EDTA), Dulbecco phosphate-buffered saline (DPBS), glycine hydrochloride, sodium acetate, sodium phosphate monobasic monohydrate, sodium phosphate dibasic, sodium borohydride, generation 2 polyamidoamine dendrimer (G2 PAMAM), bis(trichloromethyl)carbonate

(triphosgene), trifluoroacetic acid (TFA), hydrobromic acid (HBr) (33% in acetic acid), anhydrous *n*-pentane, fluorescein isothiocyanate (FITC), paraformaldehyde, phenazine methosulfate (PMS), anhydrous dimethyl sulfoxide (DMSO), *N,N*-dimethylformamide (DMF), 2-mercaptoethanol, fluorescein isothiocyanate-dextran (FITC-dextran; 3–5, 20, 59–77, 250, 500 and 2000 kDa) and Mueller–Hinton broth (MHB) were purchased from Sigma-Aldrich (Australia). Dulbecco's modified Eagle's medium (DMEM), RPMI-1640 media, fetal bovine serum (FBS), wheat germ agglutinin Alexa Fluor 594 conjugate (WGA-594), Hoechst 33342 and LysoTracker Red DND-99 were obtained from Life Technologies (Australia). 2,3-Bis(2-methoxy-4-nitro-5-sulphophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT) was obtained from Invitrogen (Australia). H-L-Lys(Z)-OH (>99%) was obtained from Mimotopes (Australia). D,L-Valine (>99%) was purchased from Acros Organics (Australia). Generation 0 polyamidoamine dendrimer (G0 PAMAM) was obtained from Dendritech Inc. (USA). Sodium bicarbonate (NaHCO₃), tetrahydrofuran (THF), diethyl ether, ethyl acetate (EtOAc) and *n*-butyl alcohol were obtained from Chem-Supply (Australia).

High-purity water with a resistivity greater than 18.2 MΩ cm, which was used in all experiments, was obtained from a three-stage Millipore Milli-Q plus 185 purification system (Millipore Corporation).

6.3.2 Synthesis of Structurally Nanoengineered Antimicrobial Peptide Polymers (SNAPPs)

6.3.2.1 Synthesis of D,L-Valine N-Carboxyanhydrides (Val NCA) and (Z)-L-Lysine N-Carboxyanhydrides (Lys NCA)

Val NCA and Lys NCA were synthesised using a previously reported method.²² Briefly, 2 g (7.14 mmol) of dried H-L-Lys(Z)-OH or 2 g (17.0 mmol) of D,L-valine was dissolved in 50 mL of anhydrous THF in an over-dried three-neck round bottomed flask under argon, followed by the addition of triphosgene (for Lys NCA: 0.85 g, 2.86 mmol, 1.2 equiv. phosgene; for Val NCA: 2.0 g, 6.74 mmol, 1.2 equiv. phosgene) to the suspension. The mixture was refluxed at 65 °C for 2 h with constant stirring. The clear solution was sparged with argon for 45 min resulting in the formation of a solid after cooling to room temperature, and then resuspended in 50 mL of anhydrous EtOAc. The suspension was placed in a separating funnel where the crude NCA solution was removed by gently washing with 50 mL of chilled saturated brine solution and 50 mL of 0.5% w/v NaHCO₃ solution. The organic phase was dried with MgSO₄

followed by filtering and concentrating into an oil under low heat, and then recrystallised twice from anhydrous EtOAc and anhydrous *n*-pentane. The as-obtained crystals were further filtered and washed with dry *n*-pentane, and subsequently redissolved in a small volume of EtOAc, followed by filtering, precipitation, rinsing with dry *n*-pentane, and finally drying at ambient temperature to obtain a white powder of Val NCA or Lys NCA (yield ~80%). Lys NCA – ^1H NMR (400 MHz, CDCl_3 , δ_{H}): 1.40–1.60 (m, 4H, $\text{NH-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-}$), 1.81–1.94 (m, 2H, $\text{NH-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-}$), 3.18 (m, 2H, $\text{NH-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-}$), 4.25 (t, 1H, $J = 4.0$ Hz, CHN), 4.97 (s, 1H, side chain NH), 5.09 (s, 2H, $\text{CH}_2\text{-ArH}$), 7.04 (s, 1H, ring NH), 7.3–7.4 (m, 5H, ArH). mp 80–82 °C (lit.²³ mp 80 °C). Val NCA – ^1H NMR (400 MHz, CDCl_3 , δ_{H}): 1.02 (d, 3H, $J = 8.0$ Hz, CH_3), 1.08 (d, 3H, $J = 8.0$ Hz, CH_3), 2.25 (m, 1H, $\text{CH}(\text{CH}_3)_2$), 4.22 (d, 1H, $J = 4.0$ Hz, CH-NH), 6.95 (s, 1H, CO-NH). mp 99–100 °C (lit.²⁴ mp 99 °C).

6.3.2.2 Synthesis of Poly(Z-L-Lysine-*r*-D,L-Valine)_{arm}PAMAM-(NH₂)_{*m*,core} Star Peptide Polymers

Lys NCA and Val NCA at a molar ratio of 2:1 were dissolved in anhydrous DMF to a total NCA concentration of ~55 mg mL⁻¹. The solution was mixed with PAMAM-(NH₂)_{*m*} (dried) which was completely dissolved in anhydrous DMSO in a volume corresponding to 10% v/v of total reaction volume under N₂. The reaction tube containing the mixture was incubated in ice with constant stirring for 24 h in a nitrogen atmosphere with a bleed for CO₂ removal. *n*-Butyl alcohol (0.86 $\mu\text{L mg}^{-1}$ of NCA_{total}) was then added to the mixture and stirred for a further 1 h to quench the remaining NCA monomer. The resulting mixture was concentrated under vacuum followed by precipitation in diethyl ether. The precipitate was then washed extensively with ether and finally dried in a vacuum to obtain poly(Z-L-Lysine-*r*-D,L-Valine)_{arm}PAMAM-(NH₂)_{*m*,core} star peptide polymers as a white solid. The average yield was ~60%.

6.3.2.3 Deprotection of Star Peptide Polymers

The star peptide polymers synthesised in Section 6.3.2.2 was completely dissolved in TFA (200 mg mL⁻¹) followed by the addition of 33% HBr in acetic acid (10 mL g⁻¹ peptide polymers). After stirring for 2 h at room temperature, the mixture was allowed to precipitate in diethyl ether and subsequently washed with ether twice followed by drying under vacuum. The resulting solid was redissolved in minimal Milli-Q water and thoroughly dialysed against Milli-

Q water in a 3.5 kDa dialysis tubing. The dialysed solution was lyophilised to obtain the deprotected SNAPP as a dried white solid. ^1H NMR (400 MHz, D_2O , δ_{H}): 0.9 (s, $2(\text{CH})_3$), 1.3–1.9 (m, $\text{NH}-\text{CH}_2-\text{CH}_2\text{CH}_2-\text{CH}_2-$), 2.0 (br s, $\text{CH}-\text{NH}$ valine), 3.0 (s, $\text{NH}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-$), 4.0–4.15 (s, $\text{CH}-\text{NH}$ backbone valine), 4.2–4.4 (s, $\text{CH}-\text{NH}$ backbone lysine).

6.3.2.4 FITC Labelling of SNAPP

SNAPP was dissolved in sodium phosphate buffer (100 mM, pH 8.0) to achieve a concentration of 2.5 mg mL^{-1} , to which FITC dissolved in DMSO (10 mg mL^{-1}) was added ($20 \text{ }\mu\text{L mg}^{-1}$ of SNAPP). The mixture was stirred for 90 min at room temperature. To remove unreacted dye, the sample was extensively dialysed in a 3.5 kDa dialysis tubing against Milli-Q water. The filtrate was lyophilised to afford the FITC-labelled SNAPP.

6.3.3 Synthesis of Calcium Carbonate Particles

CaCO_3 particles with an average diameter of $1.8 \pm 0.1 \text{ }\mu\text{m}$ were synthesised by a fast precipitation method in the presence of PSS, followed by removal of organic material by calcination.¹⁷ To prepare PSS-stabilised CaCO_3 particles, 5 mL of $\text{Ca}(\text{NO}_3)_2$ solution (1 M), 20 mL of PSS solution (10 mg mL^{-1}) and 175 mL of Milli-Q water were mixed in a 200 mL beaker under mild stirring. Next, 1 mL of Na_2CO_3 solution (1 M), 0.5 mL of PSS solution (10 mg mL^{-1}) and 3.5 mL of Milli-Q water were mixed in a 50 mL tube. These two solutions were rapidly mixed in a 200 mL beaker under vigorous stirring for 1 min. After static incubation for 20 min, the mixed solution was further vigorously stirred for 1 min. The obtained particles were washed three times with Milli-Q water by centrifugation ($2000 g$, 2 min), dried in an oven, and calcined in the presence of air at $450 \text{ }^\circ\text{C}$ for 6.5 h.

6.3.4 Preparation of SNAPP-Tannic Acid (SNAPP-TA) Capsules

For the assembly of SNAPP-TA capsules, $5 \text{ }\mu\text{L}$ of TA solution (40 mg mL^{-1}) was added to 2 mg of CaCO_3 particles that were dispersed in $140 \text{ }\mu\text{L}$ of Milli-Q water. After 15 min of incubation under shaking (1400 rpm), $50 \text{ }\mu\text{L}$ of SNAPP solution (2 mg mL^{-1}) was added to the mixture followed by incubation for 6 h at $37 \text{ }^\circ\text{C}$ with shaking (1400 rpm). The particles were washed three times with Milli-Q water to remove excess TA and SNAPP ($1000 g$, 1.5 min).

The SNAPP-TA capsules were obtained after removal of CaCO_3 templates using EDTA (100 mM, pH 8.0). For capsule imaging and the quantification of the loading and release of SNAPP, FITC-labelled SNAPP was used.

6.3.5 Preparation of SNAPP-loaded MPN (SNAPP- Fe^{III} -TA) Capsules

To assemble SNAPP- Fe^{III} -TA capsules, 20 μL of CaCO_3 particle suspension (100 mg mL^{-1}) was diluted to 100 μL with Milli-Q water and incubated with 150 μL of SNAPP solution (2 mg mL^{-1}) for 2 h under shaking (1400 rpm) to allow the adsorption and infiltration of SNAPP into the CaCO_3 particles. The particles were then washed three times with Milli-Q water to remove free SNAPP. Then, 5 μL of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution (10 mg mL^{-1}) and 5 μL of TA solution (40 mg mL^{-1}) were sequentially added to the SNAPP-loaded CaCO_3 particle suspension to form metal–polyphenol complexes. The mixture was vigorously vortexed for 10 s, after which 500 μL of MOPS buffer (50 mM, pH 8.0) was added to raise the pH. The particles were washed three times with Milli-Q water to remove excess TA and metal (1000 g , 1.5 min). The SNAPP- Fe^{III} -TA capsules were obtained after removal of CaCO_3 templates using EDTA (100 mM, pH 8.0). For the preparation of thick SNAPP- Fe^{III} -TA capsules, a slightly modified protocol was applied: 20 μL of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution (10 mg mL^{-1}), 40 μL of TA solution (40 mg mL^{-1}) and 500 μL of MOPS buffer (50 mM, pH 8.0) were sequentially added to the SNAPP-loaded CaCO_3 particle suspension, followed by vigorous stirring for 15 min. The resultant particles were washed six times with Milli-Q water (1000 g , 1.5 min). The templates were removed following the same method as described above. To enable capsule imaging and quantification of the loading and release of SNAPP, FITC-labelled SNAPP was used.

6.3.6 Capsule Characterisation

Fluorescence microscopy images were taken by using a differential interference contrast (DIC) (U-DICT, Olympus) / inverted fluorescence combined microscope (Olympus IX71) equipped with a 60 $\times$ or 100 $\times$ oil immersion objective. Transmission electron microscopy (TEM) images were taken with an FEI Tecnai TF20 instrument operating at an operation voltage of 120 kV. The TEM samples were prepared by dropping an aqueous capsule suspension onto a plasma-treated formvar-coated copper grid and subsequent drying in air. Atomic force microscopy (AFM) images were acquired using a NanoWizard II AFM instrument (JPK Instruments, Berlin,

Germany). An aqueous suspension of the capsules was placed onto Piranha-precleaned glass slides and then dried in air. *Caution! Piranha solution is extremely corrosive and reacts violently with organic materials. It should be handled with great care.* Scanning electron microscopy (SEM) images were taken with a Philips XL30 instrument at an operation voltage of 15 kV. The SEM samples were prepared by dropping a capsule suspension onto a silicon wafer, followed by sputter-coating with gold. The zeta potential of the capsules was measured by a Zetasizer Nano-ZS instrument (Malvern Instruments, Malvern, UK). Capsule counting was performed by flow cytometry (Apogee Flow, UK). Image analysis was conducted using ImageJ software.

6.3.7 Nebulisation of Capsules

To investigate the amenability of capsules to nebulisation and effect of this process on the capsule properties, an aliquot of 2 mL of SNAPP-TA or SNAPP-Fe^{III}-TA capsule suspension was subjected to nebulisation for 5 min using a PARI air-jet nebuliser. The outlet of the nebuliser was attached to a pipette tip to direct the flow of aerosol into a 1.5 mL Eppendorf tube for the aerosol collection.

6.3.8 SNAPP Loading Capacity of SNAPP-TA and SNAPP-Fe^{III}-TA Capsules

To assess the SNAPP loading capacity of SNAPP-TA and SNAPP-Fe^{III}-TA capsules, FITC-labelled SNAPP solutions of varying concentrations were examined for the assembly of SNAPP-TA and SNAPP-Fe^{III}-TA capsules. The UV absorbance at 498 nm of the diluted SNAPP-TA or SNAPP-Fe^{III}-TA capsule suspensions was measured on an Infinite M200 microplate reader (Tecan, Switzerland), and the concentration of immobilised SNAPP was calculated using a standard curve, which was calibrated by measuring SNAPP solutions with different known concentrations. Capsule counting was performed by flow cytometry. The loading capacity was expressed in picograms of SNAPP per capsule, and the loading efficiency was expressed as a percentage of the amount of immobilised SNAPP compared to the amount of SNAPP in the feed solution used during loading.

6.3.9 *In Vitro* SNAPP Release

In vitro time-dependent release of SNAPP from SNAPP-TA and SNAPP-Fe^{III}-TA capsules was measured as follows: SNAPP-TA or SNAPP-Fe^{III}-TA capsules prepared from FITC-labelled SNAPP were suspended in 150 μ L of glycine hydrochloride buffer (50 mM, pH 3.0), sodium acetate buffer (50 mM, pH 4.5), sodium phosphate buffer (50 mM, pH 7.4) or borate buffer (50 mM, pH 9.0) under shaking (1000 rpm) at 37 °C. At predefined time points, capsules in the buffers were centrifuged (SNAPP-TA: 3000 *g*, 5 min; SNAPP-Fe^{III}-TA: 1000 *g*, 2.5 min) and 100 μ L of supernatant was carefully collected for fluorescence intensity measurements by an Infinite M200 microplate reader (Tecan, Switzerland) at an excitation wavelength of 490 nm and an emission wavelength of 525 nm. The SNAPP concentration in the supernatant was calculated according to the standard curve established from measuring the absorbance of SNAPP solutions at varying known concentrations. The capsule suspensions were topped up with 100 μ L of fresh corresponding buffer solutions to maintain a total volume of 150 μ L for the subsequent release study.

6.3.10 Capsule Permeability Measurements

Capsule permeability was evaluated according to a previously reported method.¹¹ The suspensions of SNAPP-TA or SNAPP-Fe^{III}-TA capsules ($\sim 1 \times 10^7$ capsules mL⁻¹) in different buffers (the same buffers as those in Section 6.3.9 at pH 3.0, 4.5, 7.4 and 9.0) were mixed with an equal volume of FITC-dextran solution (5 mg mL⁻¹) in the corresponding buffers. Confocal microscopy images of the capsules were taken after incubating the capsules with FITC-dextran solutions for 15 min in the dark. The capsules with dark interiors were considered to be impermeable, whereas capsules with interiors of similar fluorescent intensity to the outer environment were considered to be permeable. At least 100 capsules were assessed for each sample.

6.3.11 Cell Culture

Mouse alveolar macrophage MH-S cells (American Type Culture Collection (ATCC), USA) were cultured in complete RPMI-1640 media containing 10% FBS and 0.05 mM 2-mercaptoethanol at 37 °C in 5% CO₂ humidified atmosphere.

6.3.12 Cell Association Analysis by Flow Cytometry

MH-S cells were seeded in a 24-well plate (8×10^4 cells per well) in RPMI-1640 media with 10% (v/v) FBS and 0.05 mM 2-mercaptoethanol and cultured overnight. SNAPP-TA or SNAPP-Fe^{III}-TA capsules were added to the cell culture at a capsule-to-cell ratio of 50:1. After incubation for 8 or 24 h at 37 °C, the cells were washed twice with DPBS and harvested by trypsinisation via centrifugation (100 g, 10 min). The cell pellets were washed with DPBS three times and finally resuspended in 250 µL of DPBS for the cell association analysis by flow cytometry (Apogee Flow). Three independent experiments were performed with at least 8000 cells analysed in each individual experiment.

6.3.13 Cell Association Imaging by Confocal Microscopy

MH-S cells were seeded in a 8-well Lab-Tek chambered coverglass slides (Thermo Fisher Scientific, USA) (4×10^4 cells per well) in RPMI-1640 media with 10% (v/v) FBS and 0.05 mM 2-mercaptoethanol and treated with SNAPP-TA or SNAPP-Fe^{III}-TA capsules at a capsule-to-cell ratio of 50:1. After incubation for 8 or 24 h at 37 °C, the cells were gently washed with DPBS and then fixed with paraformaldehyde (4 % in DPBS) for 15 min at room temperature. The fixed cells were then stained with WGA-594 ($5 \mu\text{g mL}^{-1}$) for 5 min in the dark and Hoechst 33342 ($1 \mu\text{g mL}^{-1}$) for 5 min at room temperature.

Cell association imaging was performed using a laser scanning confocal microscope (Nikon Corporation, Japan) equipped with a Plan Apo λ 60 $\times$ 1.4 NA oil immersion objective and 405, 488, 561 and 640 nm lasers, and analysed by ImageJ software.

6.3.14 Cell Internalisation Analysis by Imaging Flow Cytometry

MH-S cells were seeded in a 6-well plate (2×10^5 cells per well) in RPMI-1640 media with 10% (v/v) FBS and 0.05 mM 2-mercaptoethanol and incubated overnight. SNAPP-TA or SNAPP-Fe^{III}-TA capsules were added to the cell culture at a capsule-to-cell ratio of 50:1. After incubation for 24 h at 37 °C, the cells were washed twice with DPBS and harvested by trypsinisation by centrifugation (100 g, 10 min). The cells were then washed with DPBS three times and fixed with paraformaldehyde (4 % in DPBS) for 15 min at room temperature. The fixed cells were then stained with WGA-594 ($5 \mu\text{g mL}^{-1}$) for 5 min in the dark at room

temperature and finally resuspended in 50 μL of DPBS for cell internalisation analysis by imaging flow cytometry (AMNIS ImageStream®X MarkII, Amnis Corporation, USA). Bright-field and fluorescence images of at least 5000 cells were obtained. The internalisation analysis was processed by Amnis ImageStream IDEAS software.

6.3.15 Cell Viability Analysis by XTT Assay

Cytotoxicity studies were performed via XTT assay. MH-S cells cultured in a 96-well plate (1×10^4 cells per well) were treated with SNAPP-TA or SNAPP-Fe^{III}-TA capsules at a capsule-to-cell ratio of 10:1 or 50:1 for 24 h at 37 °C. After incubation, the cell culture media was replaced with 100 μL of fresh RPMI-1640 media containing XTT and PMS (9 mL of 0.2 mg mL⁻¹ XTT in RPMI-1640 media + 22.5 μL of 0.6 mg mL⁻¹ PMS in DPBS). After further incubation for 4 h, the absorbance of the mixture at 475 nm was measured using an Infinite M200 microplate reader (Tecan, Switzerland). The relative cell viability (%) was determined as a percentage of the absorbance of the treated cells compared to the untreated cells.

6.3.16 Co-localisation by Confocal Laser Scanning Microscopy

MH-S cells were seeded in an 8-well Lab-Tek chambered coverglass slides (Thermo Fisher Scientific, USA) (4×10^4 cells per well) in RPMI-1640 media with 10% (v/v) FBS and 0.05 mM 2-mercaptoethanol and allowed to incubate overnight. SNAPP-TA or SNAPP-Fe^{III}-TA capsules were added to the culture media at a capsule-to-cell ratio of 50:1. After incubation for 24 h at 37 °C, the cells were gently washed three times with DPBS, and then incubated with LysoTracker Red DND-99 which was in a final concentration of 100 nM for 1 h for endo/lysosome staining according to the manufacturer's protocol. Cells were then gently washed twice with DPBS and incubated with Hoechst 33342 (1 $\mu\text{g mL}^{-1}$) for 10 min for nuclei staining. The imaging of live cells were carried out by using a laser scanning confocal microscope (Nikon A1R) equipped with a 60 $\times$ oil immersion objective. Pearson's correlation coefficient (PCC) values and colour scatter plots were analysed by using WCIF ImageJ software. Three independent experiments were performed and the representative images with more than 50 cells were analysed for the calculation of PCC values. The results are presented as mean $\pm$ standard deviation.

6.3.17 *In Vitro* Antimicrobial Activity

To assess the antimicrobial activity of the released SNAPP compared to free SNAPP, *E. coli* was used as a model for the antimicrobial assay based on a previously published methods with slight modifications.²² *E. coli* (ATCC, 14948) was incubated in MHB at 37 °C under orbital shaking (200 rpm) for 5 h to allow bacteria growth. The bacteria were then dispersed in DMEM to achieve a concentration of 2.5×10^6 cells mL⁻¹, and 150 µL of this *E. coli* suspension was added to a 96-well microplate. Then, 150 µL of serial two-fold dilutions of free SNAPP solutions (100–6.25 mg mL⁻¹) or released SNAPP collected from capsule suspensions at pH 7.4 at different time points was added to the microplate, resulting in a mixture (300 µL) of bacteria and SNAPP. A sample of bacteria that was not treated with SNAPP was set as a negative control. After incubation for 90 min at 37 °C and undisturbed, 100 µL of the bacteria and SNAPP mixture was incubated with the same volume of MHB on a microplate. The microplate was placed in an Infinite M200 microplate reader (Tecan, Switzerland) incubating at 37 °C under orbital shaking (180 rpm), and the optical density at 600 nm (OD₆₀₀) of the samples was measured every 20 min for at least 7 h. The OD₆₀₀ was plotted against the time point to generate growth curves of *E. coli* in the presence of SNAPP in different concentrations. The OD₆₀₀ of the samples at the time point at which bacteria exponential growth of the negative control was completed was normalised as a relative percentage compared to the OD₆₀₀ of the negative control, expressed as relative bacterial growth (%). The relative bacterial growth (%) was plotted against SNAPP concentration and fitted into an exponential regression. The minimum inhibitory concentration (MIC), which is the lowest concentration of SNAPP that leads to 99% inhibition of bacterial growth, was determined as the concentration corresponding to 1% relative bacteria growth in the fitted exponential regression. The experiments were repeated three times.

6.4 Results and Discussions

6.4.1 Preparation and Characterisation of SNAPP-TA and SNAPP-Fe^{III}-TA Capsules

SNAPPs were synthesised following a previously reported method via ROP of cationic Lys NCA and hydrophobic Val NCA monomers (Scheme 6.1a).²¹ Initiated by the terminal primary amines from a PAMAM dendritic core, NCA monomers were randomly polymerised for the formation of the star-shaped arms with a theoretical lysine-to-valine molar ratio of 2:1. The

lysine residues were then deprotected by hydrobromic acid, resulting in water-soluble polymers. Variations in the arm number and overall molecular weight were achieved by using PAMAM dendrimer cores of different generations with a specific number of reactive surface sites (Scheme S6.1). Large SNAPP (^LSNAPP) (M_w 54.0 kDa) and small SNAPP (^SSNAPP) (M_w 3.7 kDa) with different structures and molecular weights were obtained from G2 PAMAM and G0 PAMAM, respectively, and exhibit similar surface charge in buffers ranging from pH 3.0 to pH 9.0 (Figure S6.1).

Two methods of SNAPP immobilisation and encapsulation were investigated by the assembly of SNAPP-TA capsules and SNAPP-loaded MPN (SNAPP-Fe^{III}-TA) capsules on sacrificial CaCO₃ particle template (Scheme 6.1b). CaCO₃ particles with a porous structure and an average diameter of $1.8 \pm 0.1 \mu\text{m}$ were prepared by a fast precipitation method in the presence of PSS followed by removal of organic material by calcination and used subsequently as templates (Figure S6.2). For the assembly of SNAPP-TA capsules, TA and SNAPP solutions were successively added to a suspension of CaCO₃ particles followed by incubation for 6 h for the formation of SNAPP-TA assemblies on the CaCO₃ particle surface. SNAPP-TA capsules were obtained following removal of the CaCO₃ template using EDTA. To encapsulate SNAPP in MPN capsules, SNAPP was first deposited onto the porous surface and interior of the CaCO₃ particles by mixing a CaCO₃ particle suspension with SNAPP solution. After extensive washing to remove excess SNAPP, FeCl₃·6H₂O and TA solutions were sequentially added to the suspension of SNAPP-loaded CaCO₃ particles, followed by the addition of MOPS buffer (50 mM, pH 8.0) to raise the pH, resulting in the cross-linking of MPNs via the formation of Fe^{III}-TA complexes. After removal of the CaCO₃ template via incubating in EDTA solution and subsequent washing with water, monodisperse SNAPP-Fe^{III}-TA capsules were obtained.

The morphology and structure of SNAPP-TA and SNAPP-Fe^{III}-TA capsules prepared from FITC-labelled ^LSNAPP were characterised using DIC microscopy, fluorescence microscopy, TEM, SEM and AFM, as shown in Figure 6.1. DIC microscopy (Figure 6.1a1, b1) and fluorescence microscopy (Figure 6.1a2, b2) images indicate that both ^LSNAPP-TA and ^LSNAPP-Fe^{III}-TA capsules were relatively monodisperse and spherical from the homogenous fluorescence detected in aqueous solution. TEM (Figure 6.1a3, b3), SEM (Figure 6.1a4, b4) and AFM (Figure 6.1a5, b5) images show the detailed morphology and structure of ^LSNAPP-TA and ^LSNAPP-Fe^{III}-TA capsules. The ^LSNAPP-TA capsules appeared fully collapsed after air-drying, featuring a shell thickness of $23 \pm 2 \text{ nm}$ and typical folds and creases that were observed for air-dried hollow MPN capsules reported previously.¹⁵ The ^LSNAPP-Fe^{III}-TA

capsules featured a much denser and rigid structure with a thicker capsule shell (39 ± 7 nm) when compared to ^{L} SNAPP-TA capsules and typical Fe^{III} -TA films²⁵ or unloaded hollow MPN capsules¹¹ (10 nm) owing to the deposition of large amounts of SNAPP and Fe^{III} -TA complexes on the highly porous surface of the CaCO_3 particles. The addition of larger amounts of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and TA for a longer incubation period resulted in a rougher particle surface, increased capsule thickness (269 ± 26 nm), and reduced collapse upon air-drying (Figure S6.3).

To demonstrate the potential application of the particles prepared herein in pulmonary delivery, the ^{L} SNAPP-TA and ^{L} SNAPP- Fe^{III} -TA capsules were aerosolised using an air-jet nebuliser, which generated fine inhalable aerosol droplets from a capsule suspension by using compressed air, following the same approach discussed in Chapter 4, Section 4.4.2. To investigate the amenability of the capsules to the nebulisation process and assess the effect of the process on the properties of the capsule, the nebulised capsules were collected for further characterisation by attaching a pipette tip to the outlet of the nebuliser to direct the flow of aerosol into an Eppendorf tube. The ^{L} SNAPP-TA and ^{L} SNAPP- Fe^{III} -TA capsules remained well dispersed and retained their internal fluorescence in aqueous solution with negligible aggregation and minimal leakage of SNAPP after nebulisation (Figure S6.4e, f). The morphology and structure of both types of capsules remained intact, with no damage or alteration observed from the TEM images (Figure S6.4g, h) as a result of nebulisation, indicating that the ^{L} SNAPP-TA and ^{L} SNAPP- Fe^{III} -TA capsules were not affected by the nebulisation process and that SNAPP immobilisation and encapsulation was maintained.

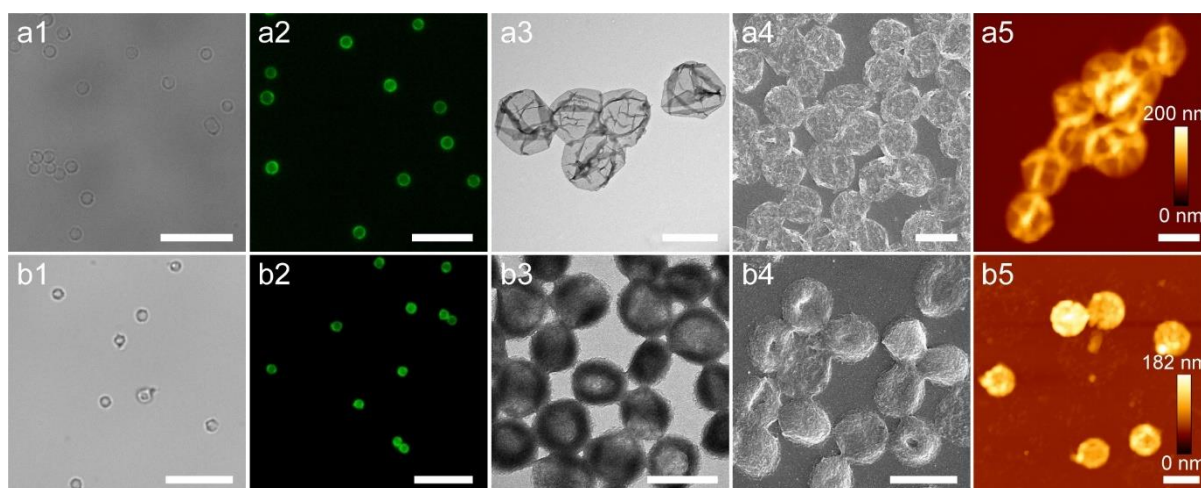


Figure 6.1. DIC microscopy, fluorescence microscopy, TEM, SEM, and AFM images of (a1–a5) ^{L} SNAPP-TA capsules and (b1–b5) ^{L} SNAPP- Fe^{III} -TA capsules. Scale bars are 10 μm in (a1, a2, b1, b2) and 2 μm in (a3–a5 and b3–b5).

6.4.2 SNAPP Loading and *In Vitro* Release

The SNAPP loading capacity of the SNAPP-TA and SNAPP-Fe^{III}-TA capsules was dependent on the SNAPP feed concentration (Figure 6.2a, b and Figure S6.6). In general, the SNAPP loading amount increased with increasing SNAPP concentration, with a maximum ^LSNAPP loading amount of 0.8 pg capsule⁻¹ for the ^LSNAPP-TA capsules and 4.4 pg capsule⁻¹ for the ^LSNAPP-Fe^{III}-TA capsules (Figure 6.2a); however, the loading efficiency decreased with increasing SNAPP concentration (Figure 6.2b). The MPN capsules encapsulated a higher amount of SNAPP (both ^LSNAPP and ^SSNAPP) than the SNAPP-TA capsules at higher SNAPP concentration (>0.4 mg mL⁻¹), probably due to the relatively stronger electrostatic interaction between positively charged SNAPP and the negatively charged CaCO₃ surface, as well as increased likelihood of the deposition of smaller-sized SNAPP onto the porous surface and interior of the CaCO₃ templates when compared with the less positively charged and bigger-sized SNAPP-TA complex (Figure S6.2).

The *in vitro* release of SNAPP from SNAPP-TA and SNAPP-Fe^{III}-TA capsules was assessed at different pH (3.0, 4.5, 7.4 and 9.0) to investigate how the encapsulated drugs can be released under different pH conditions and understand the possible interactions between drugs and carriers. As shown in Figure 6.2c and d, sustained release of ^LSNAPP was achieved at pH 7.4 and pH 9.0 for both ^LSNAPP-TA and ^LSNAPP-Fe^{III}-TA capsules, with a faster release observed at pH 9.0. Although the formation of coordination bonds between polyphenols and metal ions is pH dependent and MPN capsules tend to disassemble at low pH,¹⁵ the presence of SNAPP stabilised the MPN capsules via complex interactions including electrostatic interaction, hydrogen bonding (derived from the lysine parts of SNAPP) and hydrophobic interaction (derived from the valine parts of SNAPP) with TA or MPN. Thus, negligible release was observed at acidic pH (3.0, 4.5) mainly due to the stronger hydrogen bonding and ionic interaction between the lysine parts of SNAPP and the hydroxyl groups of TA at lower pH. When incubated at pH 7.4, ~28% of ^LSNAPP was released from ^LSNAPP-TA capsules after 12 h, which was higher than the amount released (~12%) for ^LSNAPP-Fe^{III}-TA capsules. SNAPP-TA capsules exhibited a faster release of SNAPP and higher overall release percentage over a 168 h period than SNAPP-Fe^{III}-TA capsules probably owing to the exposure of SNAPP on the surface. Coating with a thicker MPN layer did not significantly slow the release of ^LSNAPP (Figure S6.5, and Figure S6.7), as the MPN is highly porous, thus allowing the diffusion of molecules its permeable shell (Figure S6.8).

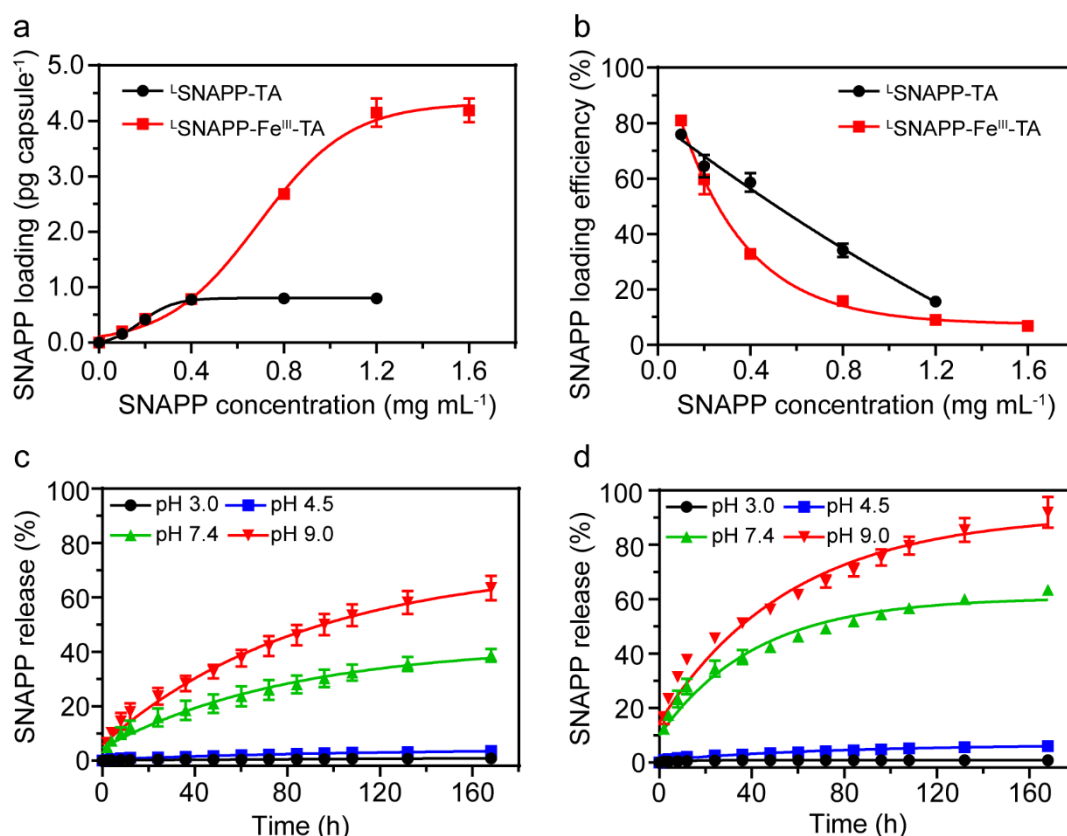


Figure 6.2. (a) Loading amount and (b) loading efficiency of FITC-labelled ^LSNAPP in ^LSNAPP-TA or ^LSNAPP-Fe^{III}-TA capsules as a function of ^LSNAPP loading concentration. *In vitro* time-dependent release of FITC-labelled ^LSNAPP from (c) ^LSNAPP-Fe^{III}-TA capsules or (d) ^LSNAPP-TA capsules at pH 3.0, 4.5, 7.4 and 9.0. Data presented are averages of three replicates, and error bars represent standard deviations.

6.4.3 *In Vitro* Cell Interactions Study

To assess the capsule–cell interactions, mouse alveolar macrophage MH-S cells were treated with SNAPP-TA or SNAPP-Fe^{III}-TA capsules over different incubation periods at 37 °C (Figure 6.3 and Figure S6.12). Both capsule systems minimally influenced cell viability after incubation for 24 h, as evaluated by XTT assay (Figure S6.13). Despite the similar sizes in aqueous solution (Figure 6.1a1, a2, b1, b2) and similar surface charge at physiological pH (^LSNAPP-TA: −28 mV, ^LSNAPP-Fe^{III}-TA: −32 mV, Figure S6.2), ^LSNAPP-TA capsules exhibited significantly higher association with MH-S cells than ^LSNAPP-Fe^{III}-TA capsules after incubation for 8 h (difference ~37%) or 24 h (difference ~52%) (Figure 6.3a), probably due to the thinner shell and resultant higher flexibility and deformability of SNAPP-TA capsules upon contact with the cell membrane, leading to higher cell membrane adhesion.²⁶

The differences in the capsule roughness and surface chemistry (presence of SNAPP on the capsule surface or interior) were also likely to affect cell association.²⁷⁻²⁸

The internalisation of the capsules by the cells was quantified by imaging flow cytometry based on an internalisation factor (IF), which was analysed from the spatial relationship between the fluorescently labelled capsules and the cell membrane. A positive IF value refers to cells containing internalised capsules, whereas a negative IF refers to cells with capsules associated on the cell membrane. As shown in Figure 6.3b and c, both ¹²⁵I-SNAPP-TA or ¹²⁵I-SNAPP-Fe^{III}-TA capsules exhibited >99% internalisation by MH-S cells.

The cell uptake of ¹²⁵I-SNAPP-TA or ¹²⁵I-SNAPP-Fe^{III}-TA capsules was further verified by confocal microscopy. After incubation for 8 h, most internalised ¹²⁵I-SNAPP-Fe^{III}-TA capsules were intact, thus retaining their original spherical shape (Figure 6.3f), whereas some of ¹²⁵I-SNAPP-TA capsules appeared deformed (Figure 6.3e). Increasing the incubation time to 24 h resulted in an increasing amount of deformed and disassembled ¹²⁵I-SNAPP-TA capsules (Figure 6.3g), whereas most ¹²⁵I-SNAPP-Fe^{III}-TA capsules remained intact (Figure 6.3h), indicating that SNAPP-Fe^{III}-TA capsules were more stable in intracellular environment than SNAPP-TA capsules. The intracellular disassembly of SNAPP-TA capsules endows them with potential applications for controllable intracellular drug release.

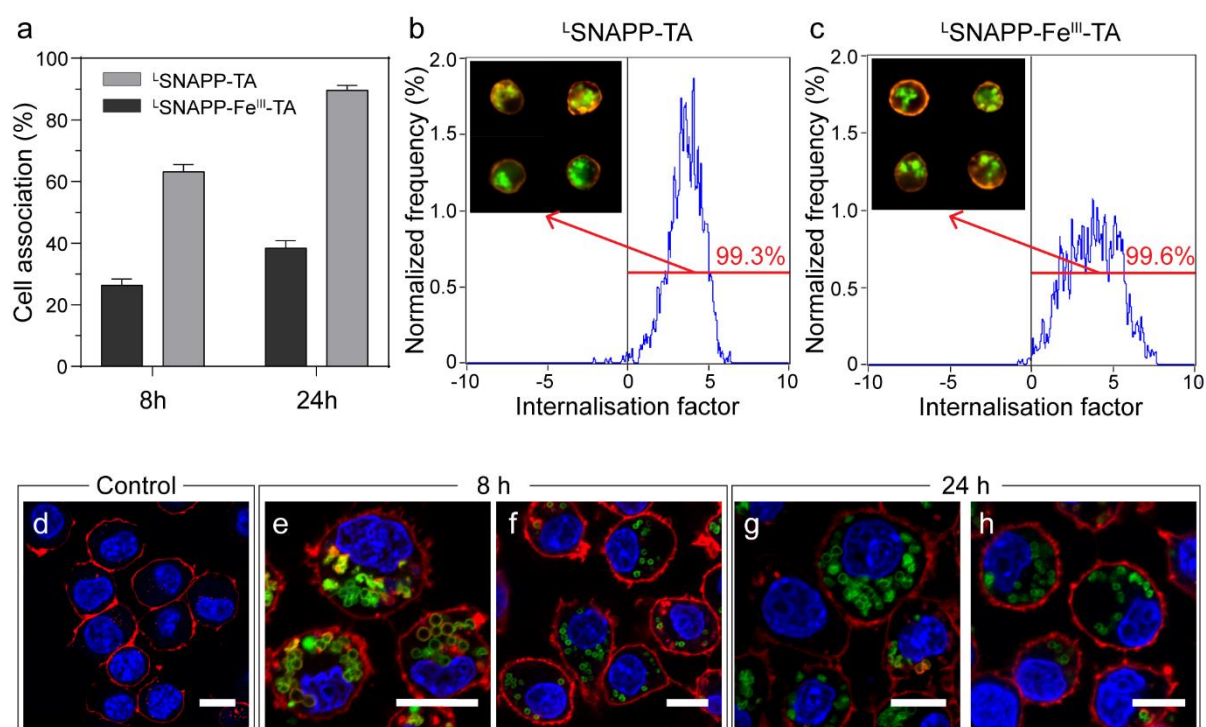


Figure 6.3. *In vitro* cell interactions study. (a) Association of ¹²⁵I-SNAPP-TA or ¹²⁵I-SNAPP-Fe^{III}-TA capsules with MH-S cells following incubation for 8 or 24 h at 37 °C and a capsule-to-cell

ratio of 50:1. Imaging flow cytometry of the quantification of the internalisation of (b) ^{L} SNAPP-TA and (c) ^{L} SNAPP-Fe^{III}-TA capsules in MH-S cells after incubation for 24 h at 37 °C at a capsule-to-cell ratio of 50:1. The capsules were fluorescently labelled by loading with FITC-labelled ^{L} SNAPP (green) and the cell membranes were stained with WGA-594 (orange). The degree of cell internalisation was expressed as an internalisation factor. The insets show the representative images of cells with internalised capsules (positive IF). Confocal microscopy images of the MH-S cells treated in the (d) absence of capsules (control) and (e, g) presence of ^{L} SNAPP-TA or (f, h) ^{L} SNAPP-Fe^{III}-TA capsules for 8 h (e, f) or 24 h (g, h) at 37 °C at a capsule-to-cell ratio of 50:1. The capsules were fluorescently labelled by loading with FITC-labelled ^{L} SNAPP (green). The cell membranes were stained with WGA-594 (red) and the nuclei were stained with Hoechst 33342 (blue). Scale bars are 10 μ m in (d–h).

6.4.4. Intracellular Delivery

To further explore the intracellular and subcellular distribution of SNAPP-TA and SNAPP-Fe^{III}-TA capsules, co-localisation analysis of SNAPP-TA and SNAPP-Fe^{III}-TA capsules with endo/lysosomes in MH-S cells was conducted. The capsules were fluorescently labelled by loading with FITC-labelled ^{L} SNAPP (green). The endo/lysosomes were stained with LysoTracker Red (red) and the nuclei were stained with Hoechst 33342 (blue). As shown in Figure 6.4a, after incubation for 24 h, ^{L} SNAPP-TA capsules exhibited relatively strong co-localisation with endo/lysosomes, indicating that most of ^{L} SNAPP-TA capsules were entrapped in endo/lysosomal compartments where the capsules were likely to be degraded by exposure to a variety of enzymes. In the contrast, ^{L} SNAPP-Fe^{III}-TA capsules displayed a smaller degree of co-localisation with endo/lysosomes, suggesting that ^{L} SNAPP-Fe^{III}-TA capsules tend to escape from endo/lysosomal compartments remaining in the cytoplasm, probably due to the ‘proton-sponge effect’ arising from the buffering capacity of MPNs, which was reported previously.²⁹ The colour scatter plots (Figure 6.4b) and corresponding PCC values (Figure 6.4c) which provide a statistical correlation between the red and green fluorescence signals in the images, reveal the stronger positive correlation and higher degree of co-localisation for ^{L} SNAPP-TA capsules with endo/lysosomes when compared with ^{L} SNAPP-Fe^{III}-TA capsules.

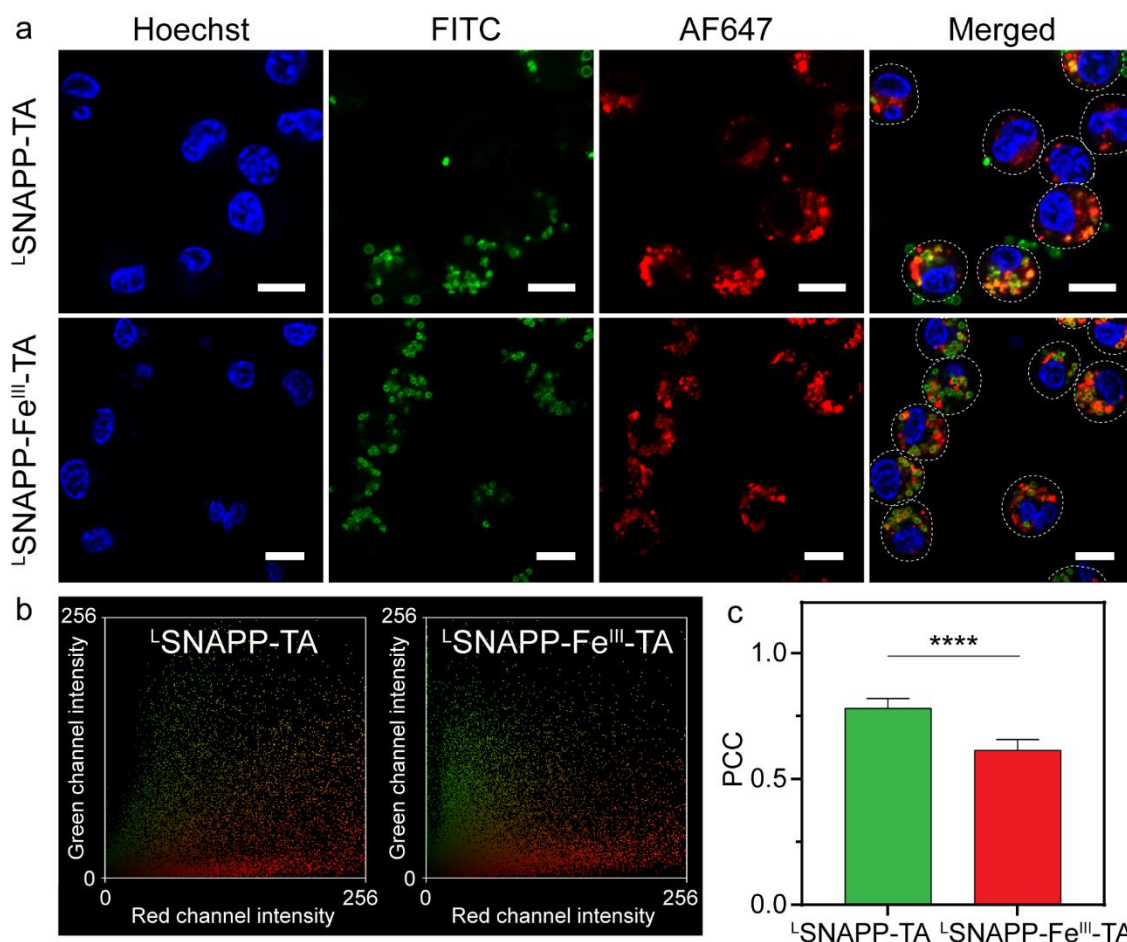


Figure 6.4. (a) Intracellular distribution of ^{L} SNAPP-TA and ^{L} SNAPP-Fe^{III}-TA capsules in MH-S cells after 24 h incubation at 37 °C at a capsule-to-cell ratio of 50:1, as evaluated by confocal microscopy. The capsules were fluorescently labelled by loading with FITC-labelled ^{L} SNAPP (green). The endo/lysosomes were stained with LysoTracker Red (red). The nuclei were stained with Hoechst 33342 (blue). The cellular boundaries were represented by dashed lines according to the corresponding bright-field images. Scale bars are 10 μ m. (b) Representative colour scatter plots of ^{L} SNAPP-TA and ^{L} SNAPP-Fe^{III}-TA capsules vs endo/lysosomes. (c) Corresponding Pearson's correlation coefficient (PCC) values of ^{L} SNAPP-TA and ^{L} SNAPP-Fe^{III}-TA capsules vs endo/lysosomes (mean $\pm$ standard deviation, $n > 50$). **** $p < 0.0001$ from the unpaired t -test.

6.4.5. In Vitro Antimicrobial Activity

The antimicrobial activity of the SNAPP released from SNAPP-TA and SNAPP-Fe^{III}-TA capsules was evaluated by determining the MIC value against a model Gram negative bacteria *E. coli* via a standard antimicrobial assay, which can correlate the increase in turbidity of a

culture with bacterial proliferation.²² The MIC indicates the lowest concentration of an antimicrobial agent that can inhibit bacterial growth without necessarily killing the pathogen, with lower MIC values corresponding to stronger antimicrobial activities.

Free SNAPP solutions of varying concentrations or released SNAPP collected from SNAPP-TA and SNAPP-Fe^{III}-TA capsule suspensions at pH 7.4 at different time points were added to the *E. coli* culture and the bacteria growth curves in the presence of SNAPP at different concentrations were obtained by plotting the OD₆₀₀ of the samples as a function of time (Figure S6.14a, c). The OD₆₀₀ of the samples at the time point at which the bacterial exponential growth of the negative control was complete was normalised as a relative percentage compared to the OD₆₀₀ of the negative control, expressed as relative bacterial growth (%). The relative bacterial growth (%) was plotted against SNAPP concentration and fitted into an exponential regression (Figure S6.14b, d, and Figure 6.5). The MIC was determined as the concentration corresponding to 1% relative bacteria growth in the exponential regression.

As shown in Figure S6.14, free ^LSNAPP displayed excellent antimicrobial activity against *E. coli* with an MIC of 16.1 µg mL⁻¹, whereas free ^SSNAPP showed a much weaker activity (MIC of 134.6 µg mL⁻¹), thereby requiring a significantly higher concentration to trigger the similar antimicrobial effect. This result can be explained by the cooperative mechanism of antimicrobial polypeptides reported previously, where in more substituted SNAPPs (i.e., with more polypeptide arms) can facilitate the cooperative action of neighbouring polypeptide arms and thus display antimicrobial activity at relatively lower concentrations.²¹ The ^LSNAPP released from ^LSNAPP-TA and ^LSNAPP-Fe^{III}-TA capsules retained antimicrobial activity against *E. coli* with an MIC of 30.5 µg mL⁻¹ for ^LSNAPP-TA capsules and 29.3 µg mL⁻¹ for ^LSNAPP-Fe^{III}-TA capsules, which is higher than that achieved by free ^LSNAPP (MIC of 16.1 µg mL⁻¹) probably due to the reduced binding of SNAPP with bacterial outer membrane via electrostatic interactions resulting from the adhesion of TA and neutralised charge.

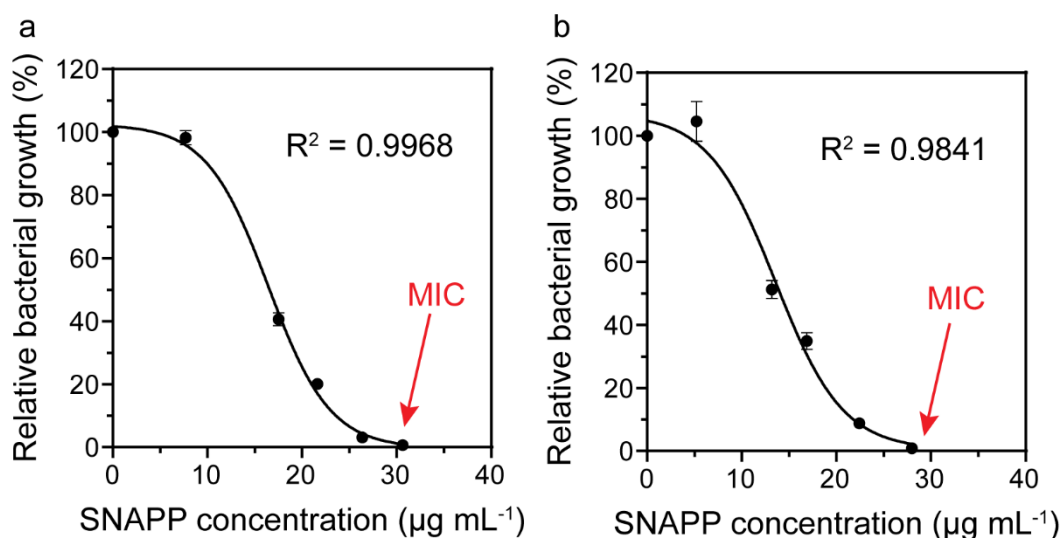


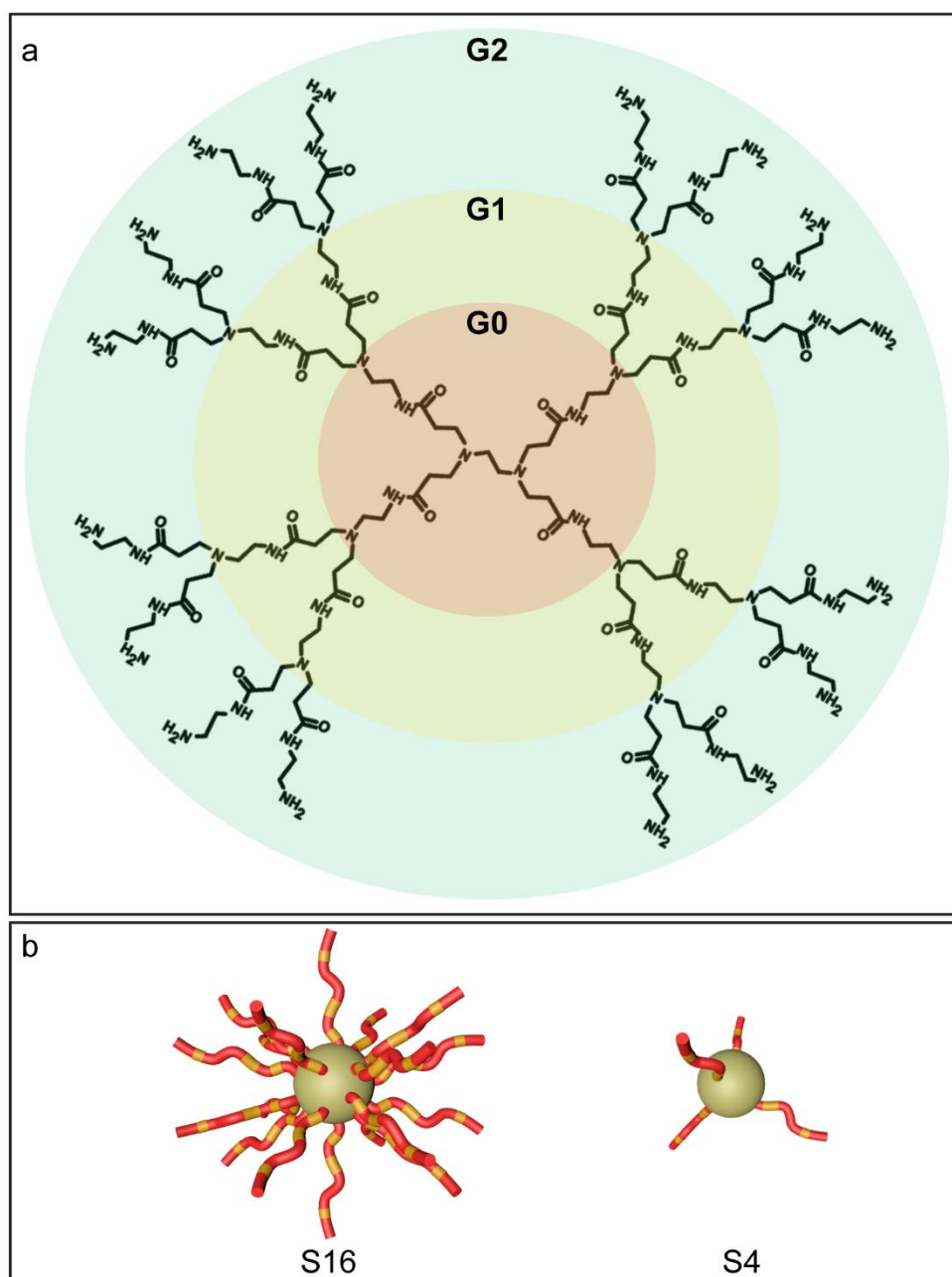
Figure 6.5. Relative growth (%) of *E. coli* as a function of the concentration of $^{\text{L}}\text{SNAPP}$ released from (a) $^{\text{L}}\text{SNAPP-TA}$ or (b) $^{\text{L}}\text{SNAPP-Fe}^{\text{III}}\text{-TA}$ capsules. The MIC, defined as the lowest concentration of SNAPP that inhibited 99% of bacterial growth (i.e., the X value at $Y = 1\%$), was determined from the fitted exponential regression. Data represent the mean value $\pm$ standard deviation ($n = 3$).

6.5 Conclusions

This chapter demonstrated the potential of drug encapsulation and intracellular delivery by using polyphenol-based capsules for the subsequent application in pulmonary delivery. SNAPPs, a new class of synthetic antimicrobial peptide mimics, were immobilised and encapsulated via the assembly of SNAPP-TA and SNAPP- $\text{Fe}^{\text{III}}\text{-TA}$ capsules with maximum loading capacities of $0.8 \text{ pg capsule}^{-1}$ and $4.4 \text{ pg capsule}^{-1}$, respectively. Sustained release of $^{\text{L}}\text{SNAPP}$ was achieved at pH 7.4 and pH 9.0 for both $^{\text{L}}\text{SNAPP-TA}$ and $^{\text{L}}\text{SNAPP-Fe}^{\text{III}}\text{-TA}$ capsules, with a faster release observed at pH 9.0. SNAPP-TA capsules exhibited a faster release of SNAPP and higher overall release percentage over a long period than SNAPP- $\text{Fe}^{\text{III}}\text{-TA}$ capsules probably owing to the exposure of SNAPP on the surface. Both SNAPP-TA and SNAPP- $\text{Fe}^{\text{III}}\text{-TA}$ capsules exhibited negligible cytotoxicity towards mouse alveolar macrophage MH-S cells and could be effectively taken up by the cells. SNAPP-TA capsules showed higher cell association than SNAPP- $\text{Fe}^{\text{III}}\text{-TA}$ capsules and exhibited relatively stronger co-localisation with endo/lysosomes, thus highlighting the potential of intracellular disassembly and degradation. The released $^{\text{L}}\text{SNAPP}$ from both $^{\text{L}}\text{SNAPP-TA}$ and $^{\text{L}}\text{SNAPP-Fe}^{\text{III}}\text{-TA}$ capsules retained high antimicrobial activity, as evaluated using an *E. coli* model. This

study provides important insights into the drug encapsulation and cellular delivery using the presently engineered polyphenol-based delivery carriers and their promising prospects for the delivery of therapeutics for lung diseases caused by bacterial infection (e.g., tuberculosis).

6.6 Supporting Information



Scheme S6.1. Schematic illustrations of (a) the structure of a generation 2 PAMAM dendrimer (G0 = generation 0; G1 = generation 1; G2 = generation 2) and (b) the structure of ^LSNAPP (M_w 54.0 kDa) and ^SSNAPP (M_w 3.7 kDa).

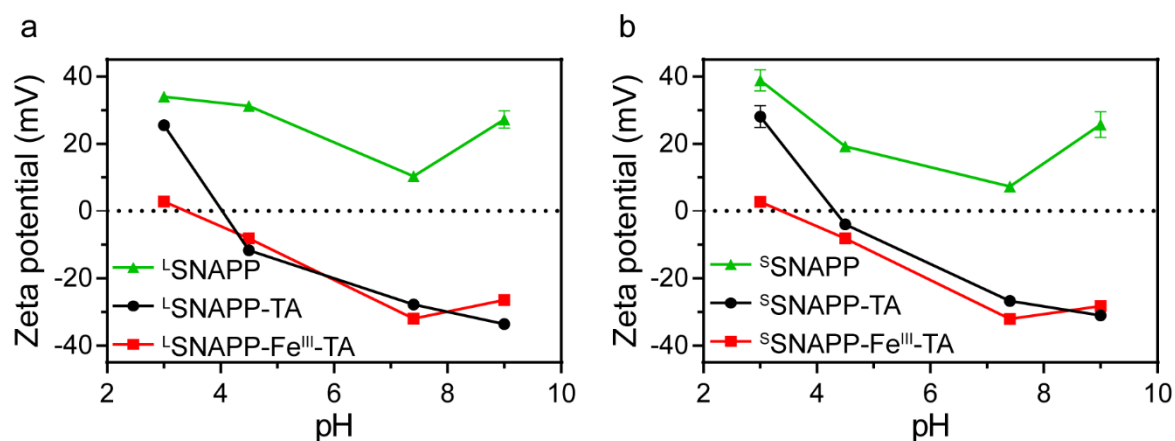


Figure S6.1. Zeta potential of SNAPP (^LSNAPP and ^SSNAPP), SNAPP-TA and SNAPP-Fe^{III}-TA capsules prepared from ^LSNAPP and ^SSNAPP at different pH (mean $\pm$ standard deviation, $n = 3$).

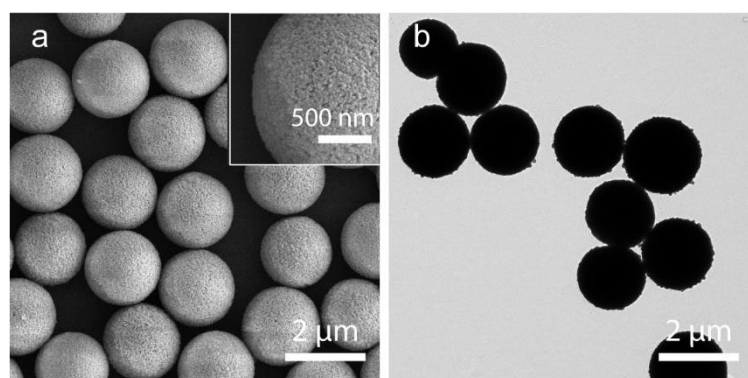


Figure S6.2. (a) SEM and (b) TEM images of CaCO₃ particles showing their porous surface structure.

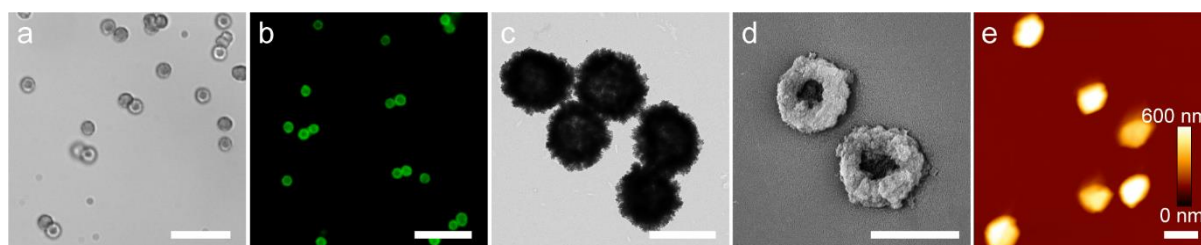


Figure S6.3. (a) DIC microscopy, (b) fluorescence microscopy, (c) TEM, (d) SEM and (e) AFM images of thick ^LSNAPP-Fe^{III}-TA capsules (thickness of 269 ± 26 nm). Scale bars are 10 μ m in (a, b) and 2 μ m in (c–e).

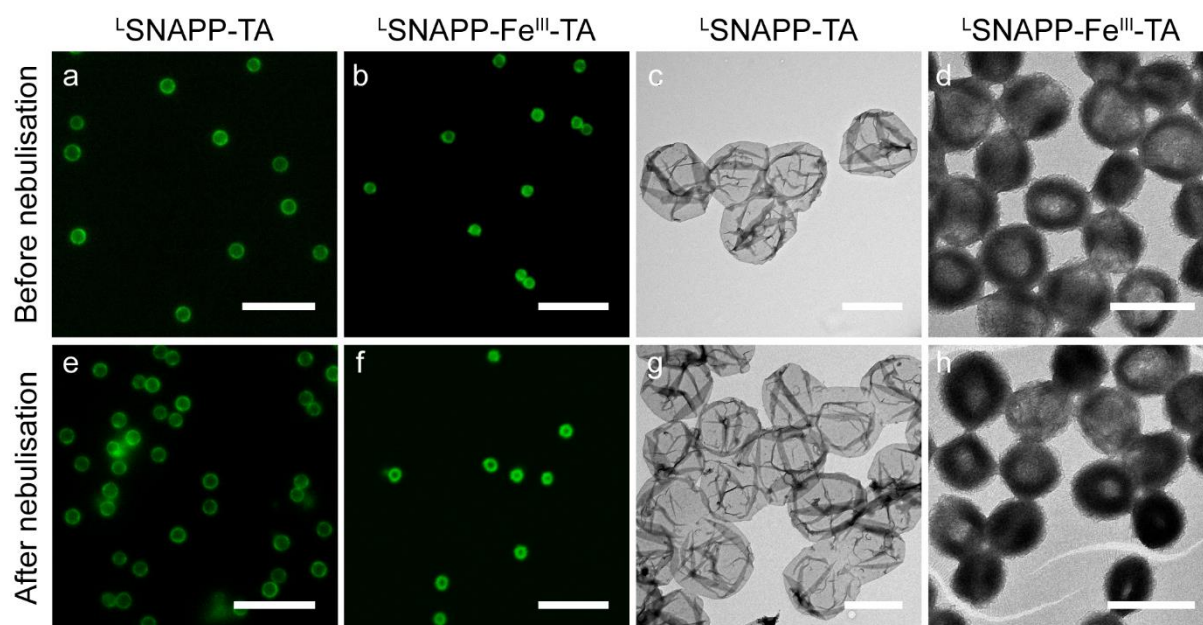


Figure S6.4. (a, b) Fluorescence microscopy and (c, d) TEM images of ^LSNAPP-TA (a, c) and ^LSNAPP-Fe^{III}-TA (b, d) before nebulisation. (e, f) Fluorescence microscopy and (g, h) TEM images of ^LSNAPP-TA (e, g) and ^LSNAPP-Fe^{III}-TA (f, h) after nebulisation. Scale bars are 10 μm in (a, b, e, f), and 2 μm in (c, d, g, h).

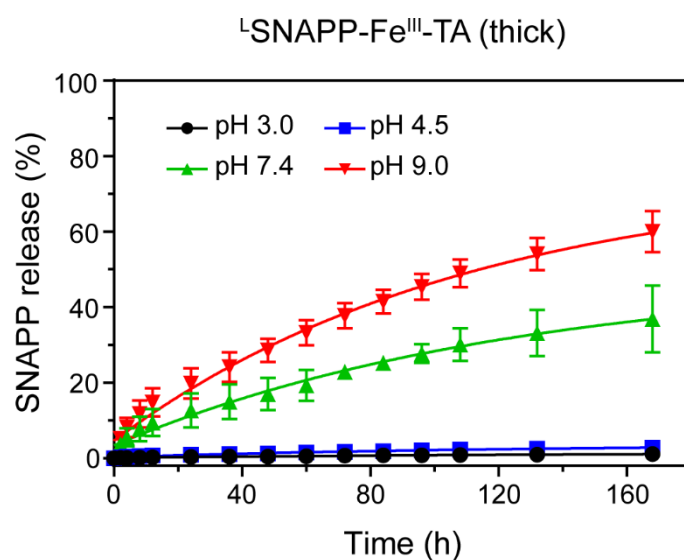


Figure S6.5. *In vitro* time-dependent release of ^LSNAPP from thick ^LSNAPP-Fe^{III}-TA capsules (thickness of 269 ± 26 nm) at pH 3.0, 4.5, 7.4 and 9.0. Data presented are averages of three replicates and error bars represent standard deviations.

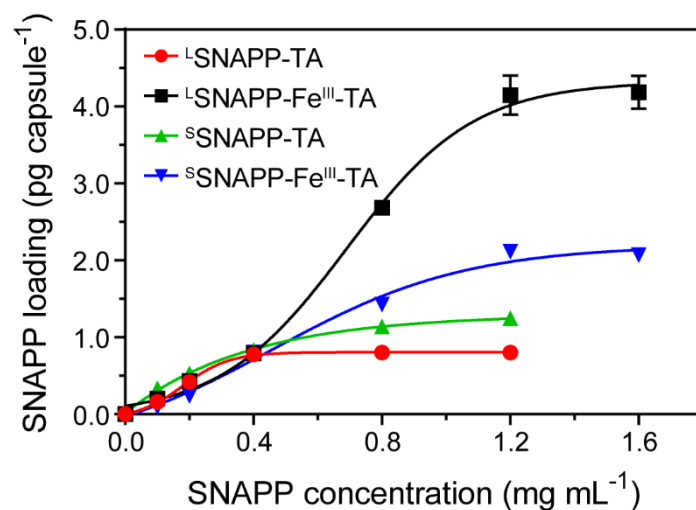


Figure S6.6. Loading amount of FITC-labelled ^LSNAPP and ^SSNAPP in SNAPP-TA and SNAPP-Fe^{III}-TA capsules as a function of SNAPP loading concentration. Data presented are averages of three replicates and error bars represent standard deviations.

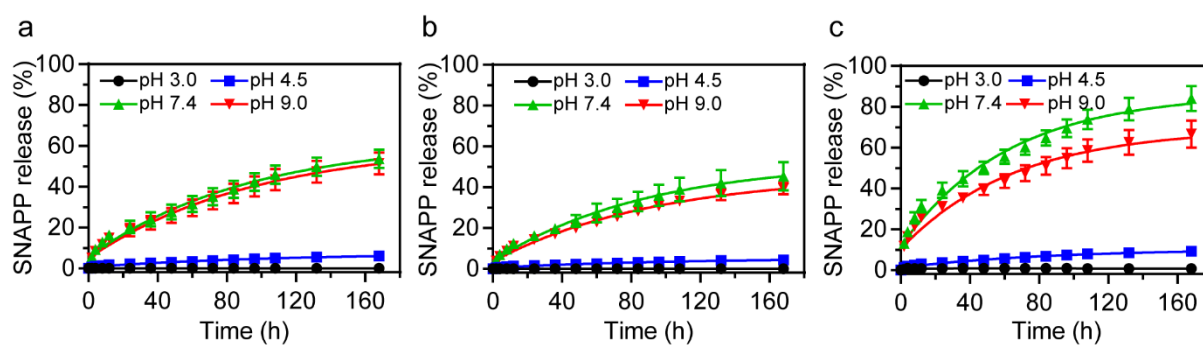


Figure S6.7. *In vitro* time-dependent release of FITC-labelled ^SSNAPP from (a) ^SSNAPP-Fe^{III}-TA capsules, (b) thick ^SSNAPP-Fe^{III}-TA capsules and (c) ^SSNAPP-TA capsules at pH 3.0, 4.5, 7.4 and 9.0. Data presented are averages of three replicates and error bars represent standard deviations.

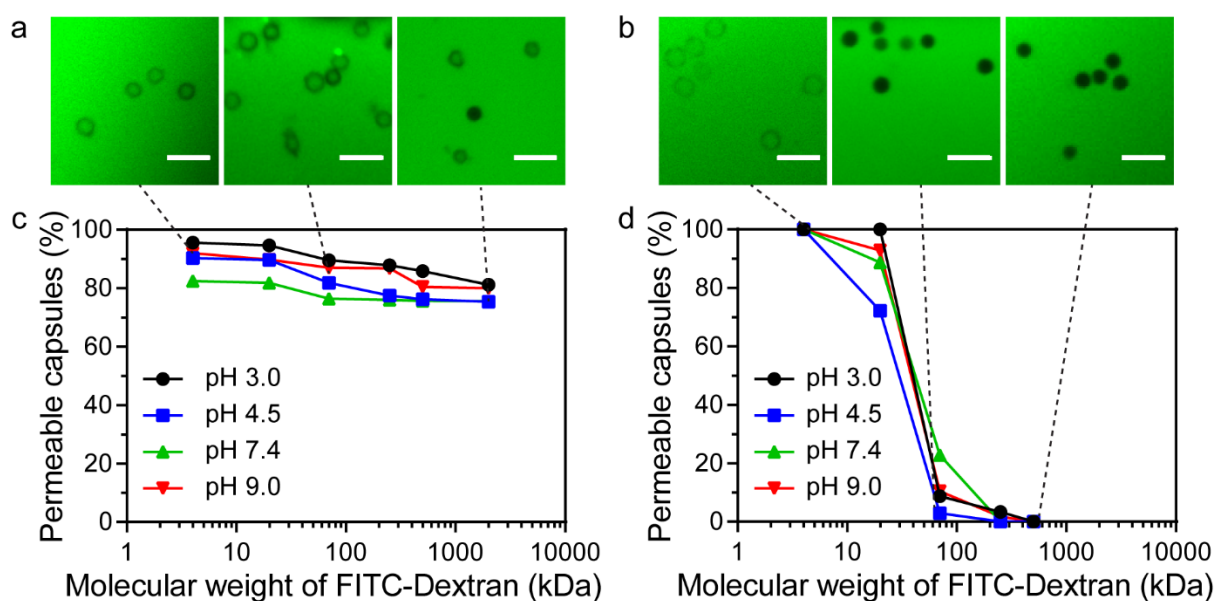


Figure S6.8. Molecular weight-dependent permeability of ^LSNAPP-Fe^{III}-TA capsules and ^LSNAPP-TA capsules at different pH. Representative confocal microscopy images of (a) ^LSNAPP-Fe^{III}-TA capsules and (b) ^LSNAPP-TA capsules incubated with FITC-dextran (3–5, 59–77 and 2000 kDa) at pH 4.5. Scale bars are 5 μ m. The percentage of permeable (c) ^LSNAPP-Fe^{III}-TA capsules and (d) ^LSNAPP-TA capsules plotted as a function of the molecular weight of FITC-dextran.

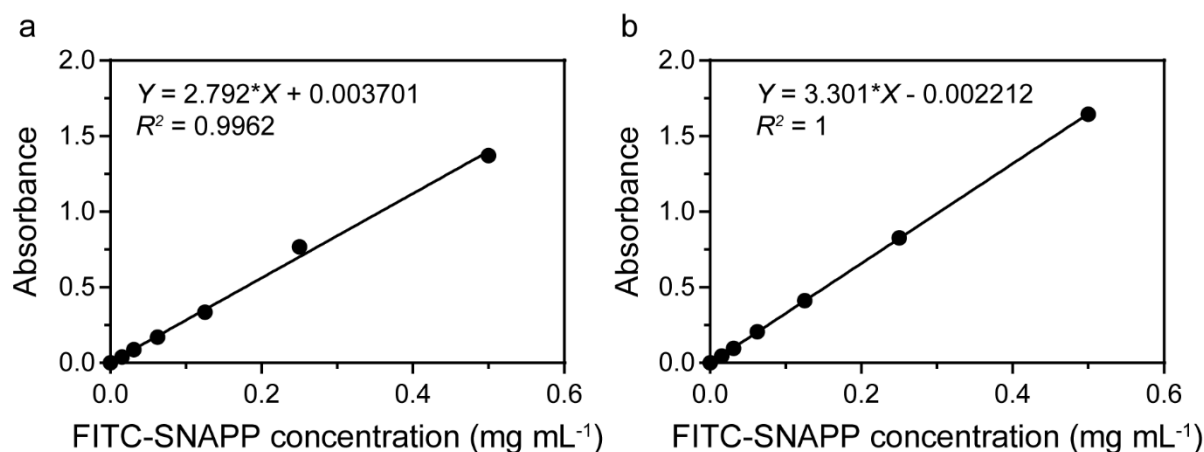


Figure S6.9. UV-vis absorbance standard curve of (a) FITC-labelled ^LSNAPP and (b) ^SSNAPP in Milli-Q water.

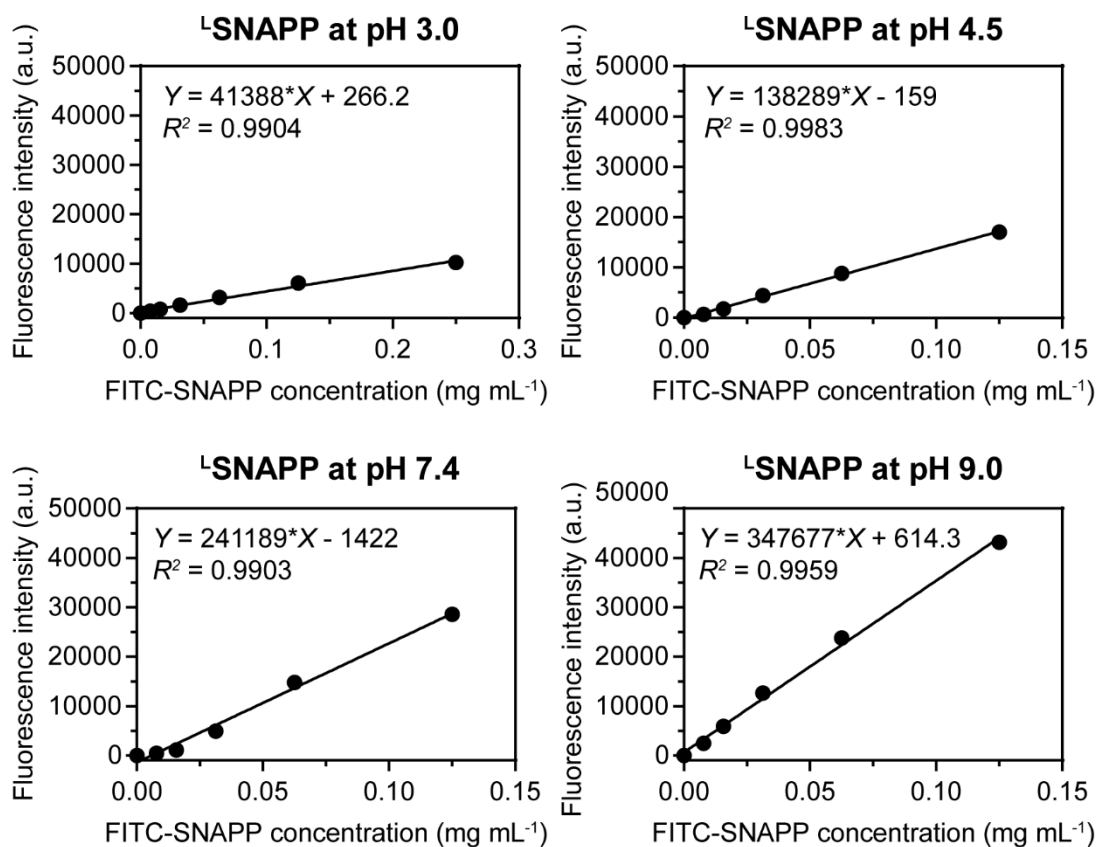


Figure S6.10. Fluorescence intensity standard curves of FITC-labelled ^LSNAPP at different pH (the measurement parameter was adjusted to achieve a reasonably high fluorescence intensity with the detectable range).

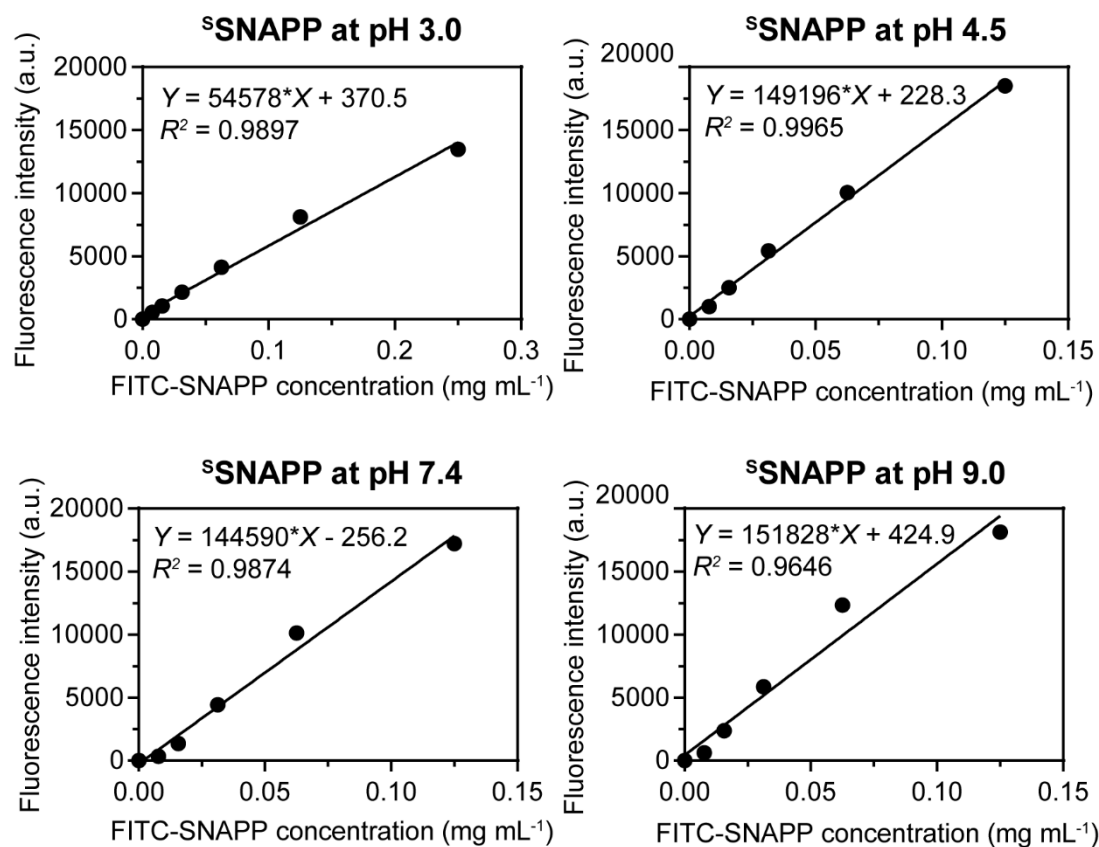


Figure S6.11. Fluorescence intensity standard curves of FITC-labelled ^sSNAPP at different pH (the measurement parameter was adjusted to achieve a reasonably high fluorescence intensity with the detectable range).

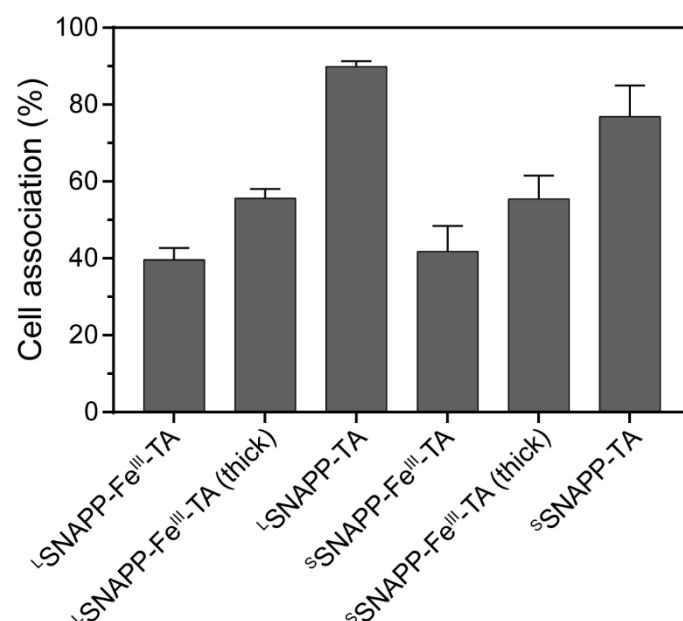


Figure S6.12. Association of SNAPP-TA and SNAPP-Fe^{III}-TA capsules prepared from ^LSNAPP or ^SSNAPP with MH-S cells after incubation for 24 h at 37 °C at a capsule-to-cell ratio of 50:1 (mean ± standard deviation, *n* = 3).

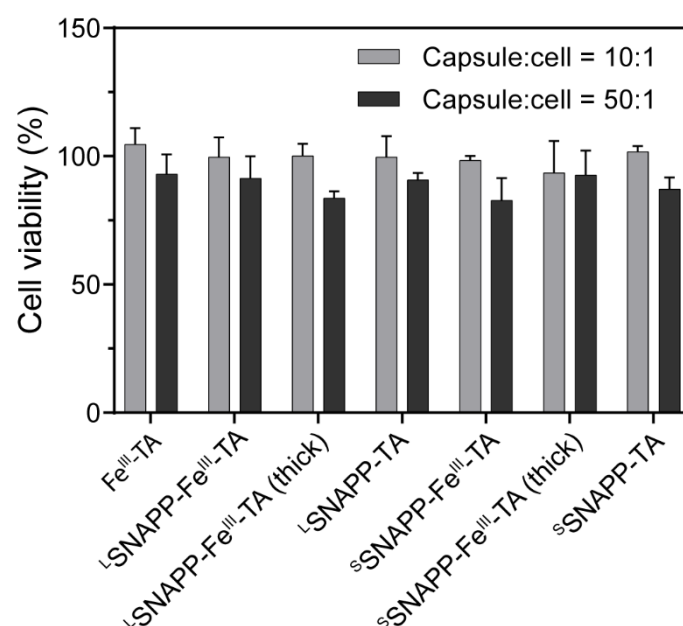


Figure S6.13. Cell (MH-S) cytotoxicity of SNAPP-TA and SNAPP-Fe^{III}-TA capsules prepared from ^LSNAPP or ^SSNAPP after incubation at 24 h at 37 °C at a capsule-to-cell ratio of 10:1 or 50:1. Cell viability was evaluated by XTT assay (mean ± standard deviation, *n* = 3). The viability of cells treated in the absence of capsules was normalised to 100%.

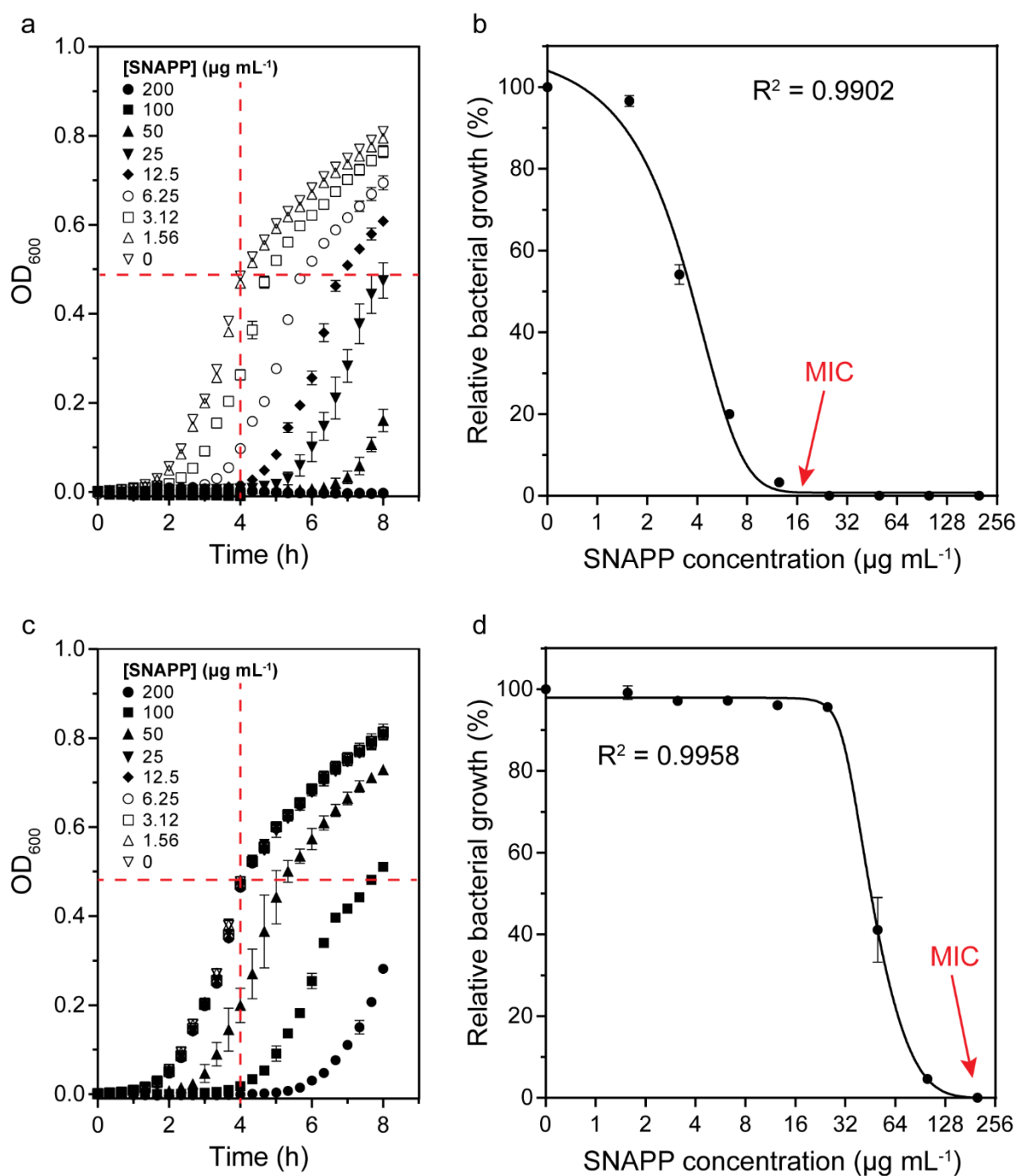


Figure S6.14. Growth curves of *E. coli* in the absence and presence of (a) ^{L} SNAPP and (c) ^{S} SNAPP at different concentrations. Relative OD_{600} at 4 h of these samples compared to the control (0 $\mu\text{g mL}^{-1}$, 100%) was fitted to an exponential function, of which the X value at $Y = 1\%$ was established as the MIC of (b) ^{L} SNAPP and (d) ^{S} SNAPP (mean $\pm$ standard deviation, $n = 3$).

6.7 References

1. Malam, Y.; Loizidou, M.; Seifalian, A. M., Liposomes and nanoparticles: nanosized vehicles for drug delivery in cancer. *Trends Pharmacol Sci* **2009**, *30* (11), 592-9.
2. Tanner, P.; Baumann, P.; Enea, R.; Onaca, O.; Palivan, C.; Meier, W., Polymeric vesicles: from drug carriers to nanoreactors and artificial organelles. *Acc Chem Res* **2011**, *44* (10), 1039-49.
3. Chudasama, V.; Maruani, A.; Caddick, S., Recent advances in the construction of antibody-drug conjugates. *Nat Chem* **2016**, *8* (2), 114-9.
4. Kedar, U.; Phutane, P.; Shidhaye, S.; Kadam, V., Advances in polymeric micelles for drug delivery and tumor targeting. *Nanomedicine* **2010**, *6* (6), 714-29.
5. Kesharwani, P.; Jain, K.; Jain, N. K., Dendrimer as nanocarrier for drug delivery. *Prog Polym Sci* **2014**, *39* (2), 268-307.
6. Cui, J.; Richardson, J. J.; Bjornmalm, M.; Faria, M.; Caruso, F., Nanoengineered Templated Polymer Particles: Navigating the Biological Realm. *Acc Chem Res* **2016**, *49* (6), 1139-48.
7. Yan, Y.; Such, G. K.; Johnston, A. P.; Lomas, H.; Caruso, F., Toward therapeutic delivery with layer-by-layer engineered particles. *ACS Nano* **2011**, *5* (6), 4252-7.
8. Patra, J. K.; Das, G.; Fraceto, L. F.; Campos, E. V. R.; Rodriguez-Torres, M. D. P.; Acosta-Torres, L. S.; Diaz-Torres, L. A.; Grillo, R.; Swamy, M. K.; Sharma, S.; Habtemariam, S.; Shin, H. S., Nano based drug delivery systems: recent developments and future prospects. *J Nanobiotechnology* **2018**, *16* (1), 71.
9. Mitragotri, S.; Burke, P. A.; Langer, R., Overcoming the challenges in administering biopharmaceuticals: formulation and delivery strategies. *Nat Rev Drug Discov* **2014**, *13* (9), 655-72.
10. Zelikin, A. N.; Ehrhardt, C.; Healy, A. M., Materials and methods for delivery of biological drugs. *Nat Chem* **2016**, *8* (11), 997-1007.
11. Ejima, H.; Richardson, J. J.; Liang, K.; Best, J. P.; van Koeverden, M. P.; Such, G. K.; Cui, J.; Caruso, F., One-step assembly of coordination complexes for versatile film and particle engineering. *Science* **2013**, *341* (6142), 154-7.
12. Shin, M.; Lee, H. A.; Lee, M.; Shin, Y.; Song, J. J.; Kang, S. W.; Nam, D. H.; Jeon, E. J.; Cho, M.; Do, M.; Park, S.; Lee, M. S.; Jang, J. H.; Cho, S. W.; Kim, K. S.; Lee, H., Targeting protein and peptide therapeutics to the heart via tannic acid modification. *Nat Biomed Eng* **2018**, *2* (5), 304-317.

13. Han, Y.; Lin, Z.; Zhou, J.; Yun, G.; Guo, R.; Richardson, J. J.; Caruso, F., Polyphenol-Mediated Assembly of Proteins for Engineering Functional Materials. *Angew Chem Int Ed Engl* **2020**.
14. Guo, J.; Tardy, B. L.; Christofferson, A. J.; Dai, Y.; Richardson, J. J.; Zhu, W.; Hu, M.; Ju, Y.; Cui, J.; Dagastine, R. R.; Yarovsky, I.; Caruso, F., Modular assembly of superstructures from polyphenol-functionalized building blocks. *Nat Nanotechnol* **2016**, *11* (12), 1105-1111.
15. Guo, J.; Ping, Y.; Ejima, H.; Alt, K.; Meissner, M.; Richardson, J. J.; Yan, Y.; Peter, K.; von Elverfeldt, D.; Hagemeyer, C. E.; Caruso, F., Engineering multifunctional capsules through the assembly of metal-phenolic networks. *Angew Chem Int Ed Engl* **2014**, *53* (22), 5546-51.
16. Ping, Y.; Guo, J.; Ejima, H.; Chen, X.; Richardson, J. J.; Sun, H.; Caruso, F., pH-Responsive Capsules Engineered from Metal-Phenolic Networks for Anticancer Drug Delivery. *Small* **2015**, *11* (17), 2032-6.
17. Ju, Y.; Cortez-Jugo, C.; Chen, J.; Wang, T. Y.; Mitchell, A. J.; Tsantikos, E.; Bertleff-Zieschang, N.; Lin, Y. W.; Song, J.; Cheng, Y.; Mettu, S.; Rahim, M. A.; Pan, S.; Yun, G.; Hibbs, M. L.; Yeo, L. Y.; Hagemeyer, C. E.; Caruso, F., Engineering of Nebulized Metal-Phenolic Capsules for Controlled Pulmonary Deposition. *Adv Sci* **2020**, *7* (6), 1902650.
18. Dickson, R. P.; Erb-Downward, J. R.; Prescott, H. C.; Martinez, F. J.; Curtis, J. L.; Lama, V. N.; Huffnagle, G. B., Cell-associated bacteria in the human lung microbiome. *Microbiome* **2014**, *2*, 28.
19. Chmiel, J. F.; Aksamit, T. R.; Chotirmall, S. H.; Dasenbrook, E. C.; Elborn, J. S.; LiPuma, J. J.; Ranganathan, S. C.; Waters, V. J.; Ratjen, F. A., Antibiotic management of lung infections in cystic fibrosis. I. The microbiome, methicillin-resistant *Staphylococcus aureus*, gram-negative bacteria, and multiple infections. *Ann Am Thorac Soc* **2014**, *11* (7), 1120-9.
20. Shrivastava, S. R.; Shrivastava, P. S.; Ramasamy, J., Much more is expected from nations to counter antimicrobial resistance: World Health Organization. *J Res Med Sci* **2015**, *20* (7), 718-9.
21. Lam, S. J.; O'Brien-Simpson, N. M.; Pantarat, N.; Sulistio, A.; Wong, E. H.; Chen, Y. Y.; Lenzo, J. C.; Holden, J. A.; Blencowe, A.; Reynolds, E. C.; Qiao, G. G., Combating multidrug-resistant Gram-negative bacteria with structurally nanoengineered antimicrobial peptide polymers. *Nat Microbiol* **2016**, *1* (11), 16162.
22. Shirbin, S. J.; Insua, I.; Holden, J. A.; Lenzo, J. C.; Reynolds, E. C.; O'Brien-Simpson, N. M.; Qiao, G. G., Architectural Effects of Star-Shaped "Structurally Nanoengineered

Antimicrobial Peptide Polymers" (SNAPPs) on Their Biological Activity. *Adv Healthc Mater* **2018**, 7 (21), e1800627.

23. Daly, W. H.; Poché, D., The preparation of N-carboxyanhydrides of α -amino acids using bis (trichloromethyl) carbonate. *Tetrahedron Lett* **1988**, 29 (46), 5859-5862.

24. Huesmann, D.; Birke, A.; Klinker, K.; Türk, S.; Räder, H. J.; Barz, M., Revisiting secondary structures in NCA polymerization: Influences on the analysis of protected polylysines. *Macromolecules* **2014**, 47 (3), 928-936.

25. Yun, G.; Besford, Q. A.; Johnston, S. T.; Richardson, J. J.; Pan, S.; Biviano, M.; Caruso, F., Self-Assembly of Nano-to Macroscopic Metal–Phenolic Materials. *Chem Mater* **2018**, 30 (16), 5750-5758.

26. Sun, H.; Wong, E. H. H.; Yan, Y.; Cui, J.; Dai, Q.; Guo, J.; Qiao, G. G.; Caruso, F., The role of capsule stiffness on cellular processing. *Chem Sci* **2015**, 6 (6), 3505-3514.

27. Prasad, B. R.; Brook, M. A.; Smith, T.; Zhao, S.; Chen, Y.; Sheardown, H.; D'Souza, R.; Rochev, Y., Controlling cellular activity by manipulating silicone surface roughness. *Colloids Surf B Biointerfaces* **2010**, 78 (2), 237-42.

28. Walkey, C. D.; Olsen, J. B.; Guo, H.; Emili, A.; Chan, W. C., Nanoparticle size and surface chemistry determine serum protein adsorption and macrophage uptake. *J Am Chem Soc* **2012**, 134 (4), 2139-47.

29. 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 (10), 11653-11664.

Chapter 7

Conclusions and Outlook

The unique structure of the lungs, including large surface area, low thickness of the epithelial barrier, extensive vascularisation and relatively low proteolytic activity in the alveolar region, makes the lungs an attractive delivery route for either local lung targeting or systemic delivery. With the development of nanotechnology and advances in materials science, various particle systems with unique properties and functions, such as polymeric particles and lipid-based particle systems have been developed as therapeutic carriers for pulmonary delivery. However, due to the presence of clearance and degradation mechanisms in the lungs, including the mucociliary clearance in airway and the alveolar macrophage clearance in deep lungs, inhaled particles are likely to have limited retention time in the target site and be rapidly eliminated. This could lead to reduced efficacy of therapeutic delivery and thus needing higher drug dosage, which could result in severe systemic side effects. For improved efficacy of therapeutic delivery and development of particle systems for biomedical applications, it is of vital importance to understand the interactions of particle systems with complex physiological environments. Therefore, in this thesis, the interactions of engineered particles with mucus, and the influence of the pulmonary corona on particle properties and cellular interactions were investigated by using distinct polymer particles thiol modified-poly(methacrylic acid) (PMA_{SH})-based particles and poly(ethylene glycol) (PEG)-based particles), which exhibit different surface properties. The potential for drug loading and intracellular delivery of therapies for lung disease or infections was also explored by using promising polyphenol-based carriers.

In Chapter 3, the link between the mechanical properties of low-fouling PEG-based particles and their interactions with immune cells was studied using a clinically relevant *ex vivo* human blood model. The PEG-based particles were prepared from different multi-arm PEG building blocks, which vary in arm number, using a mesoporous silica-templated method. The resultant PEG replica particles (template removed) showed different morphology and structures, and their elastic modulus increased from 1.5 kPa to 14.9 kPa with increase in PEG arm numbers. The mechanical properties of the particles influenced their biological interactions in a physiological system. Increasing the arm numbers (higher molecular weight) of PEG particles led to increased association with phagocytic blood cells and B cells. However, the opposite was observed for PEGylated mesoporous silica (MS@PEG) particles—those composed of PEG building blocks with increasing number of arms resulted in lower immune cell associations, probably due to a denser PEG coating and less negative particle surface charge at higher PEG arm numbers. A previous study reported that having the same arm number, higher

molecular weight PEG (longer arms) contributed to reduced immune cell associations and enhanced stealth property for assembled PEG particles, while in this study, higher molecular weight PEG led to increased cell association of PEG particles due to greater number of PEG arms. These findings suggest that higher molecular weight PEG does not necessarily contribute to better stealth property for PEG replica particles. This work indicates that the architecture of building blocks can affect the mechanical properties of the assembled particles with identical composition, and further alter their interactions with biological systems, which should be taken into account in the design of functional nanoengineered materials for biomedical applications. It also shows how the particle synthetic properties can be engineered to modulate their biological identities and interactions with cells in complex biological environments.

In Chapter 4, particle–mucus interactions in the lungs were investigated by comparing how PMA_{SH}-based particles and PEG-based particles, which exhibit different structure and surface properties, interact with artificial mucus and an air-liquid interface (ALI) cell culture. MS@PMA_{SH}, PMA_{SH}, MS@PEG and PEG particles were found to be amenable to the nebulisation process maintaining their original structure and morphology without aggregation, with a droplet size of ~5 µm, which was in the desirable size range for deposition of particles in the deep lung region. PMA_{SH} particles exhibited strong adhesion to the artificial mucus as evaluated by QCM-D, probably due to the interactions between the thiol groups of PMA_{SH} particles and the cysteine groups of mucin, while PEG particles showed negligible adsorption onto the mucus layer, confirming the low-fouling property of PEG particles. The particle behaviours in ALI cell culture were found to be consistent with the QCM-D results. MS@PMA_{SH} and PMA_{SH} particles were mostly stuck in the mucus layer, while no mucus adhesion of MS@PEG and PEG particles was detected. Taken together, this study demonstrates the potential of drug-loadable PMA_{SH}-based particles and PEG-based particles for pulmonary delivery via nebulisation and provides a preliminary understanding of the interactions of these particles with airway mucus.

Chapter 5 demonstrated the influence of the pulmonary corona on the particle surface properties and their interactions with cells. The interplay of both protein corona from blood (plasma protein corona) and pulmonary corona on the composition of a mixed protein corona, and the resultant influence on macrophage uptake of polymer particles were also investigated. Plasma protein corona, lung protein corona and the mixed coronas had different influence on the particle properties and their interactions with macrophages. The presence of a protein corona reduced macrophage uptake of MS@PMA_{SH} and PMA_{SH} particles, but increased

macrophage uptake of MS@PEG particles, and negligible effect on the PEG particles. The plasma+lung protein corona-coated particles and the lung+plasma protein corona-coated particles showed different levels of cell association under the same experimental conditions, mainly due to the different composition of the eventual mixed protein corona as evaluated by mass spectrometry. The results indicate that the features of the protein corona including the protein composition and proportions are dependent on both biological environments and the particle properties. When particles are transferred from one biological environment into another, previously adsorbed proteins may be partially replaced by the proteins from the new environment, and partially retained. The interplay of both protein coronas determines the composition of the eventual mixed protein corona, and further affects the particle–cell interactions. The addition of protein corona B to protein corona A is not necessarily equal to the addition of protein corona A to protein corona B. This study provides improved understanding of the role of the protein corona in particle–cell interactions as a result of the transport of particles from one biological site to another, which is helpful for the design of particle systems to overcome the biological barriers and achieve promising biological outcomes.

Chapter 6 explored the potential of drug encapsulation and intracellular delivery using polyphenol-based capsules for applications in pulmonary delivery. Structurally nanoengineered antimicrobial peptide polymers (SNAPPs), a new class of synthetic antimicrobial peptide mimics, were immobilised and encapsulated via the assembly of SNAPP-TA and SNAPP-Fe^{III}-TA capsules with the maximum loading amount of 0.8 pg capsule⁻¹ for SNAPP-TA capsules and 4.4 pg capsule⁻¹ for SNAPP-Fe^{III}-TA capsules. Sustained release of SNAPP was achieved at pH 7.4 and pH 9.0 for both SNAPP-TA and SNAPP-Fe^{III}-TA capsules with faster release at pH 9.0. SNAPP-TA capsules exhibited faster release of SNAPP and higher overall release percentage over a long period than SNAPP-Fe^{III}-TA capsules. Both SNAPP-TA and SNAPP-Fe^{III}-TA capsules exhibited negligible cytotoxicity and showed high internalisation by mouse alveolar macrophage MH-S cells. SNAPP-TA capsules showed higher cell association than SNAPP-Fe^{III}-TA capsules, and exhibited relatively stronger co-localisation with endo/lysosomes, revealing the potential of intracellular disassembly and degradation. The released SNAPP from both SNAPP-TA and SNAPP-Fe^{III}-TA capsules maintained high antimicrobial activity as evaluated in *E. coli*. This study provides strategies for drug encapsulation and cellular delivery using promising delivery carriers against bacterial infections in the lungs (e.g., tuberculosis, cystic fibrosis).

In summary, this thesis provides an overview of engineering particle systems for pulmonary delivery, including understanding bio-nano interactions in lung physiological environments and the applications of therapeutic delivery of potential therapies for lung diseases. PEG as low-fouling material that is commonly used for ‘stealth’ coating can minimise the adhesion of particles to mucus, while modification with thiol groups can result in strong interactions with mucus as consistent with published literature. The stealth property of PEG replica particles and PEGylated mesoporous silica (MS@PEG) particles can be influenced by the architecture of the PEG building blocks by tuning the mechanical property and surface charge of assembled particles. The synthetic or chemical properties of particles can be engineered to allow modulation of their biological identities to influence interactions with cells in complex biological environments. Upon contacting the pulmonary surfactant environment, the pulmonary corona formed on particles played an important role in modulating particle properties and their interactions with cells. When particles are transferred from one biological environment into another, previously adsorbed proteins may be partially replaced by the proteins from the new environment, and partially retained. The interplay of both protein coronas determines the composition of the eventual mixed protein corona, and further affects the particle–cell interactions. Furthermore, polyphenol-based capsules can be engineered as therapeutic carriers for pulmonary drug delivery. Overall, these findings provide an insight of the bio-nano interactions of potentially inhalable particles for pulmonary delivery and provide promising prospects for future development of templated polymer particles and polyphenol-based materials for respiratory applications.

Given the low-fouling property and poor mucoadhesive characteristic of PEG-based particles, they are potential candidates for drug delivery with long retention time in the lungs. Future work investigating the permeation performance of PEG-based particles through mucus, subsequent interactions with underlying cells, and particle distribution *in vivo* after inhalation is important to better understand the behaviour of mucus-penetrating particles in a real physiological environment for clinical applications. Besides, further tailoring of PEG-based particles such as functionalisation with specific targeting ligands can potentially expand their biomedical applications in the lungs, achieving a balance of targeting and ‘stealth’ ability to evade clearance mechanism. SNAPP-loaded polyphenol-based capsules are promising candidates for targeting therapy of the diseases that are caused by bacteria localised in endosomes of alveolar macrophages (e.g., tuberculosis). Future work evaluating the performance of SNAPP-loaded polyphenol-based capsules by using a more relevant *ex vivo*

model (e.g., bacteria-infected cells) or *in vivo* model is essential to explore their potential use as inhalable antimicrobials. Encapsulation and intracellular delivery of other therapeutic agents, including small drugs and nucleic acid drugs, using polyphenol-based capsules with tailorable properties can also be investigated as potential therapies for lung diseases. It should be noted that the polyphenol-based capsules developed in this thesis are in the micrometer scale, which usually results in relatively lower bioavailability than smaller particles especially those in a size range between 50 nm and 200 nm. Therefore, one challenge of extending the applications of polyphenol-based materials is to reduce their size to the nanometer scale (50–200 nm). Self-assembly or template-mediated methods will be explored in future work to fabricate nano-sized polyphenol-based materials for pulmonary delivery.

In this thesis, proteomics analysis by mass spectrometry was performed to identify the proteins in the pulmonary corona. Due to the presence of high contents of phospholipids in pulmonary surfactant, large amounts of lipid can be adsorbed onto particles together with proteins. Therefore, it is essential to analyse the lipid composition of the pulmonary corona in addition to the protein composition to comprehensively characterize the complex composition of the pulmonary corona. Furthermore, it is important to study the specific roles of four major surfactant associated proteins (SP-A, -B, -C and -D) in biological responses, especially in macrophage uptake to better understand the interactions of particles with the highly complex pulmonary surfactant fluid. The effect of the pulmonary corona on cell apoptosis/death and inflammatory cytokine secretion should also be studied in the future.