Capsosomes: En Route Toward Synthetic Cellular Systems

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Abstract

Engineering artificial cells is currently an emerging area of research that involves constructing mimics of biological cells. These biomimetic cellular structures hold tremendous promise for the creation of next-generation therapeutic tools due to their ability to restore lost cellular functions. Amongst their potential applications, replenishing absent or malfunctioning enzymatic activities to degrade waste products or to support the synthesis of medically relevant biomolecules is a chief goal, which can provide long-term therapeutic solutions for chronic diseases. Artificial cells do not require the complex multifunctionality of their biological counterparts and can be more simply designed to perform a specific activity. A key approach in designing a cell-like system is a subcompartmentalized assembly, which is one of the features of biological cells that enable the performance of multiple complex biochemical reactions within confined environments.

This thesis focuses on developing a bottom-up approach to assemble micron-sized vessels with a controlled number of enzyme-loaded subcompartments toward cell mimicry. Capsosomes, polymer hydrogel capsules containing controlled amounts of intact cargo-loaded liposomal subcompartments, were developed in this thesis and they represent a novel class of carrier system toward the design of bioinspired vehicles. Polymer capsules, assembled via the sequential deposition of interacting polymers onto particle templates (layer-by-layer technique, LbL) followed by core removal, serve as structurally stable scaffolds with tunable permeability that allow exchange of reagents and nutrients between the internal and external milieu – resembling cell membranes. On the other hand, liposomes divide the interior of the capsules into subcompartments and can stably encapsulate fragile hydrophobic and hydrophilic cargo, e.g., enzymes in order to conduct encapsulated catalysis – resembling cell organelles.

The creation of (bio)degradable capsosomes is based on the sequential assembly of cargo-loaded liposomes and polymers onto sacrificial particle templates, followed by
the LbL deposition of poly(N-vinylpyrrolidone) (PVP) and thiol-functionalized poly(methacrylic acid) (PMA\textsubscript{SH}) via hydrogen bonding. Upon crosslinking the thiols of the PMA\textsubscript{SH} and dissolution of the particle templates, colloidal stable capsosomes are obtained. The coassembly of polymers and liposomes was optimized via novel noncovalent linkage concept using tailor-made cholesterol-modified polymers and this unique approach facilitates stable incorporation of intact liposomes into polymer films. Spatial position of the subcompartments can be controlled, which yields capsosomes containing membrane-associated or “free-floating” subunits.

Capsosomes exhibit size-dependent retention of the encapsulated cargo within the liposomal subunits. To prolong the stability of the liposomes in the compartmentalized assembly against degradative enzymes, the outer membrane of the capsosomes was surface functionalized with poly(ethylene glycol) (PEG). The functionality of capsosomes was demonstrated by triggered encapsulated (two-step) enzymatic catalysis. Capsosomes encapsulating glutathione reductase were able to generate glutathione, a potent antioxidant, while simultaneously releasing small therapeutic molecules, which highlights the ability of this subcompartmentalized assembly in addressing the complexity in therapeutic cell mimicry. The phase transition temperature of the liposomes was used as a trigger to initiate the enzymatic reactions, allowing capsosomes to be repeatedly used for multiple subsequent catalysis. Capsosomes with tailored properties present new opportunities en route to the development of functional cell mimics and the presented studies highlight crucial aspects for the successful applications of capsosomes as therapeutic artificial cells.
Declaration

This is to certify that:

(i) The thesis comprises only my original work towards the PhD except where indicated in the Preface;

(ii) Due acknowledgement has been made in the text to all other material used;

(iii) The thesis is less than 100,000 words in length, exclusive of tables, bibliographies, appendices, and footnotes.

Rona Chandrawati
Preface

Chapters 2, 4, 5, 6, 7, and 8 of this thesis have been published in a different form in the following articles:


In Chapter 4, cholesterol-modified poly(L-lysine) (PLL<sub>c</sub>) was synthesized by Dr Luke A. Connal (The University of Melbourne, Australia). Poly(methacrylic acid)-co-(cholesteryl methacrylate) (PMA<sub>c</sub>) and poly(methacrylic acid)-co-(oleyl methacrylate) (PMA<sub>oa</sub>) were synthesized by Dr Almar Postma (CSIRO Materials Science and Engineering, Australia).

In Chapter 5, poly(hydroxypropyl methacrylamide) with pyridyl disulfide-activated thiol group (HPMA<sub>PD</sub>) was synthesized by Dr Volga Bulmus (The University of New South Wales, Australia). Poly(N-vinylpyrrolidine)-block-(cholesteryl acrylate) (PVP<sub>c</sub>) was synthesized by Dr Almar Postma (CSIRO Materials Science and Engineering, Australia). TEM images (Figure 5.5) were captured by Dr Brigitte Städler (The University of Melbourne, Australia).

In Chapter 7, poly(methacrylic acid)-poly(ethylene glycol) (PMA-PEG) copolymer was prepared by Dr Siow-Feng Chong (The University of Melbourne, Australia).

In Chapter 8, PMA-KP9 conjugates were prepared by Dr Siow-Feng Chong (The University of Melbourne, Australia).
Publications

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


   *This publication was featured on the cover of the journal issue*


   *This publication was featured on the cover of the journal issue*


*LH and RC contributed equally to this work*


*This publication was featured on the cover of the journal issue*


Presentations

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


5. Chandrawati, R.; Städler, B.; Caruso, F. “Capsosomes: Liposomal Subcompartments within Polymer Hydrogel Capsules”, *Particulate Fluids Processing Centre (PFPC) Seminar*, oral presentation, **September 2010**, The University of Melbourne, Australia.


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<tr>
<td>AF488</td>
<td>Alexa Fluor 488</td>
</tr>
<tr>
<td>AF633</td>
<td>Alexa Fluor 633</td>
</tr>
<tr>
<td>AIBN</td>
<td>2,2'-azoisobutyronitrile</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BM(PEG)₂</td>
<td>1,8-bis(maleimido)diethylene glycol</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Calcium chloride</td>
</tr>
<tr>
<td>CaCO₃</td>
<td>Calcium carbonate</td>
</tr>
<tr>
<td>CaT</td>
<td>Chloramine T</td>
</tr>
<tr>
<td>CF</td>
<td>Carboxyfluorescein</td>
</tr>
<tr>
<td>CLSM</td>
<td>Confocal laser scanning microscopy</td>
</tr>
<tr>
<td>CPADB</td>
<td>4-(cyanopentanoic acid)-4-dithiobenzoate</td>
</tr>
<tr>
<td>CR</td>
<td>Carboxyrhodamine</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>Dextran&lt;sub&gt;4-FITC&lt;/sub&gt;</td>
<td>10 kDa dextran fluorescein isothiocyanate</td>
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<tr>
<td>Dextran&lt;sub&gt;10-FITC&lt;/sub&gt;</td>
<td>4 kDa dextran fluorescein isothiocyanate</td>
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<tr>
<td>DIC</td>
<td>Differential interference contrast</td>
</tr>
<tr>
<td>DMAc</td>
<td>Dimethylacetamide</td>
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<tr>
<td>DMPC</td>
<td>1,2-dimyristoyl-sn-glycero-3-phosphocholine</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleotide triphosphate</td>
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<td>DOPC</td>
<td>1,2-dioleoyl-sn-glycero-3-phosphocholine</td>
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<tr>
<td>DOPS</td>
<td>1,2-dioleoyl-sn-glycero-3-(phospho-L-serine)</td>
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<td>DPPC</td>
<td>1,2-dipalmitoyl-sn-glycero-3-phosphocholine</td>
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<td>DTT</td>
<td>DL-dithiothreitol</td>
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<tr>
<td>EDC</td>
<td>1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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</table>
FITC  Fluorescein isothiocyanate
GFP   Green fluorescent protein
GOX   Glucose oxidase
GR    Glutathione reductase
GSH   Reduced L-glutathione
GSSG  Oxidized L-glutathione
HA    Hyaluronic acid
HCl   Hydrochloric acid
HEPES 4-(2-hydroxyethyl)piperazine-1-ethane-sulfonic acid
HF    Hydrofluoric acid
HPMA Poly(hydroxypropyl methacrylamide)
HPMA$_{PD}$ Poly(hydroxypropyl methacrylamide) with pyridyl disulfide-activated thiol groups
$L^{s,zw}_{(NBD)}$ (Fluorescently labeled) saturated zwitterionic liposomes
$L^{s,-}_{(NBD)}$ (Fluorescently labeled) saturated negatively charged liposomes
$L^{u,zw}_{(NBD)}$ (Fluorescently labeled) unsaturated zwitterionic liposomes
$L^{u,-}_{(NBD)}$ (Fluorescently labeled) unsaturated negatively charged liposomes
$L_{DMPC/DPPC-\beta}$ β-lactamase-loaded DMPC/DPPC liposomes
$L_{DPPC-\beta}$ β-lactamase-loaded DPPC liposomes
$L_{DPPC-GR}$ Glutathione reductase-loaded DPPC liposomes
LB    Langmuir-Blodgett
LbL   Layer-by-layer
Luc$_{FITC}$ Luciferase fluorescein isothiocyanate
MAL-PEG-NHS Maleimide polyethylene glycol succinimidyl ester
MES   2-(N-morpholino)ethanesulfonic acid
MgSO$_4$ Magnesium sulphate
MOPS  3-(N-morpholino)propanesulfonic acid
NaCl  Sodium chloride
NADPH β-nicotinamide adenine dinucleotide 2′-phosphate
NaHCO$_3$ Sodium bicarbonate
NaOAc Sodium acetate
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Name</th>
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<tr>
<td>NBD-PC</td>
<td>1-myristoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>NH₄F</td>
<td>Ammonium fluoride</td>
</tr>
<tr>
<td>NPC</td>
<td>4-nitrophenol chloroformate</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>P4VP</td>
<td>Poly(4-vinylpyridine)</td>
</tr>
<tr>
<td>PAA</td>
<td>Poly(acrylic acid)</td>
</tr>
<tr>
<td>PAAm</td>
<td>Polyacrylamide</td>
</tr>
<tr>
<td>PAH</td>
<td>Poly(allylamine hydrochloride)</td>
</tr>
<tr>
<td>PAni</td>
<td>Polyaniline</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDA-HCl</td>
<td>Pyridine dithioethylamine hydrochloride</td>
</tr>
<tr>
<td>PDADMAC</td>
<td>Poly(diallyldimethylammonium chloride)</td>
</tr>
<tr>
<td>PDMS</td>
<td>Poly(dimethylsiloxane)</td>
</tr>
<tr>
<td>PDSM</td>
<td>2-(2-pyridyldisulfide)ethylmethacrylate</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
</tr>
<tr>
<td>PEI</td>
<td>Poly(ethylenimine)</td>
</tr>
<tr>
<td>PEO</td>
<td>Poly(ethylene oxide)</td>
</tr>
<tr>
<td>PIAT</td>
<td>Poly-(L-isocyanoalanine(2-thiophen-3-yl-ethyl)amide)</td>
</tr>
<tr>
<td>PLA₂</td>
<td>Phospholipase A₂</td>
</tr>
<tr>
<td>PLL</td>
<td>Poly(L-lysine)</td>
</tr>
<tr>
<td>PLLₜ</td>
<td>Cholesterol-modified poly(L-lysine)</td>
</tr>
<tr>
<td>PMA</td>
<td>Poly(methacrylic acid)</td>
</tr>
<tr>
<td>PMAₜ</td>
<td>Poly(methacrylic acid)-co-(cholesteryl methacrylate)</td>
</tr>
<tr>
<td>PMAₒa</td>
<td>Poly(methacrylic acid)-co-(oleyl methacrylate)</td>
</tr>
<tr>
<td>PMAPD</td>
<td>Poly(methacrylic acid) with pyridyl disulfide-activated thiol groups</td>
</tr>
<tr>
<td>PMAₕSH</td>
<td>Thiol-functionalized poly(methacrylic acid)</td>
</tr>
<tr>
<td>PMOXA</td>
<td>Poly(2-methylloxazoline)</td>
</tr>
<tr>
<td>POD</td>
<td>Peroxidase</td>
</tr>
<tr>
<td>Poly(AAc-co-DSA)</td>
<td>Poly(acrylic acid)-co-poly(distearin acrylate)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PS</td>
<td>Polystyrene</td>
</tr>
<tr>
<td>PSS</td>
<td>Poly(styrenesulfonate)</td>
</tr>
<tr>
<td>PVA</td>
<td>Poly(vinyl alcohol)</td>
</tr>
<tr>
<td>PVP</td>
<td>Poly(N-vinylpyrrolidone)</td>
</tr>
<tr>
<td>PVPₖ</td>
<td>Poly(N-vinyl pyrrolidone)-block-(cholesteryl acrylate)</td>
</tr>
<tr>
<td>QCM-D</td>
<td>Quartz Crystal Microbalance with Dissipation Monitoring</td>
</tr>
<tr>
<td>RAFT</td>
<td>Reversible addition-fragmentation chain transfer</td>
</tr>
<tr>
<td>SAM</td>
<td>Self-assembled monolayer</td>
</tr>
<tr>
<td>TCEP</td>
<td>Tris(2-carboxyethyl)phosphine</td>
</tr>
<tr>
<td>TCI</td>
<td>Transcutaneous immunization</td>
</tr>
<tr>
<td>TEA</td>
<td>Triethylamine</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>$T_m$</td>
<td>Phase transition temperature</td>
</tr>
<tr>
<td>TNB</td>
<td>2-nitro-5-mercaptobenzoic acid</td>
</tr>
<tr>
<td>TTx</td>
<td>Tetanus toxoid</td>
</tr>
<tr>
<td>TX</td>
<td>Triton X</td>
</tr>
<tr>
<td>UV-Vis</td>
<td>Ultraviolet-Visible</td>
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Chapter 1

Introduction

Over the past few decades, technological innovation has resulted in remarkable advances in healthcare. Most notably in the field of biomedical engineering, the development of state of the art strategies and techniques to exploit the unique properties and functions of novel materials opens up new avenues to a wide range of biomedical applications, such as encapsulated catalysis, sensing, drug delivery, and diagnostics. A chief example is the engineering of artificial cells,\textsuperscript{1-6} which is currently an emerging area of research that involves constructing simplified synthetic cells to resemble living cells. The study of such artificial cell models has garnered significant interest because it would contribute to a better understanding of biological cells. These synthetic cells are designed to mimic cellular functions and biological responses within small-scale domains and are expected to have an impact on next-generation therapeutic concepts as a powerful biomedical tool for potential therapeutic applications, such as enzyme therapy, gene therapy, drug delivery, and diagnostics. The role of these biomimetic structures in enzyme therapy predominantly focuses on enzymatic activities to degrade waste products or to support the synthesis of medically relevant biomolecules. The lost or absence of a specific enzymatic activity in humans is often a lifelong condition and requires regular medication, which can be cost intensive, and patient compliance. Artificial cells have the potential to replenish missing or deficient cellular enzymatic activities and therefore hold tremendous promise for treating diseases on the cellular and subcellular levels. The development of this biomedical platform would not only be beneficial for individual patients but also for the healthcare system.

Designing artificial cells requires reconstruction of the structure and function of biological cells (Scheme 1.1). Biological cells are self-contained, living entities. They
are equipped with multiple highly optimized and specialized internal subcompartments known as organelles that have the ability to carry out specific metabolic activities and are surrounded by a selectively permeable biological membrane that serves as a structural scaffold and partitions the cell interior from the external milieu. The movement of reactive species across these membranes is controlled by specific transmembrane protein channels, allowing precise regulation over the chemical species present within the cells and organelles. Assembling such nature-inspired structures therefore presents a significant challenge; however, artificial cells do not require the complex multifunctionality of their biological counterparts, but rather can be simply designed to perform a specific activity.

**Scheme 1.1.** Schematic illustration of a biological cell and its synthetic mimic, artificial cell. A key feature of artificial cells is the design of a compartmentalized assembly; the minimal requirements for the successful design of artificial cells are: (i) a structural scaffold with specific permeability to allow controlled interaction between the internal and external milieu – resembling cell membranes and (ii) encapsulated machinery as specialized subcompartments to allow multiple, spatially separated enzymatic reactions within a confined area – resembling cell organelles.
Due to the unique subcompartmentalized structure, biological cells are able to spatially separate and precisely regulate thousands of enzymatic (cascade) reactions, hence making the mimicry of this feature a key aspect in designing artificial cells. There are minimal requirements that have to be fulfilled for subcompartmentalized approach to artificial cells: (i) a structural scaffold with specific permeability that allows controlled interaction between the cell interior and the external milieu – resembling cell membranes and (ii) encapsulated machinery as specialized subunits that allow for successive enzymatic reactions within a confined area – resembling cell organelles (Scheme 1.1). This compartmentalized design therefore allows control over the permeability of (bio)molecules across the membrane of the scaffold while entrapping various machinery, hence facilitating cell-like parallel and/or sequential enzymatic reactions with the aim to mimic metabolic processes.

In recent years, considerable progress has been achieved in designing and developing functional bioinspired systems, with the most successful examples to date based on liposomes, polymersomes, and polymer capsules. Liposomes and polymersomes are self-assembled vessels made from amphiphilic lipid and block copolymers, respectively, and are able to encapsulate diverse cargo. Polymer capsules are prepared via the layer-by-layer (LbL) technique, i.e., the sequential adsorption of interacting polymers onto sacrificial template particles followed by core removal, and have been widely used to design carrier systems for biomedical applications due to facile control over size, shape, composition, and permeability of the capsule membrane. Several artificial designs of subcompartmentalized assemblies incorporating the aforementioned building blocks include vesosomes (liposomes within a liposome), polymersomes within a polymersome, polymer subcompartments within a polymer capsule, and shell-in-shell microcapsules – all based on single-component systems.

This thesis therefore describes a novel subcompartmentalized assembly, termed capsosomes, which unite two fundamentally different systems: polymer capsules and liposomes. The key advantages of polymer capsules and liposomes are maintained by combining these two assemblies. Polymer capsules serve as
colloidally stable scaffolds and allow for fine control over the membrane permeability. On the other hand, liposomes can stably encapsulate small, fragile hydrophobic and hydrophilic cargo, and divide the interior of the capsule into subcompartments that enable confined specific reactions to be conducted. Selective access of (bio)molecules across the machinery, successive encapsulated enzymatic catalysis, and controlled release of therapeutic molecules are key prerequisites for the successful development of artificial cell-based therapeutic carriers. The architecture of the capsosomes therefore represents a promising platform for constructing therapeutic cell mimics. This system combines physicochemical properties that are not readily achievable using single-component assemblies and would lead to a marked improvement in the performance of nanoengineered assemblies for confined (bio)chemical reactions.

In this thesis, a brief historical perspective on the development of polymer capsules and liposomes as carrier systems for biomedical applications, such as encapsulated catalysis and therapeutic delivery, is provided as a literature review in Chapter 2. Current status of the development of artificial cells and recent advances en route to designing biomimetic compartmentalized assemblies toward cell mimicry are also discussed. The chapters following the literature review describe the development of capsosomes toward functional therapeutic artificial cells – from assembly to applications. A description of the instrumental techniques employed throughout this thesis is outlined in Chapter 3. Chapter 4 focuses on fundamental aspects underpinning the stable and high loading of intact liposomal subcompartments into polymer films. The optimized polymer/liposome assembly is then followed by the creation of (bio)degradable capsosomes in Chapter 5. Chapter 6 discusses the size-dependent retention and stability of encapsulated cargo within the liposomal subcompartments of capsosomes. In Chapter 7, surface functionalization of capsosomes is demonstrated and the effect of surface modification on the stability of the liposomal subcompartments is assessed. The functionality of capsosomes is demonstrated by triggered encapsulated enzymatic catalysis and is detailed in Chapter 8. Chapter 9 provides the main conclusions of the research and outlines
future development of these biomimetic assemblies toward next-generation therapeutic tools.
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Chapter 2

Literature Review

Chapter 2

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2.1 Introduction

Engineering micro- and nanoparticle systems has generated significant research interest due to their potential application in diverse fields, such as encapsulated catalysis, sensing, and drug delivery.\(^1\) As mentioned in Chapter 1, polymer capsules, liposomes, and polymersomes have emerged as versatile carrier systems for biomedical applications. Through judicious choice of building blocks, these synthetic carriers can be readily engineered with tailored interfacial properties, offering new possibilities for the design of advanced assemblies with specific permeability, stability, and stimuli-response. Advances in engineering such carriers over the past decades have led to the development of sustained therapeutic delivery systems; however, there are certain critical challenges yet to be met. Exploiting novel approaches to prepare functional carrier systems therefore remains a key facet of current biomedical research, in particular in the field of therapeutic cell mimicry, which is expected to open new opportunities in biomedicine and lead to markedly improved performance of therapeutic administration. In recent years, interest in mimicking the complex hierarchical architectures and functionalities of biological systems and the associated spatially confined enzymatic reactions has led to the design and synthesis of a range of compartmentalized assemblies.\(^2\)\(^{-5}\) This literature review aims to: (i) present a historical perspective on the development of polymer capsules and liposomes – the building blocks for the assembly of capsosomes – in biomedical applications; (ii) provide an overview of the current development status of artificial cells; and (iii) highlight recent advances en route to designing biomimetic compartmentalized assemblies and discuss their applications in the area of encapsulated catalysis and therapeutic delivery toward the creation of therapeutic cell mimicry.

2.2 Layer-by-Layer (LbL)-Assembled Polymer Capsules

2.2.1 LbL Assembly Technique

Polymer capsules prepared by layer-by-layer (LbL) assembly technique are promising carriers for biomedical applications.\(^6\)\(^{-11}\) The concept of LbL assembly was first
proposed by Iler in 1966, who suggested alternating deposition of oppositely charged particles to create a film of multiple layers.\textsuperscript{12} This concept was later rediscovered and developed by Decher and Hong in 1991 to create multilayers by the alternate deposition of oppositely charged polyelectrolytes.\textsuperscript{13} LbL is a simple, affordable, and versatile process that does not require any expensive or specialized equipment and allows a wide variety of components to be used as building blocks on a range of substrates, offering a number of key advantages while overcoming the limitations of the Langmuir-Blodgett (LB) approach\textsuperscript{14-18} and the self-assembled monolayer (SAM) method.\textsuperscript{19-22} For these reasons, the LbL technique has been used to fabricate functional thin films with tailored properties and controlled architectures.

Electrostatic binding is the main approach used in the formation of the multilayers.\textsuperscript{23,24} A general LbL procedure to form polyelectrolyte multilayer films via electrostatic interactions is illustrated in Scheme 2.1. A charged substrate, for example with a negatively charged surface, is first immersed into a polycation solution. The positively charged polyelectrolyte will adsorb onto the surface, leading to formation of a single layer. When the polyelectrolyte adsorption reaches equilibrium, the substrate is rinsed to remove any excess polymer. The reversal charge of the surface allows the adsorption of subsequent oppositely charged polyelectrolyte, i.e., negatively charged polyelectrolyte can then be adsorbed onto the positively charged surface. The sequential adsorption of these oppositely charged polyelectrolytes can be repeated to form multilayer films with desired thickness depending on the number of deposited layers. Thickness of the film can also be tailored by varying the LbL assembly conditions, including salt and pH of the solution,\textsuperscript{25-28} solvent polarity,\textsuperscript{27,29} and temperature of adsorption.\textsuperscript{30}
Scheme 2.1. Schematic illustration of LbL assembly of multilayer films based on electrostatic interaction. A negatively charged substrate is immersed into a polycation solution (i), followed by a series of washes to remove any non-adsorbed polymer (ii). The now positively charged substrate is then immersed into a polyanion solution (iii) with subsequent washing (iv). The sequential adsorption of these oppositely charged polyelectrolytes can be repeated to form multilayer films with desired thickness.

Besides electrostatic force, other molecular interactions such as hydrogen bonding, covalent interactions, DNA hybridization, hydrophobic interactions, charge-transfer interactions, metal-ligand coordination, and stereocomplexation have also been used to drive the LbL assembly process. Due to the choice of interactions, this assembly technique is applicable to a wide range of materials, including a variety of charged and noncharged polymers and other components such as nucleic acids, proteins, polypeptides, polysaccharides, viruses, and nanoparticles. LbL can be performed on planar surfaces (e.g., glass slides, quartz slides, gold substrates, and silicon wafers) and three-dimensional templates (e.g., porous and nonporous silica particles, polystyrene particles, gold nanoparticles, liquid crystal droplets, nanotubes, enzymes, model drugs, liposomes, and red blood cells). As such, LbL
assembly technique has proved to be a versatile and most common approach today for the formation of functional thin films.

2.2.2 LbL Capsule Engineering

In 1998, Donath et al. extended the LbL assembly technique to the formation of polymer capsules by the use of charged colloidal templates. The sequential adsorption of interacting polymers onto these substrates followed by the subsequent removal of the sacrificial cores leads to the formation of hollow polymer capsules (Scheme 2.2), which typically have a size range of 0.1 to 5 µm in diameter.

Scheme 2.2. Schematic illustration of the formation of hollow polymer capsules by LbL assembly technique. Interacting polymers (i and ii) are adsorbed onto a particle template; this step can be repeated until the desired number of layers is achieved (iii). Dissolution of the sacrificial template of the core-shell particle (iv) results in the formation of a hollow polymer capsule.

Polymer capsules provide a versatile platform for the encapsulation of different types of cargo, such as drugs, oligonucleotides, molecular dyes, enzymes, and peptides. There are two different strategies developed for encapsulating different types of substances within polymer capsules, namely pre-loading and post-loading (Scheme 2.3). The first method involves adsorption of cargo onto a template followed by subsequent deposition of polymer multilayers. As an
alternative, cargo-loaded emulsions and crystals or aggregates of the molecule of interest, e.g., crystallized small molecule dyes and drugs,\textsuperscript{58} protein crystals,\textsuperscript{56} protein aggregates,\textsuperscript{73} and condensed DNA\textsuperscript{74} can be used as the template themselves. Otherwise, cargo can be post-loaded to preformed capsules through diffusion driven by a concentration gradient between the external environment and the hollow cores of the capsules. This method usually involves changing the permeability of the capsule wall, which can be done by changes in pH\textsuperscript{75,76} or temperature.\textsuperscript{77} The encapsulation efficiency of the post-loading technique is typically low in comparison to the previously described pre-loading method; this is due to the lack of affinity of the cargo for the capsule interior.

\textbf{Scheme 2.3.} Strategies for cargo encapsulation in LbL-assembled polymer capsules. a) Pre-loading method: cargo is adsorbed onto the template prior to the LbL assembly of the membrane of the polymer capsule and b) post-loading method: cargo is infiltrated to the preformed capsule, which requires changes in the permeability of the capsule wall.

LbL-assembled capsules have attracted great interest in the past few years due to the simplicity of the assembly coupled with facile control over capsule chemical and structural properties, and have emerged as multifunctional carrier systems in a range of different applications, including drug/gene delivery, catalysis, optics, imaging, and sensing. The composition and permeability of the capsule membrane can be tailored through the choice of the deposited polymer and the number of layers adsorbed. The shell characteristics of the capsules can be engineered and
adapted to suit a wide range of applications, for example the surface of the capsules can be functionalized to include poly(ethylene glycol) (PEG) for low-fouling properties\textsuperscript{43,78,79} or antibodies for targeted therapeutic delivery.\textsuperscript{80,81} Furthermore, polymer capsules can also be custom-designed to respond to specific stimuli, such as pH, ionic strength, temperature, and presence of a chemical or enzyme, allowing triggered controlled release of the loaded substances.\textsuperscript{1}

As mentioned in Section 2.2.1, many different intermolecular interactions such as electrostatic interactions, hydrogen bonding, DNA hybridization, or covalent linking have been used to drive the multilayer buildup by LbL assembly. The most commonly investigated polyelectrolytes for electrostatic assembly include strong polyelectrolytes such as poly(ethyleneimine) (PEI), poly(styrenesulfonate) (PSS), poly(diallyldimethylammonium chloride) (PDADMAC) and weak polyelectrolytes such as poly(allylamine hydrochloride) (PAH) and poly(acrylic acid) (PAA) (Scheme 2.4).\textsuperscript{7}

![Scheme 2.4. Chemical structures of commonly used charged polyelectrolytes.](image-url)
Although electrostatics has been extensively used in the formation of LbL films, it is only applicable to charged materials. Furthermore, as polyelectrolyte multilayers are often highly charged, they typically exhibit high biofouling and are unsuitable for biomedical applications. One of the most commonly studied non-electrostatic interactions used to facilitate the assembly of uncharged polymers is hydrogen bonding. The first examples of multilayers assembled through hydrogen bonding were reported independently by Stockton and Rubner,\textsuperscript{82} and Zhang and co-workers\textsuperscript{83} in 1997. The study by Stockton and Rubner described a system based on the interaction of polyaniline (PAni) with poly(N-vinylpyrrolidone) (PVP), poly(vinyl alcohol) (PVA), polyacrylamide (PAAm), or poly(ethylene oxide) (PEO) (Scheme 2.5). On the other hand, Zhang and co-workers formed a multilayer film by the alternate deposition of PAA and poly(4-vinylpyridine) (P4VP) (Scheme 2.5).

**Scheme 2.5.** Chemical structures of polymers used to assemble LbL films based on hydrogen bonding.
To design hydrogen-bonded LbL films that can recognize specific stimuli, Sukhishvili and Granick prepared “erasable” multilayer systems based on poly(methacrylic acid) (PMA)/PVP, PMA/PEO, and PAA/PEO, which respond to changes in environmental pH. These polymer pairs remain stable via hydrogen bonding when the polyacids are protonated. As pH increases, the degree of ionization of these polyacids increases and when a critical charge density in the polyacids is reached, repulsion of the charged polymers and disruption in hydrogen bonds result in deconstruction of the film. It was found that the critical pH at which deconstruction takes place varies for different polymer pairs; the critical pH for PMA/PVP, PMA/PEO, and PAA/PEO is 6.9, 4.6, and 3.6, respectively.

Among the multilayer systems described above, hydrogen-bonded polymer pair based on PMA and PVP stands as one of the prominent polymer capsule systems with high colloidal stability. In addition, they are biocompatible, responsive over a biologically relevant pH range, and have been widely used in biomedical applications; therefore, the PMA/PVP system is selected as the driving force for the LbL assembly of polymer capsules for the formation of capsosomes. Polymer capsules based on PMA/PVP system is discussed in more detail in the next section (Section 2.3).

### 2.3 Poly(Methacrylic Acid) (PMA) Hydrogel Capsules

#### 2.3.1 Preparation and Properties of PMA Hydrogel Capsules

Polymer capsules formed by the assembly of PMA/PVP multilayer films owe their structural ability to intermolecular hydrogen bonds that form when PMA is protonated in acidic pH. These capsules, however, will spontaneously disintegrate at physiological conditions due to the ionization of PMA at pH above the $pK_a$ of PMA (i.e., above pH 6.5) and hence disruption of the hydrogen bonds. To endow these hydrogen-bonded capsules with stability at elevated pH, the constituent polymers need to be crosslinked. Nature-inspired thiol-disulfide interconversion was chosen to stabilize the PMA/PVP multilayers due to the biodegradability and reversible chemistry. To facilitate this crosslinking approach, PMA was functionalized with thiol
moieties \((\text{PMA}_{\text{SH}})\) and sequentially deposited with PVP onto particle templates.\(^{32,85}\) Upon completion of the polymer deposition steps, the thiol groups on the \(\text{PMA}_{\text{SH}}\) chains were converted into bridging disulfide linkages via oxidative crosslinking, e.g., with hydrogen peroxide or chloramine T,\(^{85}\) or via thiol-disulfide exchange,\(^{86,87}\) which resulted in films that remained stable at physiological pH. Removal of the core particles and release of uncrosslinked PVP at physiological conditions yield colloidally stable, (bio)degradable, single-component PMA hydrogel capsules (Scheme 2.6).\(^{32,72,85,88}\)

**Scheme 2.6.** Schematic illustration of the assembly of disulfide-stabilized PMA hydrogel capsules. A particle template is coated with LbL assembly of \(\text{PMA}_{\text{SH}}/\text{PVP}\) (red/blue) multilayer films via hydrogen bonding and this film is stabilized by crosslinking the thiols groups on the \(\text{PMA}_{\text{SH}}\) chains into disulfide linkages (i). Core dissolution (ii) and dispersion at physiological pH (iii) result in a single-component PMA hydrogel capsule.

Disulfide-stabilized PMA capsules have been used to encapsulate DNA,\(^{67-70}\) antigenic peptides,\(^{71,72}\) intact proteins,\(^{32}\) and lipophilic drugs.\(^{64,66}\) The LbL fabrication technique allows facile control over the wall thickness, permeability, stability and degradability of the capsules. The thickness and permeability of the capsules can be controlled by the number of deposited layers.\(^{89}\) In addition, the crosslinking process is highly versatile and permits physicochemical properties to be tailored by varying...
the crosslinking density – recent studies showed that the permeability of PMA capsules can also be tuned by varying the thiol content of the PMA\( _{\text{SH}} \) chains as well as the type of crosslinkers used to stabilize the multilayer film.\(^\text{87}\) PMA hydrogel capsules exhibit excellent colloidal stability over an entire physiological range of pH (pH 4-9) and no aggregation was observed in the presence of serum.\(^\text{85}\) These capsules can be deconstructed in the presence of a reducing agent, e.g., dithiothreitol or glutathione, which converts the disulfide linkages into thiol moieties.\(^\text{32,85,89}\) Furthermore, intracellular reducing environment also favors the cleavage of the disulfide linkages and allows the capsules to disintegrate, verifying their (bio)degradable nature. In the extracellular oxidizing environment, the disulfide bonds will preserve the capsule integrity. This makes PMA hydrogel capsules attractive candidates for biomedical applications, where triggered release of encapsulated cargo is highly desirable.

### 2.3.2 PMA Hydrogel Capsules in Encapsulated Catalysis

PMA hydrogel capsules are semipermeable in nature, that is, they are typically permeable to small molecules (monomers, ions, small organics, etc), but show size-dependent permeability to macromolecules, allowing confinement of catalytic enzymes and controlled communications with external milieu.\(^\text{69,70}\) These multilayer polymer capsules therefore are particularly suitable as vessels for encapsulated catalysis as they permit the continuous exchange of small substrate and product molecules.

The semipermeable nature of PMA hydrogel capsules was exploited by Price et al., who reported that by encapsulating both DNA and the endonuclease DNase I within the interior of the crosslinked PMA capsules, DNA degradation could be triggered through the external introduction of divalent cations (Ca\(^{2+}\) and Mg\(^{2+}\)) across the capsule walls, which activated the endonuclease (Figure 2.1a).\(^\text{69}\) Successful degradation of encapsulated fluorescently labelled DNA was confirmed via disappearance of the fluorescent signal from the capsule interior; while intact DNA is confined within PMA capsules, the small products of digestion diffuse across the
capsule walls (Figure 2.1b). This represents an advance toward developing cellular mimics in which continuous biochemical reactions can be confined and controlled. The selective retention of DNA, permeability to smaller nucleotides, and external triggering of the reaction were facilitated by the use of semipermeable PMA hydrogel capsules. Price et al. also utilized these capsules for continuous RNA synthesis from an encapsulated DNA template. Externally introduced RNA polymerase permeated the capsule walls and bound to the corresponding promoter sequence on the encapsulated double-stranded DNA, triggering RNA synthesis via transcription.

Figure 2.1. Triggered degradation of DNA in PMA hydrogel capsules. a) Degradation of DNA by DNase I and diffusion of the degraded products from PMA capsules. b) Red fluorescently labelled DNA coencapsulated with DNase I in green fluorescently labelled PMA capsules. Triggering the enzymatic activity causes digestion of the DNA and release of the low molecular weight products.

2.3.3 PMA Hydrogel Capsules in Therapeutic Delivery

The continuous development of new drug delivery systems is driven by the intrinsic issues associated with free drugs, such as poor stability and undesirable toxicity. To overcome these limitations, scientific interest has been dedicated toward the incorporation of these drugs into macromolecular and colloidal carriers, which not only increases the payload of the therapeutics but also has the potential to protect the therapeutic molecules from degradation in vivo, control the rate of drug release at a specific site of action, and lower the exposure of healthy cells to toxic side effects. As mentioned in Section 2.3.1, PMA hydrogel capsules exhibit excellent colloidal stability, drug loading capability, and degradation mechanism in respond to physiological triggers to facilitate cargo release under intracellular reducing
environment. Furthermore, recent studies have shown that 300 nm-, 500 nm-, and 1 µm-diameter PMA capsules were effectively internalized by a panel of mammalian cells (neuronal, muscle, ovary, kidney, macrophages, etc) and they exhibit negligible inherent cytotoxicity. These characteristics therefore make PMA capsules superior candidate carriers for applications in drug delivery.

PMA capsules have proven to be unique carriers for anticancer therapeutics. Sivakumar et al. reported successful encapsulation of oleic acid-solubilized anticancer drugs, such as doxorubicin and 5-fluorouracil, into 500 nm- and 1 µm-diameter PMA capsules and demonstrated their in vitro drug release and anticancer effect on human colorectal cancer cell line LIM1215. It was shown that while non-loaded PMA capsules were non-toxic to cells, oil-solubilized doxorubicin loaded-PMA capsules exhibited a more pronounced antiproliferation activity in comparison to an equivalent amount of doxorubicin administered onto cultured cells in its free form, which did not significantly affect cell viability (Figure 2.2). This confirms that the drug-loaded PMA capsules are more effective in killing the cancer cells than the free drugs, highlighting their therapeutic benefits, and therefore supports the underlying rationale behind the development of drug carriers.

In the area of vaccination, PMA hydrogel capsules were loaded with antigenic epitopes (KP9 oligopeptides, whole ovalbumin (OVA) protein, or immunogenic OVA-derived peptide sequence) and their ability to activate specific T cells was investigated. Due to the low molecular weight, the encapsulation strategy for short KP9 oligopeptide antigens in PMA capsules was achieved via their conjugation to a carrier polymer. For overall success in vaccination, these carrier capsules need to be internalized by antigen presenting cells (APCs). Upon intracellular processing, the capsules can subsequently release the encapsulated immunogenic peptides and proteins to the APCs. Recent studies have shown that the loaded capsules were effectively internalized by APCs, and effective stimulation of the specific CD4 and CD8 T cells was demonstrated in vitro and in vivo, implying the successful delivery of therapeutic payload by PMA capsules. These results therefore highlight the utility of PMA hydrogel capsules as multifunctional therapeutic carriers.
Figure 2.2. Cell viability assay of LIM1215 colon cancer cells after incubation with drug-loaded PMA capsules or drug in its free form. a) untreated cells; b) doxorubicin-oleic acid loaded 1 µm PMA capsules; c) doxorubicin-oleic acid loaded 500 nm PMA capsules; d) 5-fluorouracil-oleic acid loaded 500 nm PMA capsules; e) free doxorubicin at 0.1 mg mL⁻¹; f) free doxorubicin at 0.05 mg mL⁻¹; g) free 5-fluorouracil at 0.1 mg mL⁻¹; h) free doxorubicin at 5 ng mL⁻¹; i) doxorubicin-oleic acid loaded 500 nm PAH/PSS capsules; j) supernatant solution of doxorubicin-oleic acid loaded 500 nm PMA capsules; k) naked oleic acid emulsions; l) oleic acid loaded 500 nm PMA capsules; and m) oleic acid loaded 1 µm PMA capsules.

2.4 Liposomes

2.4.1 Preparation and Properties of Liposomes

Liposomes are supramolecular assemblies of amphiphilic lipids, which self associate to enclose an aqueous compartment surrounded by a lipid bilayer (Scheme 2.7). A number of different approaches have been used to assemble these lipid vesicles, including thin-film hydration, solvent injection, reverse-phase evaporation, sonication, and membrane extrusion. The first method – thin-film hydration – remains the most widely used method for the preparation of liposomes. In this case,
Lipids must first be dissolved in an organic solvent, e.g., chloroform, and the solvent is then evaporated under nitrogen, yielding a thin lipid film on the sides of a round bottom flask. When these thin films are hydrated, lipid vesicles are formed. The size of these vesicles can be reduced through sonication or extrusion (forcing the vesicles through polycarbonate membranes with defined pore sizes). Once formed, liposomes are typically 20 nm to 10 μm in diameter with the phospholipid bilayer about 4-5 nm thick. Their charge and surface properties can be easily tuned simply by varying the composition and chemistry of the phospholipids (e.g., lipid headgroup charge and saturation of the lipid alkyl chains) or adding new components to the lipid mixture during the preparation methods.

Due to the presence of hydrophobic and hydrophilic domains, a variety of therapeutic cargo (e.g., anti-cancer drugs, DNA, peptide vaccines, enzymes, and imaging agents) can be loaded into this assembly; hydrophobic drugs are loaded into the liposome membrane while hydrophilic molecules can be encapsulated in the aqueous core. Liposomal properties have been continuously improved to make them more robust and to overcome the lack of control over degradability through the addition of cholesterol to the lipid bilayer membrane; coating their surface with polymers, e.g., PEG or PVP; and incorporating polymerizable lipid amphiphiles into the membrane. A number of different stimuli have been exploited to trigger the release of the encapsulated cargo from liposomes, including temperature, pH.
light, redox potential, magnetic fields, near IR, and ultrasound each with its specific advantage depending on the applications.

2.4.2 Liposomes in Encapsulated Catalysis

Liposomes are intensively studied in different areas of biomimetic chemistry and cell mimicry, and have been widely used as compartment structures for encapsulated enzymatic catalysis. Enzyme molecules can be encapsulated inside liposomes without chemical modification, therefore preserving their affinity to cofactor and substrate molecules. Like cellular bilayer membranes, liposomal membranes are impermeable to macromolecules, ionic species, and many small organic molecules. However, controlled interaction between the liposome interior and external environment can be achieved by the aforementioned stimuli (temperature, pH, light, redox potential, magnetic fields, near IR, and ultrasound; Section 2.4.1) and also by the formation of transient pores in the lipid membranes, e.g., by incorporating gramicidin or by interaction with certain polymers. These properties allow spatial separation of different chemical environments and make liposomes excellent candidates as encapsulated reaction vessels.

Several studies have been reported on confined enzymatic reactions in liposomes, especially resembling those that typically occur in biological cells. These include synthesis of nucleic acids and proteins inside the lipid vesicles, accomplished by encapsulating the components necessary for the biosynthesis within the liposome compartments. Shohda and Sugawara incorporated DNA template, deoxynucleotide triphosphates (dNTPs – monomer of DNA), and DNA polymerase (an enzyme that catalyzes the polymerization of dNTPs) into liposomes and observed the generation of new DNA strands on the inner surface of the vesicles. In another study, Mansy and co-workers encapsulated bacterial DNA replication components derived from thermophilic bacteria into liposomes to perform intravesicle DNA amplification. Additional examples of DNA synthesis inside liposomes include amplification of DNA strands via polymerase chain reaction (PCR). PCR components such as DNA polymerase, DNA template, primers, and dNTPs were encapsulated inside lipid
vesicles and thermal cycles allowed successful amplification of DNA strands. A recent report described by Sugawara and co-workers showed that 1229 bp DNA fragment encoding green fluorescent protein (GFP) can be enzymatically replicated in the lipid vesicles using a real-time PCR technique and the PCR product was confirmed by polyacrylamide gel electrophoresis.

The first study on protein expression inside liposomes is pioneered by Luisi and co-workers, who reported the successful synthesis of polyphenylalanine inside conventional submicrometric lipid vesicles by coencapsulating poly(U) template, phenylalanine, ribosomes, tRNA, and translational factors within the aqueous compartment of the liposomes. Other studies have also shown the confined expression of functional GFP, Qβ replicase, or β-glucuronidase; two-step cascade synthesis of T7-RNA polymerase and GFP; synthesis of α-hemolysin and GFP (synthesized α-hemolysin formed a membrane pore that made the vesicle membrane more permeable to allow entrance of fresh nutrients leading to GFP production); and synthesis of glycerol-3-phosphate acyltransferase and lysophosphatidic acid acyltransferase, demonstrating the flexible nature of liposome nanoreactors.

Overall, the successful intravesicle enzymatic catalysis for synthesis of biomolecules demonstrated that liposomes do not inhibit the activity of the enzymes and the molecular components, and therefore are suitable as compartment structures to reconstitute cellular functions.

2.4.3 Liposomes in Therapeutic Delivery

Liposomes have also been widely used as therapeutic carriers because they are biocompatible, degradable in vivo, and have low immunogenicity and excellent safety profiles in humans. The lipid bilayer surface can be functionalized with low-fouling polymers, e.g., PEG, to form stealth liposomes. Surface functionalization of liposomes with PEG can be achieved in several ways, for example, by physical adsorption or covalent attachment of PEG chains onto the surface of the preformed
liposomes, or by incorporation of PEG-lipid conjugates to the lipid mixture during liposome preparation.\textsuperscript{120} The presence of the PEG chains on the liposome surface provides steric repulsion between the lipid vesicles, thus improving their stability. Moreover, the surface modification of liposomes with PEG improves their blood circulation half-life due to reduced interaction with plasma and cell-surface proteins and this feature is desirable to improve the bioavailability of the therapeutic payload.\textsuperscript{93,120}

Active targeting of the drug delivery vector can be achieved by functionalizing liposomes with specific ligands or antibodies that bind to receptors or antigens on the target cells.\textsuperscript{93} The liposomes can be internalized into target cells before the drug is released, which therefore minimizes toxicity to other healthy cells. Due to these attractive features, liposomes are among the first and one of the most successful delivery systems to date. Developments in this area have resulted in the approval of several liposomal drugs for clinical applications, which include doxorubicin (Doxil and Myocet) or the chemically similar daunorubicin (DaunoXome) for cancer treatment, and anti-fungal amphotericin B (AmBisome).\textsuperscript{120} Other drugs, such as antibiotic ciprofloxacin and chemotherapy agent vincristine, have been successfully encapsulated in liposomes with excellent retention from weeks to months in buffer; however, they are released from the liposomes within minutes in physiological environments\textsuperscript{121,122} – this aspect severely limits the efficacy of unilamellar liposomes as drug carriers, hence development of structures that can enhance retention of therapeutic molecules in liposomes is required.

2.5 Current Development of Artificial Cells

Engineering artificial cells is a relatively young field of research and includes several different concepts to reconstruct cellular components into synthetic compartments.\textsuperscript{123-130} Recent remarkable achievements include the successful creation of artificial red blood cells\textsuperscript{131,132} and the first self-replicating, synthetic bacterial cell containing man-made, chemically synthesized genome.\textsuperscript{133} Designing cell-like structures therefore has attracted increasing attention as it provides
Chapter 2

potential methodologies that will open up new and unique prospects for therapeutic applications in the future – from enzyme therapy to new biomedical devices and self-regulating bioreactors. Mimicking biological cells requires the understanding of the cells’ key features, some of them include: (i) cell membrane, which separates the cell interior from the external environment and controls the passage of nutrient molecules and waste into and out of the cells; (ii) cell organelles, which are specialized subunits within a cell with the ability to carry out specific metabolic activities; (iii) cascades of chemical reactions in cells, which are catalysed by active enzymes; and (iv) self-regulating system to respond to specific stimuli. With the complexity of biological cells, the ability to mimic cellular functions currently still lags behind the understanding of the underlying mechanism and this has prompted many researchers to simplify the objectives of cellular biomimicry into smaller, readily achievable goals; hence, the current synthetic cell systems fulfil only typically one simple cellular function.

Artificial cells can be assembled via two methods; top-down or bottom-up approaches. The former concept employs biological components and involves the encapsulation of whole cells or organelles into a system. For example, cells were immobilized within a semipermeable polymer membrane, which allows for the transport of nutrients, oxygen, and therapeutic agents. The latter concept on the other hand refers to an encapsulation strategy where (macro)molecular building blocks are assembled into functional components via directed- and/or self-assembly techniques to mimic a living cell. This bottom-up approach not only allows nanoscale control over material properties, but also provides the ability to construct artificial cells with specific functionality without the inherent complexities of biological cells. The bottom-up approach starts with the construction of a cell model in a suitable compartment to allow the encapsulation of biochemical reactions. To date, lipid vesicles have been widely used as the compartment structures for artificial cells because: (i) the boundary of unilamellar lipid vesicles is similar to the lipid matrix of the cell membrane; (ii) the size of these vesicles is in the range of the size of cells; and (iii) liposomes can undergo budding and fission, similar to biological cells. It is therefore straightforward to use liposomes as cell-mimicking
compartments to perform encapsulated enzymatic reactions that resemble those occurring in biological cells. Examples of enzymatic catalysis in liposomes were described in Section 2.4.2 and they include synthesis of nucleic acids, DNA amplification via PCR, and protein expression inside the lipid vesicles. As liposome membranes are impermeable to many molecules, fabrication of advanced cell mimics typically requires the incorporation of protein transport channels into the liposome membranes to allow the controlled movement of substrate and products across the membrane.\(^{103,118}\)

A novel biomimetic system was recently reported by Li and co-workers, describing early and important examples of cell mimics based on LbL-assembled polymer capsules.\(^{144-146}\) Adenosine triphosphate (ATP) synthase, an important enzyme that drives the production of ATP (energy) for the cell, was incorporated within a lipid bilayer membrane that coated the shell of polymer capsules, mimicking the molecular motors in biological cell membranes. The transport of protons across the capsule walls was facilitated by the enzymes and the resulting proton gradients drove the ATP synthesis inside the capsules. These ATP synthase-containing polymer capsules serve as an energy storage container and this study will contribute to a better understanding of the function of ATP synthase in biology.

Although the aforementioned cell-mimicking systems are already remarkably complex, they are still based on single-compartment structures. Biological cells contain a large number of organelles that provide a confined environment able to effectively facilitate division and regulation among thousands of enzymatic (cascade) reactions. This hierarchical structure is a prerequisite for biological organization, hence a compartmentalized system has increasingly become recognized as an essential key feature in designing artificial cells.\(^{128}\) These cell mimics are engineered for specific tasks related to therapeutic applications and this research direction has been on the ultimate agenda of synthetic biology.
2.6 Biomimetic Compartmentalized Assemblies

Mimicking biological cells using bottom-up assembly has led to the development of several compartmentalized assemblies, which is a key requirement for the development of artificial cells. Designing hierarchical systems that mimic the compartmental organization of biological cells not only sequesters diverse chemical reactions within a single carrier but also allows coencapsulation of diverse molecules that are spatially confined.

2.6.1 Subcompartmentalized Polymer Capsules

Multilayer polymer capsules fabricated by the LbL assembly technique have emerged as versatile components for the assembly of multicompartment particles due to their desirable physicochemical properties. The compartmentalization facilitates segregation of multiple enzymes for spatial control of enzymatic cascade reactions. For example, Kreft et al. assembled concentric two-compartment polyelectrolyte capsules (Scheme 2.8a) containing glucose oxidase (GOX) and peroxidase (POD), confined to the outer and inner compartments, respectively. In the presence of appropriate substrates, GOX and POD catalyze a cascade reaction producing resorufin (Figure 2.3a), which can be detected using fluorescent techniques. The two-compartment capsules were fabricated by sequential coprecipitation of the enzymes with calcium carbonate (CaCO₃) followed by LbL assembly onto the particles and core dissolution. Glucose was oxidized by GOX to produce H₂O₂ that diffused through the polymer multilayers into the inner compartment. Following the addition of amplex red, an increase in red fluorescence due to production of resorufin was observed in the inner capsule compartment. Formation of resorufin could not occur in the outer compartment or free solution because of the confinement of POD to the inner compartment. It was proposed that the eventual increase in fluorescence in the outer compartment was due to steady state diffusion of resorufin from the inner compartment. Although qualitative in character, the biomimetic confinement of a cascade enzymatic reaction was demonstrated.
Scheme 2.8. a) Concentric two-compartment capsule showing inner (white) and outer (red) capsules. b) Pericentric capsule showing a carrier capsule (red) decorated with multiple subcompartments (white). Subcompartments may be contained within the carrier capsule or adsorbed onto the surface. c) A Janus capsule formed via anisotropic deposition of subcompartment capsules (white) to the surface of the carrier capsule.

Using the enzyme-CaCO$_3$ coprecipitation approach, Bäumler et al. fabricated a three-compartment enzyme particle system containing β-glucosidase (βGLU), GOX, and POD confined to outer, middle, and inner compartments, respectively. As illustrated in Figure 2.3a, the start of the cascade reaction was detected by increased green fluorescence in the outer compartment, caused by βGLU catalyzed hydrolysis of a nonfluorescent substrate into glucose and fluorescein. Glucose thus triggered the GOX-POD cascade, the end of which was detected by increased red fluorescence confined to the inner compartment. Particles with barrier compartments of bovine serum albumin (BSA) located between the enzyme compartments showed a retarded reaction rate in comparison to particles without barrier compartments (Figure 2.3b). This was attributed to the additional diffusion resistance for substrate molecules resulting from the barrier layers. By tailoring barrier thickness, these structures may allow individual kinetic parameters in complex enzyme cascades to be determined.
Figure 2.3. a) Enzymatic cascade reaction performed in three-compartment enzyme particles using fluorogenic substrates to allow monitoring of reaction kinetics. b) Time-dependent increase in resorufin fluorescence for three-compartment three-enzyme particles and five-compartment three-enzyme particles, i.e., particles containing BSA barrier compartments between enzyme compartments. Green represents fluorescein located in the outer compartment, and blue represents Alexa Fluor 680-labeled BSA (not enzymatically active).

Alternatively, pericentric-type multicompartment capsules can be produced by adsorbing small capsules onto template particles followed by additional multilayer assembly on these coated particles (Scheme 2.8b). Pericentric assembly facilitates incorporation of a greater number of subcompartments compared with concentric assembly, potentially leading to higher enzyme loading. Using this approach, Kulygin et al. assembled disulfide-stabilized PMA capsules containing smaller PMA capsule subcompartments. 300 nm-diameter crosslinked PMA capsules were first adsorbed onto 3 μm-diameter aminated silica (SiO$_2$) particles, followed by the assembly of PVP and PMA$_{5H}$ multilayers onto the capsule-coated SiO$_2$ particles. Control over the
number of subcompartments was achieved by varying the ratio of 300 nm capsules to 3 μm SiO$_2$ particles. Crosslinking of the outer PMA$_{SH}$ multilayers with a bifunctional thiol-reactive crosslinker and subsequent silica dissolution afforded PMA carrier capsules subcompartmentalized with smaller PMA capsules. Selective degradation of the subcompartments or carrier capsules could be achieved by employing either degradable or nondegradable crosslinkers. Moreover, subcompartments responsive to different stimuli incorporated into the capsules could be selectively degraded without damaging the integrity of the carrier capsules, thus demonstrating the potential for triggered mixing of macromolecular substrates and enzymes encapsulated within the subcompartments.

Fabrication of capsules with anisotropic composition, or Janus capsules (Scheme 2.8c), is attracting increasing interest as they may act as large amphiphiles, allowing the self-assembly of supramolecular aggregate structures.$^{149,150}$ To fabricate anisotropic multicompartment capsules, Delcea et al. first produced small (500 nm-diameter) and large (4.8 μm-diameter) polyelectrolyte capsules.$^{151}$ The large capsules were then embedded in a multilayer film of hyaluronic acid (HA) and poly(L-lysine) (PLL) supported on a glass slide. Partial embedding of the large capsules was observed, thus allowing the anisotropic adsorption of small capsules to the surface of the large capsules by electrostatic interactions. Multicompartment Janus capsules were isolated by inversion of the glass slide followed by the addition of sodium hydroxide, causing disassembly of the HA/PLL film without compromising the integrity of the Janus capsules.

2.6.2 Vesosomes (liposomes-in-liposomes)

Zasadzinski and co-workers have developed multicomartment liposome architectures, termed vesosomes, where small liposomes are entrapped within a larger liposome (Scheme 2.9a).$^{2,152,153}$ These assemblies are formed via a temperature-induced approach to form vesicles from lipid bilayer sheets. Below phase transition temperature ($T_m$), the addition of ethanol to aqueous dispersions of saturated phospholipids, e.g., dipalmitoylphosphatidylcholine, leads to the
formation of interdigitated bilayer sheets. When these bilayers are heated at elevated temperature above the $T_m$, the membrane sheets become less rigid and spontaneously close to form unilamellar lipid vesicles. During the transition from bilayer sheets to closed vesicles, these interdigitated membranes can simultaneously encapsulate preformed cargo-loaded liposomes, thus form a multicompartment liposome assembly. The encapsulation process does not require specific recognition or other chemical or mechanical processing. In addition, the newly generated vesicles are capable of maintaining their structural integrity, even at temperatures below the $T_m$.

Scheme 2.9. Liposome- and polymersome-based subcompartmentalized assemblies. a) Liposomes-in-liposomes (vesosomes) and b) polymersomes-in-polymersomes.

The potential of vesosomes to act as therapeutic carriers is evidenced by the successful encapsulation of DNA, a model drug prochlorperazine, or antibiotic ciprofloxacin within the compartments as well as the sustained release profile of the encapsulated cargo.\textsuperscript{152,153} As the bilayer-within-a-bilayer structure forms a physical barrier to direct interaction between the internal compartments and external environment, vesosomes efficiently protect the internal vesicles from degradation under biomedically relevant conditions, i.e., in the presence of serum or degradative
enzymes, such as phospholipases, that can otherwise lyse the vesicular structures and result in premature release of the encapsulated cargo. The additional bilayer membrane therefore delays cargo leakage. In serum, enhanced ciprofloxacin retention was reported (from <10 min in unilamellar liposomes to ~6 h in vesosomes with more than 40% of the encapsulated cargo still retained even after 10 h incubation in serum at 37 °C). Upon exposure to phospholipases, the multicompartiment liposome assembly extends cargo retention by two orders of magnitude in comparison to unilamellar liposomes of the same composition. Vesosomes therefore possess several advantages compared to unilamellar liposomes because: (i) release profiles of encapsulated molecules can be controlled and extended over a longer period of time; (ii) different cargo can be encapsulated in well-defined ratios within a single carrier and delivered simultaneously; and (iii) the interior vesicles can be prepared from entirely different phospholipid compositions from one another and from the exterior vesicles – affording this system with tunable stability and degradation properties. Given the multicompartiment nature of vesosomes, this carrier system has been proven to overcome the major limitation of unilamellar liposomes, i.e., the premature cargo release in physiological environments. Hence, it is envisaged that vesosomes would be a viable alternative for the delivery of weakly basic drugs, which leak too rapidly from liposomes. Vesosomes also retain the essential features of conventional unilamellar liposomes; one of which is the possibility to functionalize the surface of the vesicles with inert, biocompatible polymers such as PEG, which remains a gold standard for preparing long-circulating liposomes. Wong et al. reported that PEG-lipid coating prevents vesosome aggregation in blood and leads to a half-life of ~2 h in mice with similar biodistribution patterns to those of conventional unilamellar liposomes.

In another study, Mishra et al. pioneered the use of vesosomes for transcutaneous immunization (TCI), where the model antigen, tetanus toxoid (TTx), was encapsulated within the internal liposomal compartments. It was observed that TCI with vesosomal systems significantly increased the level of anti-TTx antibodies in an in vivo model in comparison to topically administered cationic unilamellar
liposomes or a free TTx formulation, suggesting a more effective stimulation of the immune response, possibly due to better localization of the internal vesicles along with the encapsulated antigen in a confined environment. These findings suggest the potential of vesosomes for effective topical delivery of vaccines.

Vesosomes incorporating different types of liposomes can also be used to conduct sequentially mixed reactions. Encapsulation of dichlorodimethylacridinium phosphate into liposomes consisting of 1,2-dimyristoyl-sn-glycero-3-phosphocholine/1,2-dimyristoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] \( (T_m = 23 \, ^\circ C) \) and fluorescein diphosphate into liposomes consisting of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine/1,2-dipalmitoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] \( (T_m = 41 \, ^\circ C) \) was performed prior to incorporation of both liposome populations into vesosomes.\(^{155}\) Increasing the temperature above the \( T_m \) of each subcompartment caused sequential release of the encapsulated cargo into the outer vesosome compartment, where conversion to fluorescent products by alkaline phosphatase occurred. Such assemblies are gaining increasing interest as they allow the study of enzymatic reactions at the single molecule level, facilitating the observation of previously unobserved enzyme behavior.

**2.6.3 Other Subcompartmentalized Assemblies**

**2.6.3.1 Polymersomes-in-Polymersomes**

Similar to vesosomes, multicompartment polymersome (synthetic mimics of liposomes formed via the self-assembly of amphiphilic block copolymers) assemblies have also recently been developed (Scheme 2.9b). Chiu et al. reported the first example of a polymersome-in-polymersome assembly prepared by a double emulsion technique in a water/oil/water system.\(^{156}\) In this system, the outer and inner compartments are comprised of poly(acrylic acid)-co-poly(distearin acrylate) (poly(AAc-co-DSA)) polymersomes equipped with pH-responsive transmembrane channels, allowing controlled release of encapsulated cargo in response to environmental pH changes; this pH-responsive process is reversible in the range of pH 5 to 8. While these polymersomes are impermeable to small hydrophilic solutes
such as calcein at pH 5, a change in the ionization state of the AAc units at pH 8 results in the formation of pores and subsequent transport of the encapsulated cargo across the membranes.

Multicompartment polymersomes prepared via a double emulsion technique were also reported by Weitz and co-workers. Using a capillary microfluidic device, polymersomes with multiple internal compartments were produced with two distinct inner phases containing different model encapsulants without the risk of cross-contamination. The novelty in this design lies in the ability to control the number as well as the size of the compartments in the assembly. The former is achieved by tuning the flow rates of the phases in the microfluidic channels, while the latter can be precisely controlled by the size of the inner droplets. The general nature of this approach is expected to enable the fabrication of liposomes or other synthetic bilayer vesicles with a controlled number of multiple compartments.

Another recent development in compartmentalized polymer vesicles is a multicompartment system based on polymersomes of different compositions for the carrier and the subcompartments, as reported by Nallani and co-workers. Non-permeable poly(2-methyloxazoline)-block-poly(dimethylsiloxane)-block-poly(2-methyloxazoline) (PMOXA-PDMS-PMOXA) polymersomes were entrapped within semi-permeable polystyrene-block-poly-(L-isocyanoadipine(2-thiophen-3-yl-ethyl)amide) (PS-PIAT) polymersomes and it was demonstrated that a model protein (cyanine-5 conjugated immunoglobulin G) could be encapsulated within PS-PIAT polymersomes without leakage into the PMOXA-PDMS-PMOXA subcompartments. These multicompartment polymersome assemblies formed using different polymersomes for the carrier and subcompartments have emerged as promising cell and cellular organelle mimics, as they afford increased levels of spatial organization and complexity. Incorporation of polymersome subcompartments responsive to different stimuli would permit sequential mixing of multiple encapsulated cargo and subsequent reactions.
While this assembly is envisaged to serve as a suitable prototype for cell mimics, it is also particularly promising as a vector in therapeutic applications because it facilitates the confinement of different therapeutic components within specific subcompartments. In addition, the multicompartment system can be designed such that different polymersomes release their encapsulated cargo in response to different chemical or environmental stimuli.

2.7 Literature Summary

LbL-assembled polymer capsules and liposomes have emerged as versatile carrier systems for biomedical applications. Multidisciplinary efforts to engineer capsule- and liposome-based subcompartmentalized assemblies have led to astonishing progress and success in creating cell-like structures; however, significant challenges still remain. The findings reported in the literature revealed that the current subcompartmentalized assemblies are based on single-component systems. While each platform, polymer capsules and liposomes, fulfil in part some of the requirements as building blocks for the synthetic cells, they have some inherent limitations in their native forms. For instance, although polymer capsules possess the desired structural integrity and control over degradation properties as well as being well-suited for the encapsulation of macromolecular cargo, the semipermeable nature makes them unsuitable for the encapsulation of small, fragile therapeutic molecules and reagents as they can freely diffuse across the capsule walls. Liposomes, on the other hand, provide effective encapsulation for small- and medium-sized cargo but can be susceptible to structural stability and are largely impermeable to their surroundings.

Multi-component compartmentalization assembly will allow the incorporation of a range of structures in a single carrier system, thus allowing the optimization of physicochemical properties of the assembly, e.g., permeability, stability, and stimuli-response, which are not achievable using single-component assemblies. In view of this, the development of novel biomimetic subcompartmentalized assembly, termed capsosomes, which unite the two fundamentally different systems – polymer
capsules and liposomes, will be presented in the remainder of the current thesis and the applications en route to functional therapeutic artificial cells are highlighted. By combining these two systems, capsosomes present an attractive biomedical platform for the creation of artificial cells because they retain the beneficial properties of both polymer capsules and liposomes, while overcoming the inherent limitations of each individual component. The structural stability of capsosomes, the ability to load diverse cargo within their subcompartments, the responsiveness of both capsules and liposomal subcompartments to specific stimuli, and the controlled interaction between the internal and external milieu make capsosomes an advanced candidate platform for the bottom-up assembly of artificial cells.
2.8 References

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Chapter 3

Instrumentation and Techniques

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3.1 Aim

This chapter describes the fundamental operating principles for each instrumental technique employed in this thesis. Primary emphasis is given here to the theoretical principles, the general setup of the corresponding instruments, and their function in characterization of materials. Detailed experimental materials and protocols will be presented in relevant chapters.

3.2 Instrumentation and Techniques

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

Quartz Crystal Microbalance is a simple and cost-effective mass sensing technique that monitors mass deposition on the surface of a quartz crystal based upon the piezoelectric effect. The piezoelectric quartz crystal produces an electric charge when subject to mechanical stress. Conversely, a mechanical oscillation is produced when an electric field is applied, which results in the generation of a transverse acoustic wave of characteristic resonance frequency \( f \) that propagates across the thickness of the crystal (Scheme 3.1a). When a thin film is adsorbed onto the surface of the crystal, the acoustic wave propagates into the film (Scheme 3.1b). Thicker film results in a longer acoustic wavelength and therefore lower resonance frequency compared to a crystal with no adsorbed layer on the surface. The direct correlation between the changes in resonance frequency and the thickness of the layer enables determination of mass changes using the Sauerbrey equation (Equation 3.1):

\[
\Delta f = \frac{-2f_0^2 \Delta m}{A\sqrt{\mu_q \rho_q}}
\]

(Equation 3.1)

where \( \Delta f \) (Hz) is the measured frequency change, \( f_0 \) (Hz) is the resonance frequency, \( \Delta m \) (g) is the mass change, \( A \) (cm\(^2\)) is the piezoelectrically active area of the quartz crystal, and \( \mu_q \) and \( \rho_q \) are the shear modulus and density of the quartz crystal (\( \mu_q = 2.947 \times 10^{11} \text{ g cm}^{-1} \text{ s}^{-2} \) and \( \rho_q = 2.648 \text{ g cm}^{-3} \)).
Scheme 3.1. Schematic illustration of an oscillating quartz crystal in QCM: (a) at its fundamental resonance frequency and (b) after the adsorption of a layer on the surface of the crystal.

This Sauerbrey equation is valid if the mass changes are uniformly distributed and the adsorbed films are thin and rigid. In some cases, the adsorbed films are soft. A soft film will not fully couple to the quartz oscillation, resulting in dampening of the oscillation. In such situation, the Sauerbrey equation will underestimate the mass of the surface and therefore becomes invalid. The damping, or dissipation of the film (D), can be measured by recording the response of a freely oscillating crystal at its resonance frequency. D gives an indication of the viscoelastic properties of the film and is defined as:

\[
D = \frac{E_{\text{lost}}}{2\pi E_{\text{stored}}} \quad \text{(Equation 3.2)}
\]

where \(E_{\text{lost}}\) is the energy lost (dissipated) during one oscillation cycle and \(E_{\text{stored}}\) is the total energy stored in the oscillator. By applying a Voigt viscoelastic model, the properties of the soft film (viscosity, elasticity, and thickness) can be characterized in detail.\(^5\)
QCM has been largely used for mass measurements in vacuum, gas, and liquid phase.\textsuperscript{1,6,7} In this thesis, QCM-D (Q-sense E4, Sweden) is used to monitor the adsorption of polymer films and liposomes on planar surfaces.

### 3.2.2 Flow Cytometry

Flow cytometry is a powerful technique that uses the principles of light scattering to count and analyze microscopic bodies suspended in a liquid stream. It has been widely used for cell analysis\textsuperscript{8} in medical research applications and has recently been extended to characterize thin films on colloidal particles or polymer capsules.\textsuperscript{9} Flow cytometer performs the analysis by passing thousands of particles per second through a laser beam and capturing the light that emerges from each particle as it passes through. By monitoring the interactions between the particles and the irradiated light beam, flow cytometer generates simultaneous multiple parameters analysis to report physical and chemical characteristics, including size, structural complexity, and fluorescence intensity.

The setup of a basic flow cytometer is presented in Scheme 3.2, which consists of four main components: (i) a flow cell, which directs liquid stream containing particles through focused laser beam; (ii) laser (the light source); (iii) optics, which gather and direct the light; and (iv) detectors. The flow cell is the central to the flow cytometer setup. Here the particles are injected into a stream of sheath fluid and hydrodynamically focused where they are positioned in line for measurement. As each particle passes through the focused laser beam, it will scatter light and the light signals (forward and side scattered) coming from individual particles are then collected by a series of detectors. A detector in line with the laser beam collects the forward scatter data, while the detector perpendicular to the laser beam collects the side scatter data. Forward and side scatters provide information about the particle size and structural complexity, respectively.
Fluorescent molecules (fluorophores) that are present in the samples are also excited by the laser beam and the fluorescence emission of the particles is collected by detectors with the appropriate filters. By analyzing the changes in fluorescence intensity, flow cytometry measurements can be used to monitor the buildup of polymer and liposome multilayers onto a particle template and to examine the encapsulation and release of biomolecules from polymer capsules and capsosomes. This technique is also used to perform accurate volumetric particle counting. All flow cytometry measurements were performed on a CyFlow Space (Partec GmbH, Germany) using an excitation wavelength of 488 or 633 nm. At least 20,000 particles or capsosomes were analyzed in each experiment.

3.2.3 Ultraviolet-Visible (UV-Vis) Absorption Spectroscopy

Ultraviolet-Visible (UV-Vis) spectroscopy allows for the quantitative determination of the concentration of specific compounds in solution via the measurement of the attenuation of light upon irradiation of a sample\(^\text{10}\). When light is passed through a sample, photons collide with the molecules in the sample and raise them to an excited state; this process is known as absorption. According to Beer-Lambert law, the amount of light absorbed is directly proportional to the sample concentration, as shown in Equation 3.3\(^\text{11}\).
\[ A = -\log_{10}\left( \frac{I}{I_0} \right) = \varepsilon cl \]  

(Equation 3.3)

where \( A \) is the measured absorbance, \( I \) is the intensity of the transmitted light, \( I_0 \) is the intensity of the incident light, \( \varepsilon \) (L mol\(^{-1}\) cm\(^{-1}\)) is the extinction coefficient of the absorbing species, \( c \) (mol L\(^{-1}\)) is the concentration of the sample in the solution, and \( l \) (cm) is the pathlength of the light through the sample.

A typical setup of a UV-Vis spectrophotometer consists of: (i) a light source; (ii) a diffraction grafting in a monochromator to separate different wavelengths of light; (iii) a sample holder; and (iv) a photodiode detector (Scheme 3.3). UV-Vis spectrophotometer uses two light sources, a deuterium arc lamp and a tungsten lamp for UV (190 – 380 nm) and visible (380 – 800 nm) measurements, respectively. In a single beam spectrophotometer, all of the light passes through the sample holder. The intensity of the transmitted light (\( I \)) is compared to the intensity of the light before it passes through the sample (\( I_0 \)). Absorbance reading is then obtained based on the ratio \( I/I_0 \) using the Beer-Lambert law. A NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) were used for all UV-Vis measurements.

Scheme 3.3. Schematic diagram of a single beam UV-Vis spectrophotometer.
3.2.4 Fluorescence Spectroscopy

Fluorescence spectroscopy is a technique used to analyze fluorescence from a sample. Fluorescent molecules absorb a photon at a characteristic wavelength when exposed to light, which raises the energy level of an electron to an excited state. The fluorophores will then emit a photon to relax the electron back to the ground state. The photon emitted is of lower energy and has a longer wavelength, therefore emitted fluorescence can be distinguished from the excitation light. Both excitation and emission wavelengths are specific characteristics for each fluorophore and the excitation and emission spectra of these fluorophores can be obtained using a fluorescence spectrophotometer.

A fluorescence spectrophotometer consists of four basic components: (i) a light source to provide the source of photons to excite the fluorophores; (ii) a sample holder; (iii) monochromators to select the wavelengths of excited and emitted light; and (iv) a detector to collect the emitted fluorescence (Scheme 3.4). A beam of light passes through an excitation monochromator to allow a single wavelength of light to strike the sample. The sample absorbs the light and generates fluorescence. The emitted fluorescence is then collected at 90° to the primary light path and filtered by the emission monochromator to a photomultiplier detector. A fluorescence emission spectrum is generated by plotting the fluorescence intensity against the measured wavelengths at a given excitation wavelength. Similarly, a fluorescence excitation spectrum is obtained by plotting the fluorescence emission over a range of excitation wavelengths. A Fluorolog-3 Model FL3-22 spectrophotometer (Jobin Yvon Inc., USA) equipped with a HgXe lamp was used in all experiments to allow quantification of encapsulated or adsorbed fluorescently labeled molecules onto the particles.
3.2.5 Differential Interference Contrast (DIC) Microscopy

Optical microscope is a device that uses visible light and a system of lenses to produce a magnified image of small objects. Some samples (e.g., hollow polymer capsules) cannot be imaged under brightfield illumination and in this case, differential interference contrast (DIC) microscopy is used to enhance the optical contrast of the transparent samples. These samples typically have an optical thickness that changes within the field of view, hence causing variations in phase in the image plane when polarized light interacts with the sample of interest. These variations can be translated to light intensity to give an accurate visualization of the materials.

As illustrated in Scheme 3.5, light from a lamp passes through a polarizing filter and then a Nomarski-modified Wollaston prism. Here, the polarized light is split into two beams (sampling and reference beams) at an angle of 90° to each other. The two beams are then focused by a condenser, allowing them to pass through two adjacent points (~0.2 μm apart) in a sample. As different areas in the sample have different refractive indices, the beams encounter different optical path lengths. This
causes a change in phase of one beam relative to another and results in an image of the sample carried by both the 0° and 90° polarized light. Nevertheless, no interference will occur at this point due to the different polarization states. The beams then pass through an objective lens and a second Nomarski-modified Wollaston prism, which combines the two beams at a polarization of 135°. This combination results in interference of the images, introducing contrast (brightening or darkening of areas) according to the optical path difference.

**Scheme 3.5.** Schematic diagram of a differential interference contrast (DIC) microscope.

### 3.2.6 Fluorescence Microscopy

Fluorescence microscopy is used to generate clear and magnified images of samples that possess inherent fluorescent characteristics or those that have been labeled with fluorophores. Typical components of a fluorescence microscope are: (i) a light source (xenon arc lamp or mercury-vapor lamp); (ii) an excitation filter; (iii) a dichroic mirror; and (iv) a barrier filter (Scheme 3.6). In a fluorescence microscope, light from the lamp passes through an excitation filter, where certain wavelengths of light that correspond to the excitation of the fluorophores are selected. A dichroic
mirror reflects the excitation light into an objective and then onto the specimen. The irradiation causes the fluorophores of the sample to be excited and subsequently emits fluorescent light. The emitted fluorescence at higher wavelength returns through the same objective that is used for the excitation and is subsequently focused through the dichroic mirror, which allows only light of wavelengths longer than the excitation wavelength to pass through and therefore filters out the remaining excitation light from fluorescent light. The light then passes through a barrier filter and travels to a detector, producing a magnified, brightly-lit object against a dark background.

All characterizations by DIC and fluorescence microscopy were performed on the Olympus IX71 digital wide-field microscope equipped with a DIC slider (U-DICT, Olympus), the corresponding filter sets, and a 60× oil immersion objective.

Scheme 3.6. Schematic diagram of a fluorescence microscope.
3.2.7 Confocal Laser Scanning Microscopy (CLSM)

Being different with conventional fluorescence microscopy, confocal laser scanning microscopy (CLSM) produces high resolution optical images from selected depth of field by using point illumination to focus an image onto one plane and a spatial pinhole to eliminate out-of-focus light. By scanning the specimen point-by-point, three-dimensional reconstructions of topologically complex objects can be obtained.

In CLSM (Scheme 3.7), being similar to fluorescence microscope, laser light reflects off a dichroic mirror and is focused by an objective lens onto a specimen, where fluorophores are excited and emit light. The emitted fluorescence is then recollected by the same objective lens and passes through the dichroic mirror, which directs solely emitted light to a pinhole. The pinhole excludes out-of-focus light (red lines) so that only light from a selected plane is received by the detector. All CLSM characterizations were performed on a Leica TCS SP2 AOBS confocal microscope (Leica, Germany) equipped with an argon laser using a 63× oil immersion objective.

Scheme 3.7. Schematic diagram of a confocal laser scanning microscope (CLSM).
3.2.8 Transmission Electron Microscopy (TEM)

Transmission electron microscopy (TEM) is a technique that operates on the same basic principles as a light microscope but uses a beam of electrons as the light source instead of light. Light microscope is limited by the wavelength of light, while electrons have wavelengths about 100,000× shorter than that of light; this results in optimal resolution of TEM images many orders of magnitude better than that from a light microscope and magnifications of up to about 10,000,000×. TEM therefore can reveal the fine detail of nanostructured materials as well as objects on an atomic level. TEM requires measurements to be performed under vacuum as air molecules would rapidly spread and attenuate electron beams. In addition, samples have to be ultrathin (< 100 nm) to allow transmission of the electrons through the specimens.

Typical components of TEM include: (i) an electron gun; (ii) a series of electromagnetic lenses; and (iii) a fluorescent screen or camera housed within a column (Scheme 3.8). Electron gun produces a beam of electrons through thermoionic emission from a tungsten filament cathode. This beam, travelling in vacuum in the column of the microscope, is then accelerated by an anode and focused onto a sample by a condenser lens. The electrons interact with the sample as they pass through, carry information about the structure of the specimen, and form an image produced by an objective lens. Apertures are used to control the size of the electron beam that passes through it. The image formed is further magnified by a projector lens and projected onto a fluorescent screen. Level of magnification can be controlled simply by adjusting the amount of current that flows through the lenses. All TEM characterizations were performed on an FEI Tecnai TF30 TEM (FEI-Company, The Netherlands).
Scheme 3.8. Schematic diagram of a transmission electron microscope (TEM).
3.3 References

Chapter 4

Coassembly of Polymers and Liposomes

Chapter 4

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4.1 Aim

The aim of this chapter is to examine fundamental aspects regarding stable incorporation of intact liposomal subcompartments into polymer films. Novel noncovalent anchoring concepts using cholesterol- or oleyl-modified polymers to stably attach liposomes to polymeric films are explored. The optimized polymer precursor layer, polymer separation layer, and polymer capping layer are identified to maximize the number of liposomal subunits in the polymer/liposome assembly while avoiding liposome displacement and/or rupturing.

4.2 Introduction

The assembly of capsosomes requires stable incorporation of intact liposomal subcompartments into polymer capsules; therefore, the anchoring between liposomes and polymers is of paramount importance. This chapter introduces novel noncovalent linkage concepts using cholesterol- or oleyl-modified polymers to stably attach liposomes to polymeric films (Scheme 4.1). Cholesterol is a non-toxic natural membrane constituent and its incorporation into lipid membrane is a fast and spontaneous process.\textsuperscript{1-5} The cholesterol/lipid membrane interaction neither relies on a specific lipid composition in the membrane, nor requires chemical modification of the lipids themselves. Furthermore, it is not affected by the ionic strength or pH of the aqueous solution and is insensitive to the presence of reactive groups. Thus, this method is well-suited for the adsorption of a range of liposomes; (un)saturated, charged, zwitterionic, synthetic or native (cell-derived).\textsuperscript{6} Oleyl moieties have also been widely incorporated into liposomes with different lipid compositions.\textsuperscript{7,8} Liposomes containing oleyl alcohol in their membrane are found to better maintain their pH-sensitive properties, especially in the presence of serum, and they facilitate more efficient cargo release in acidic conditions.\textsuperscript{7} If these features are maintained in the polymer form used in this study, it could be beneficial for the stability of liposomal subcompartments in capsosomes in physiological environments and subsequently allow better control of cargo release for applications aimed at therapeutic artificial cells and drug delivery.
In this study, fundamental aspects regarding stable incorporation of intact liposomal subcompartments into polymer films are examined, in particular the loading efficiency and stability of the liposomal cargo into the polymer film. Suitable polymer precursor layers are characterized to maximize liposome adsorption and various polymer capping layers are examined to stably anchor liposomes into the polymer films. The upper limit of liposome multilayering onto particle templates is also identified with the aim to maximize the number of subcompartments.

**Scheme 4.1.** Polymer/liposome assembly on a planar surface or on a particle template. Liposomes are adsorbed onto the polymer surface via novel noncovalent anchoring concept using cholesterol- (a) or oleyl-modified (b) polymers.
4.3 Experimental Section

4.3.1 Materials

Methacryloyl chloride, cholesterol, oleyl alcohol, 4-nitrophenol chloroformate (NPC, 95%), poly(L-lysine) (PLL, \( M_w \) 40 - 60 kDa), methacrylic acid, 2,2'-azoisobutyronitrile (AIBN), triethylamine (TEA, 99%), dichloromethane (DCM), hydroquinone, hydrochloric acid (HCl), sodium bicarbonate (NaHCO₃), magnesium sulphate (MgSO₄), methanol, diethyl ether, chloroform, dimethyl sulfoxide (DMSO), 1,4-dioxane, tetrahydrofuran (THF), hexane, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), sodium chloride (NaCl), sodium dodecyl sulfate were purchased from Sigma-Aldrich (Sydney, Australia). 4,4'-Azobis(4-cyanopentanoic acid) (Vazo 68) was obtained from Fluka AG (Sydney, Australia) and recrystallized from methanol before use. Zwitterionic lipids, 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC, phase transition temperature -20 °C), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC, phase transition temperature 23 °C), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC, phase transition temperature 41 °C), negatively charged lipids, 1,2-dioleoyl-sn-glycero-3-(phospho-L-serine) (DOPS, phase transition temperature -11 °C), and fluorescent lipids, 1-myristoyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn-glycero-3-phosphocholine (NBD-PC), were purchased from Avanti Polar Lipids (Alabama, USA). Silica particles (3.25 μm in diameter) were obtained from Microparticles GmbH (Berlin, Germany). Reversible addition-fragmentation chain transfer (RAFT) agent 4-cyano-4-(dodecylsulfanylthiocarbonyl)sulfanyl pentanoic acid was synthesized as described in the literature.⁹

HEPES buffer consisting of 10 mM HEPES and 150 mM NaCl (pH 7.4) was used for all of the experiments. The buffer solutions were made with ultrapure water (Milli-Q gradient A 10 system, resistivity ≈ 18 MΩ cm, TOC < 4 ppb, Millipore Corporation, Massachusetts, USA).
4.3.2 Monomer Synthesis

4.3.2.1 Cholesteryl Methacrylate

Cholesterol (4.15 g, 10.7 mmol) and TEA (10 mL) were dissolved in DCM (10 mL) with the addition of 2 mg hydroquinone. The solution was cooled in an ice bath while a DCM (10 mL) solution of methacryloyl chloride (1.68 g, 16.1 mmol) was added dropwise. The reaction was allowed to warm up to room temperature and left to stir overnight. The reaction mixture was dried down to remove excess TEA, redissolved in DCM (20 mL), and washed twice with 50 mL of a solution containing 0.5 M HCl, 5 wt % NaHCO$_3$, deionised water, and brine. It was subsequently dried over MgSO$_4$, filtered, and the solvent was removed to give 4.9 g of crude material. This was precipitated twice from DCM into methanol to give 2.52 g (52% yield) of cholesteryl methacrylate.

4.3.2.2 Oleyl Methacrylate

To an ice-cooled solution of freshly distilled oleyl alcohol (10.00 g, 37 mmol) and TEA (10.20 g, 14.10 mL, 100 mmol) in dry DCM (250 mL), methacryloyl chloride (8.76 g, 8.18 mL, 84 mmol) was added dropwise under argon. The solution was stirred with ice cooling for 30 min and was then left to stir for an additional 12 h at room temperature. The solvent was evaporated under vacuum, the residue was redissolved in diethyl ether (200 mL) and washed successively with 0.5 M HCl, water, 5 wt % NaHCO$_3$, water and brine. The organic layer was dried with MgSO$_4$ and the solvent was evaporated under vacuum. The crude product was purified by column chromatography (silica gel (Kieselgel Merck 60, 0.040-0.063 mm), 1:9 ethyl acetate/hexane) giving the product as a clear oil (9 g, 72%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$(ppm): 0.85 (t, 3H, CH$_3$), 1.25-1.29 (m, 22H, CH$_2$), 1.64-1.69 (m, 2H, CH$_2$), 1.93 (br s, 3H, CH$_3$C=C), 1.98-2.02 (m, 4H, 2 × CH$_2$CH=CH), 4.2 (t, 2H, J 6.7 Hz, CH$_2$-O), 5.29-5.36 (m, 2H, CH=CH oleyl), 5.53 (br s, 1H, CH$_3$=C), 6.10 (br s, 1H, CH$_2$=C).
4.3.3 Polymer Synthesis

4.3.3.1 Cholesterol-Modified Poly(L-lysine) (PLL<sub>c</sub>, M<sub>w</sub> 40 - 60 kDa)

Cholesterol (0.10 g, 0.26 mmol) and TEA (0.14 mL, 1.00 mmol) were dissolved in chloroform. NPC (0.05 g, 0.25 mmol) in chloroform (1 mL) was added to this solution and stirred at room temperature for 16 h. Chloroform was then removed by rotary evaporation and the reactive cholesterol was used without further purification. PLL (0.1 g, c.a. 0.7 mmol of NH<sub>2</sub>) was dissolved in 2 mL of DMSO and NPC-modified cholesterol (65 mg, 0.15 mmol) in DMSO (1 mL) was subsequently added to this solution. The mixture was allowed to react for 16 h and the cholesterol-modified PLL was precipitated (twice) into diethyl ether (100 mL). Solvents were removed under vacuum. By <sup>1</sup>H NMR analysis the polymer had approximately 8 mol% cholesterol functionality. <sup>1</sup>H NMR (CD<sub>3</sub>2SO), δ(ppm): 1.6–2.52 (br m, CH<sub>2</sub> & CH, PLL<sub>c</sub> polymer backbone and PLL side chains), 4.37 (m, OCH–, cholesteryl), 5.34 (tr, C=CH–, cholesteryl).

Scheme 4.2. Schematic illustration of the synthesis of cholesterol-modified poly(L-lysine) (PLL<sub>c</sub>). PLL was modified with cholesterol by reacting cholesterol with NPC followed by subsequent reaction of this compound with PLL.
4.3.3.2 Poly(methacrylic acid)-co-(cholesteryl methacrylate) (PMA<sub>c</sub>, M<sub>w</sub> 11.5 kDa)

RAFT agent 4-cyano-4-(dodecylsulfanylthiocarbonylsulfonyl)pentanoic acid (27.00 mg, 0.067 mmol), AIBN (0.60 mg, 0.004 mmol), methacrylic acid (1.06 g, 12.3 mmol), and cholesteryl methacrylate (0.53 g, 1.16 mmol) were combined with 1,4-dioxane (1.5 mL) in a Young vessel. The solution was degassed with four freeze-pump-thaw cycles and transferred to a temperature controlled oil bath heated at 60 °C for 3 h, followed by subsequent quenching of the polymerization by rapid cooling in liquid nitrogen. Samples of the monomer/polymer mixture were diluted with CDCl<sub>3</sub> for NMR analysis. The monomer/polymer mixture was twice precipitated from THF into diethyl ether, followed by removal of the polymer by filtration and dried under suction. The amount of cholesteryl methacrylate copolymerized was calculated as 7 mol% by NMR: \(^1\text{H NMR} ([\text{CD}_3]_2\text{SO}), \delta (\text{ppm}): 0.30–2.45 (\text{br m, CH}_3 \& \text{CH}_2, \text{PMA}_c \text{ polymer backbone}), 3.25 (\text{tr, } -\text{CH}_2\text{S, RAFT end-group}), 4.35 (\text{m, OCH}→, \text{cholesteryl } 3\text{H}), 5.35 (\text{tr, } \text{C=CH}→, \text{cholesteryl } 6\text{H}), 12.3 (\text{br s, COOH, PMA}).

4.3.3.3 Poly(methacrylic acid)-co-(oleyl methacrylate) (PMA<sub>oa</sub>, M<sub>w</sub> 25 kDa)

RAFT agent (26.30 mg, 0.065 mmol), Vazo 68 (3.60 mg, 13.0 mmol), methacrylic acid (1.01 g, 12.0 mmol), and oleyl methacrylate (0.44 g, 1.29 mmol) were combined with 1,4-dioxane (3.0 mL) in an ampule. The solution was degassed with four freeze-pump-thaw cycles, flame-sealed, and transferred to a temperature-controlled oil bath at 60 °C for 7 h. The polymerization was then quenched by rapid cooling in liquid nitrogen. Samples of the monomer/polymer mixture were diluted with CDCl<sub>3</sub> for NMR analysis. The monomer/polymer mixture was twice precipitated from a workable dioxane/THF mixture into diethyl ether/hexane (3:1). The polymer was removed by filtration and dried under suction to isolate 1.24 g (85%) of polymer.
Coassembly of Polymers and Liposomes

Scheme 4.3. Schematic illustration of the synthesis of poly(methacrylic acid)-co-(cholesteryl methacrylate) (PMA₇) (a) and poly(methacrylic acid)-co-(oleyl methacrylate) (PMAₒ₉) (b). PMA₇ was synthesized via the copolymerization of methacrylic acid and cholesterol methacrylate, while PMAₒ₉ was synthesized via the copolymerization of methacrylic acid and oleyl methacrylate. The polymer synthesis is controlled by an acid functional RAFT agent 4-cyano-4-(dodecylsulfanythiocarbonyl)sulfanyl pentanoic acid.
A small sample of each copolymer, poly(methacrylic acid)-co-(oleyl methacrylate), was modified by methylation of carboxylic acid groups with trimethylsilyldiazomethane, using a procedure reported in the literature.\textsuperscript{10} These samples were analyzed using gel permeation chromatography (GPC) (Figure 4.1) performed on a Waters 515 HPLC pump and Waters 717 Plus Autosampler, equipped with a Waters 2414 refractive index detector, 3× Mixed-C (7.5 × 300 mm, 5 μm particle size, linear molecular weight range 200-2,000,000), and 1× Mixed E PL gel column (7.5 × 300 mm, 3 μm particle size, linear molecular weight range up to 30,000) from Polymer Laboratories. THF with a flow rate of 1.0 mL min\textsuperscript{-1} was used as eluent at 22 ± 2 °C. Molecular weights were calculated via calibration with narrow polydispersity polymethyl methacrylate standards (Polymer Laboratories) ranging from 600 to $7.5 \times 10^6$ g mol\textsuperscript{-1}. Molecular number ($M_n$) and weight-average ($M_w$) molecular weights were evaluated using Waters Millennium/Empower software. A third-order polynomial was used to fit the log $M$ versus time calibration curve, which was linear across the molecular weight ranges.

![Figure 4.1. THF-GPC curves of methylated poly(methacrylic acid)-co-(oleyl methacrylate): 2.0% (light gray), 3.4% (gray), and 8.0% (dark gray) oleyl content.](image)
$^1$H (400 MHz) and $^{13}$C (100 MHz) NMR spectra were obtained with a Bruker Av400 spectrometer at 25 °C. Spectra were recorded for samples dissolved in deuterated solvent and chemical shifts were reported as parts per million from external tetramethylsilane. Monomer conversions were obtained from the $^1$H NMR spectra of crude poly(methacrylic acid)-co-(oleyl methacrylate) samples. The compositional ratio of the methylated copolymers was calculated by $^1$H NMR via the integrated peak intensity ratio of oleyl CH=CH (5.3 ppm) versus that of methyl methacrylate OCH$_3$ (3.6 ppm) (Figure 4.2). The amount of oleyl methacrylate copolymerized was calculated as 2.0, 3.4, and 8.0 mol% by NMR.

![Figure 4.2](image)

**Figure 4.2.** $^1$H NMR spectra of poly(methacrylic acid)-co-(oleyl methacrylate): 2.0% (3), 3.4% (2) and 8.0% (1) oleyl content. Labeled peaks: (a) -COOH, (b) -CH=CH, (c) -CH$_2$O-, (d) 2 × -CH$_2$CH=CH, and (e) -CH$_2$, -CH$_3$ polymer backbone signals and aliphatic oleyl signals, in dimethyl sulfoxide-d$_6$. 
4.3.4 Liposome Formation

Unilamellar liposomes were prepared by evaporation of the chloroform of the lipid solution (2.5 mg of DOPC (L\textsubscript{u,zw}), DOPC/DOPS = 4:1 wt % (L\textsuperscript{u}), DMPC/DPPC = 4:1 wt % (L\textsubscript{z}w), or DMPC/DPPC/DOPS = 7:1:2 wt % (L\textsuperscript{z})) under nitrogen for 1 h, followed by hydration with 1 mL of HEPES buffer and extrusion through 50 nm filters (31 times). For fluorescently labeled liposomes, 1 wt % of NBD-PC was added to the lipid mixture to obtain liposomes with the following composition of lipids: DOPC/NBD-PC (L\textsubscript{u,zw}NBD\textsuperscript{+}), DOPC/DOPS/NBD-PC (L\textsuperscript{u}-NBD\textsuperscript{+}), DMPC/DPPC/NBD-PC (L\textsubscript{z}wNBD\textsuperscript{+}), or DMPC/DPPC/DOPS/NBD-PC (L\textsuperscript{z}-NBD\textsuperscript{+}).

4.3.5 Polymer/Liposome Assembly on Planar Surfaces

Characterization of the polymer/liposome assembly on planar surfaces was performed using Quartz Crystal Microbalance with Dissipation Monitoring (QCM-D, Q-sense E4, Sweden). Silica-coated crystals (QSX300, Q-sense) were cleaned in a 2 wt % sodium dodecyl sulfate solution overnight, followed by rinsing with Milli-Q water, drying with nitrogen, and exposure to UV (Bioforce Nanosciences, U.S.A.) for 30 min. Cleaned crystals were then mounted into the flow chambers of the instrument. HEPES buffer was used in all of the adsorption steps. Unless otherwise mentioned, the mass and dissipation measurements were monitored at 24 ± 0.02 °C. In all experiments, after obtaining a stable HEPES buffer baseline, a polymer solution (1 mg mL\textsuperscript{-1}, 15 min) was introduced into the measurement chamber and left to adsorb onto the crystal to form a polymer precursor layer. After the surface was saturated, the chamber was rinsed with buffer solution to remove the excess polymer. The resulting polymer-coated surface was then exposed to L\textsubscript{u,zw}, L\textsuperscript{u}-, or L\textsubscript{z}w (2.5 mg mL\textsuperscript{-1}, 1 h), and left to incubate until the surface was saturated. The liposome solution was replaced with buffer solution and the film assembly was continued by adsorbing a polymer capping layer (1 mg mL\textsuperscript{-1}, 15 min). When the surface was saturated, the chambers were washed with buffer solution. Normalized frequencies using the third overtone are presented. The increase of mass on the surface (adsorption of polymers/liposomes) is reflected by a negative ΔF. Liposome desorption or other phenomenon leading to mass loss results in a positive ΔF.
4.3.6 Polymer/Liposome Assembly on Particle Substrates

A suspension of 3.25 μm-diameter SiO$_2$ particles (5 wt %) in HEPES buffer was incubated with the polymer precursor layer (1 mg mL$^{-1}$, 15 min) and washed with three centrifugation/redispersion cycles (1060 g, 30 s). Fluorescently labeled unsaturated (L$_{u,zw}^{NBD}$ or L$_{u,-}^{NBD}$) or saturated (L$_{s,zw}^{NBD}$ or L$_{s,-}^{NBD}$) liposomes (2.5 mg mL$^{-1}$, 1 h) were allowed to interact with the polymer-coated particles, washed three times, and polymer separation layers were subsequently adsorbed (1 mg mL$^{-1}$, 15 min). The adsorption of liposomes and polymer separation layer(s) was repeated until the required number of layers was deposited, followed by the adsorption of a polymer capping layer (1 mg mL$^{-1}$, 15 min). The fluorescence of the particles was analyzed by flow cytometry after each liposome adsorption step and after deposition of the polymer capping layer.

4.3.7 Characterization Methods

4.3.7.1 Flow Cytometry

A Cyflow Space (Partec GmbH, Germany) flow cytometer using an excitation wavelength of 488 nm was used to measure the intensity of the liposome-coated particles. At least 20,000 particles were analyzed in each experiment. The alternating deposition of polymer(s) and liposomes onto silica particles (with the aim to assemble liposome multilayers) was characterized using flow cytometry. After each liposome deposition step, the fluorescence intensity of the particles was monitored, and when there was no increase in fluorescence intensity, an increase lower than 50%, or severe aggregation, no further deposition steps were performed. At least two independent experiments were performed.
4.4 Results and Discussion

4.4.1 Noncovalent Linkage Concept Using Cholesterol-Modified Polymers

To optimize the stable incorporation of intact liposomes into polymer films, a new approach of sandwiching liposomes between two polymer layers by using cholesterol as a noncovalent linker is exploited (Scheme 4.1a). Biodegradable positively charged PLL was synthesized by attaching cholesterol to the amines of the polymer via NPC (Scheme 4.2), while controlled radical copolymerization via RAFT was chosen to synthesize negatively charged PMA due to its monomer compatibility, good end-group purity, and control afforded over the chain composition and architecture (Scheme 4.3a). This approach allows the design and synthesis of a polymer with a homogeneous composition of reactive monomers. Suitable polymer precursor and capping layers for the liposomes were identified on planar silica surfaces by QCM-D.

Depending on the target application, different types of liposomal subcompartments might be employed: saturated or unsaturated as well as charged or zwitterionic liposomes. For instance, the encapsulation of negatively charged or positively charged cargo is likely to be facilitated by using zwitterionic or oppositely charged liposomes to prevent unfavorable interactions between the cargo and the subcompartments. Therefore, both the lipid composition of the liposomes and the effect of the cholesterol moieties on the polymers (in comparison to the unmodified polymers) on liposome incorporation were investigated.

4.4.1.1 Adsorption of Unsaturated Liposomes

With the aim to optimize the amount of intact liposomes stably incorporated into the polymer films, the frequency changes of QCM crystals pre-coated with different polymer precursor layers upon the addition of liposomes were monitored using QCM-D. However, the polymer precursor layer is not the only parameter that governs the polymer/liposome assembly. Therefore, to gain a more comprehensive understanding of the immobilization of liposomes onto polymer films, liposomes consisting of two different types of lipids were employed, i.e., unsaturated
zwitterionic DOPC liposomes (L\textsuperscript{u,zw}) and unsaturated negatively charged DOPC/DOPS liposomes (L\textsuperscript{u,−}). The QCM crystals were first coated with different polymer precursor layers (PLL, PLL\textsubscript{c}, PLL/PMA, or PLL/PMA\textsubscript{c}; Table 4.1), and it was found that the cholesterol moieties did not hinder polymer adsorption.

Table 4.1. QCM-D frequency changes upon binding of four different polymer precursor layers to SiO\textsubscript{2} crystals.

<table>
<thead>
<tr>
<th>Polymer precursor layer</th>
<th>-ΔF [Hz]</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLL</td>
<td>4.6 ± 0.3</td>
</tr>
<tr>
<td>PLL\textsubscript{c}</td>
<td>6.2 ± 0.6</td>
</tr>
<tr>
<td>PLL/PMA</td>
<td>11.7 ± 1.6</td>
</tr>
<tr>
<td>PLL/PMA\textsubscript{c}</td>
<td>35.0 ± 1.1</td>
</tr>
</tbody>
</table>

Figure 4.3 compares the frequency changes of the QCM crystal upon the addition of L\textsuperscript{u,zw} or L\textsuperscript{u,−} to the four different polymer precursor layers. Similar frequency changes for both types of liposomes (ΔF = -181 ± 10 Hz) were observed, independent of whether PLL or PLL\textsubscript{c} was used as the precursor layer. This frequency change is similar to the values measured for 50 nm DOPC liposomes adsorbed onto positively charged PAH pre-coated SiO\textsubscript{2} crystals\textsuperscript{13} or gold coated crystals,\textsuperscript{14} and corresponds to the adsorption of a monolayer of liposomes. In contrast, only ~5% of a liposome layer was adsorbed onto a PMA precursor layer. This finding is in agreement with previous results on the largely electrostatic interaction of liposomes with the polymers: the adsorption onto positively charged surfaces produces a monolayer of liposomes, while negatively charged surfaces do not support adsorption.\textsuperscript{13,15} The approach in anchoring the liposomes to the polymer multilayers based on a noncovalent cholesterol linker developed here enables the adsorption of a monolayer of intact liposomes onto a negatively charged PMA\textsubscript{c} precursor layer. The affinity of cholesterol to the lipid membrane proved to be highly efficient, with cholesterol-functionalized PMA supporting the adsorption of a monolayer of both zwitterionic and negatively charged liposomes.
Chapter 4

Figure 4.3. QCM-D frequency changes upon binding of unsaturated zwitterionic liposomes (DOPC, L\textsuperscript{u,zw}) or unsaturated negatively charged liposomes (DOPC/DOPS, L\textsuperscript{u,-}) to four different polymer precursor layers (PLL, PLL\textsubscript{c}, PLL/PMA, or PLL/PMA\textsubscript{c}).

The stable, high loading of liposomes into a polymer film not only depends on the polymer precursor layer, but also on the polymer capping layer. The capping layer has to stably incorporate the liposomes into the assembly while avoiding/minimizing displacement or rupturing of the liposomes. A suitable capping layer was identified, depending on the precursor layer and the different types of liposomes immobilized. Figure 4.4a and 4.4b shows the frequency changes upon the adsorption of the different polymer capping layers to QCM crystals primed with PLL, PLL\textsubscript{c}, or PLL/PMA\textsubscript{c} as precursor layers and L\textsuperscript{u,zw} or L\textsuperscript{u,-}, respectively. For both zwitterionic and negatively charged liposomes electrostatically adsorbed onto PLL and capped with PLL\textsubscript{c} or PMA, a loss of mass from the surface was observed, possibly through competitive displacement of the liposomes via preferential interaction of PLL\textsubscript{c} with the liposomes (former case) or PMA with PLL (latter case). The data suggested that PLL\textsubscript{c} only bound to the liposomes and the repulsive interaction between PLL\textsubscript{c} and the PLL precursor layer dominates over the electrostatic interaction between the liposomes and the PLL precursor layer, resulting in displacement of loosely bound liposomes. The displacement was larger for the negatively charged liposomes, indicating that the charge attraction between the PLL\textsubscript{c} polymer and the liposomes supported the
incorporation of the cholesterol moieties into the lipid membrane and subsequently led to a higher amount of detached liposomes from the surface. On the other hand, when PMA_c was introduced as the capping layer, unlike unmodified PMA, its cholesterol moieties enabled the adsorption of the polymer to the liposomes. Furthermore, PMA_c electrostatically interacts with the PLL precursor layer, which enabled the stable incorporation of liposomes between a polymer precursor layer and a polymer capping layer.

In contrast to PLL, the use of PLL_c as a precursor layer avoided liposome desorption upon addition of a PLL or PLL_c capping layer (Figure 4.4a and 4.4b). This indicates that both polymer capping layers adsorbed only to the liposomes, whilst the cholesterol-modified polymer precursor layer provided stable anchoring of the liposomes, avoiding the previously described charge repulsion-induced desorption of the liposomes. Adding PMA as a capping layer to a PLL_c/liposome film resulted in a much lower positive frequency change as compared to a PLL/liposome assembly, indicating that the affinity between the liposomes and the underlying PLL_c precursor layer via a cholesterol linker was more pronounced compared with the electrostatic interaction of PMA and PLL. On the other hand, capping the PLL_c/liposome assembly with PMA_c resulted in the stable incorporation of liposomes, again due to the binding of the PMA_c to both parts of the film. This notion further highlights the importance of cholesterol as an anchoring molecule for the adsorption of liposomes.

A similar trend to the PLL_c/liposome assembly was observed when a PLL/PMA_c/liposome assembly was allowed to interact with the different polymer capping layers (Figure 4.4a and 4.4b). When a PLL, PLL_c, or PMA_c capping layer was introduced, polymer binding was observed for all cases, but it was more pronounced for the cholesterol-modified polymers. However, the frequency change for a PMA_c capping layer was lower than that observed for the PLL_c capping layer since PMA_c only bound to the liposomes but not to the PMA_c precursor layer. Moreover, PMA_c also bound to the negatively charged liposomes (Figure 4.4b), further demonstrating the usefulness of the presented noncovalent linkage concept for the liposome assembly based on cholesterol-functionalized polymers. As expected, a PMA capping
layer did not show any binding or liposome displacement due to charge repulsion between PMA and the liposomes as well as the PMAc precursor layer.

In summary, a PLLc precursor layer and a PMAc capping layer were found to be the most suitable combination for the stable incorporation of unsaturated zwitterionic or negatively charged liposomes into the polymer film.

Figure 4.4. QCM-D frequency changes upon binding of different polymer capping layers (PLL, PLLc, PMA, or PMAc) to a layer of unsaturated zwitterionic liposomes (DOPC, Lu,zw) (a) or unsaturated negatively charged liposomes (DOPC/DOPS, Lu−) (b) adsorbed onto different polymer precursor layers (PLL, PLLc, or PLL/PMAC).
4.4.1.2 Adsorption of Saturated Liposomes

The choice of liposomes to be used in the polymer assemblies strongly depends on the required purpose of the subcompartments. Although unsaturated liposomes are interesting in their own right and provide relevant fundamental information about the polymer/liposome interaction, saturated liposomes are predominantly used for cargo loading. During storage or at room temperature, the saturated liposomes are in their ordered gel phase and cargo leakage can be minimized. In addition, the phase transition of the liposomes can be used as a trigger to facilitate cargo release due to the enhanced permeability of the membrane during the lipid rearrangement at the phase transition temperature ($T_m$). In this study, 50 nm saturated zwitterionic liposomes ($L_{5,2w}$) consisting of a mixture of zwitterionic DMPC and DPPC lipids with $T_m$ of about 28 °C were employed. Figure 4.5 compares the frequency changes upon adsorption of $L_{5,2w}$ to different polymer precursor layers at an adsorption temperature below ($T = 24 \, ^\circ\text{C}$) and above ($T = 32 \, ^\circ\text{C}$) $T_m$. As expected, the electrostatically driven adsorption of the liposomes to a PLL or PLL/PMA pre-coated crystal was independent of the adsorption temperature. The effect of the cholesterol moieties attached to PLL on the liposome immobilization was moderate: when the temperature was increased from 24 °C to 32 °C, a higher amount of adsorbed liposomes (~25%) onto PLL$_c$ was detected. Above $T_m$, a 28% increase in the amount of adsorbed liposomes to PLL$_c$ was observed as compared to unmodified PLL. In contrast, when the liposomes were adsorbed onto cholesterol-modified PMA, a pronounced difference (a two-fold increase) in adsorption above $T_m$ was exhibited. Since pristine PMA only results in negligible adsorption of the liposomes, the observed effect is attributed solely to the presence of cholesterol.
Figure 4.5. QCM-D frequency changes upon binding of saturated zwitterionic liposomes (DMPC/DPPC, L\(^{5,zw}\)) to four different polymer precursor layers (PLL, PLL\(_c\), PLL/PMA, or PLL/PMA\(_c\)) at an adsorption temperature below \((T = 24 \, ^\circ\text{C})\) or above \((T = 32 \, ^\circ\text{C})\) the phase transition temperature \((T_m)\).

PLL\(_c\) was chosen as a capping layer because it was found to be the most successful candidate to stably anchor the unsaturated liposomes to the polymer precursor layer. Figure 4.6 shows QCM-D frequency changes upon binding of PLL\(_c\) to L\(^{5,zw}\) adsorbed onto different polymer precursor layers at two different adsorption temperatures. Below \(T_m\) of the liposomes, the interaction of cholesterol with the lipid membrane is minimized. The adsorption behavior of a PLL\(_c\) capping layer was similar to the adsorption of PLL to a layer of unsaturated liposomes in their liquid state (Figure 4.4a), causing the partial displacement of the liposomes, possibly via dominant electrostatic interaction between PLL\(_c\) and PLL or PLL\(_c\). The stable incorporation of the saturated liposomes between the two polymer layers was only observed when the PLL\(_c\) capping layer was introduced at a temperature above \(T_m\) of the liposomes. The increase in temperature has driven the liposomes through their phase transition, facilitating the incorporation of the cholesterol into the lipid membrane.
Figure 4.6. QCM-D frequency changes upon binding of PMAć to saturated zwitterionic liposomes (DMPC/DPPC, Ls,zw) adsorbed onto different polymer precursor layers (PLL, PLLć, or PLL/PMAć) at an adsorption temperature below (T = 24 °C) or above (T = 32 °C) the phase transition temperature (Tm). The stable incorporation of the saturated liposomes between the two polymer layers was enhanced when the liposome adsorption and capping was performed above Tm allowing the incorporation of the cholesterol into the lipid membrane.

Based on the above findings, it is concluded that PLLć and PMAć (precursor and capping layer, respectively) are suitable for the incorporation of saturated liposomes into a polymer film when the assembly is performed above Tm of the liposomes. Taken together, these QCM-D findings illustrate that the cholesterol-driven anchoring between the liposomes and the polymer is in many aspects superior over a covalent linkage or electrostatic adsorption.
4.4.2 Noncovalent Linkage Concept Using Oleyl-Modified Polymers

In Section 4.4.1, novel noncovalent anchoring concept using cholesterol-modified polymers was introduced and this approach facilitates stable incorporation of liposomes to polymeric films. In the current study, the feasibility of using oleyl units in comparison to cholesteryl as noncovalent linkages to anchor liposomes to a polymer layer is explored.

PMA\textsubscript{oa} copolymers with different percentages of oleyl methacrylate (2.0, 3.4, and 8.0 mol \%) were synthesized and denoted as PMA\textsubscript{oa-2\%}, PMA\textsubscript{oa-3.4\%}, and PMA\textsubscript{oa-8\%}, respectively (Scheme 4.3b). RAFT polymerization was chosen to synthesize the oleyl-modified PMA copolymers. Due to the disparity in reactivity ratios and the living polymerization processes, this produces copolymers with similar gradient structures, that is, where the composition of the monomers drift with conversion, as compared to purely random copolymers.\textsuperscript{12,18} The polymerization produced polymers with narrow molecular weight distributions (PDI < 1.2) throughout, as shown in Table 4.2. Recent studies have indicated very low to no cytotoxicity for RAFT synthesized polymers; hence, the RAFT moiety was not removed postpolymerization.\textsuperscript{19-22}

Table 4.2. RAFT-controlled copolymers of methacrylic acid with oleyl methacrylate.

<table>
<thead>
<tr>
<th>Polymer\textsuperscript{a}</th>
<th>$M_n$\textsuperscript{b}</th>
<th>$M_n$\textsuperscript{c}</th>
<th>PDI\textsuperscript{b}</th>
<th>Oleyl : MA\textsuperscript{d} (mol %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24,200</td>
<td>20,920</td>
<td>1.20</td>
<td>2.0 : 98.0</td>
</tr>
<tr>
<td>2</td>
<td>24,800</td>
<td>21,450</td>
<td>1.17</td>
<td>3.4 : 96.6</td>
</tr>
<tr>
<td>3</td>
<td>25,200</td>
<td>21,980</td>
<td>1.19</td>
<td>8.0 : 92.0</td>
</tr>
</tbody>
</table>

\textsuperscript{a} For each polymerization $[\text{monomer}_{\text{total}}] : [\text{RAFT}] : [\text{Vazo 68}] = 200 : 1 : 0.001; for 1 [MAA] : [OMA] in polymerization mixture = 97 : 3, for 2 [MAA] : [OMA] = 95 : 5, and for 3 [MAA] : [OMA] = 90 : 10. \textsuperscript{b} Molecular weights of methylated polymer obtained using THF GPC calibrated with PMMA standards. \textsuperscript{c} Corresponding molecular weight of acid copolymer (original nonmethylated). \textsuperscript{d} Calculated from $^1$H NMR of methylated copolymer from ratio of integrated peaks corresponding to the oleyl and methacrylate components.
The stable incorporation of \( \text{L}^{u,zw}, \text{L}^{u,-}, \text{and L}^{s,zw} \) to the polymer films was previously demonstrated with PMA\(_{c}\) as a capping layer (Section 4.4.1). Herein, the ability of these oleyl-modified polymers, in comparison to PMA\(_{c}\) (7 mol% cholesterol), to serve as polymer precursor or capping layers for liposome anchoring to polymer films is examined both on planar surfaces and particles templates, and the effect of different percentage modification of oleyl moieties on PMA is compared.

### 4.4.2.1 Assembly on Planar Surfaces

The suitability of using PMA\(_{oa}\) as a precursor or capping layer was examined by QCM-D and compared with results previously obtained for PMA\(_{c}\). Figure 4.7a summarizes the frequency changes of the QCM crystals upon addition of \( L^{u,zw} \) or \( L^{s,zw} \) to the different polymer precursor layers (PMA\(_{c}\), PMA\(_{oa-2\%}\), PMA\(_{oa-3.4\%}\), and PMA\(_{oa-8\%}\)) adsorbed onto PLL-coated surfaces. The same amount of \( L^{u,zw} \) (yellow bar) was adsorbed (\( \Delta F = -194 \pm 51 \) Hz), irrespective of the different percentages of anchor molecules available. This demonstrates that the oleyl unit, similar to cholesterol, supports liposome adsorption onto the polymer film, since it was previously demonstrated that \( L^{u,zw} \) does not adsorb onto pristine PMA (Figure 4.3).

For \( L^{s,zw} \), two adsorption temperatures were examined: below (\( T = 24 ^\circ C \)) and above (\( T = 37 ^\circ C \)) its \( T_m \). When the adsorption step was performed below \( T_m \), only \( \sim 25\% \) of \( L^{s,zw} \) was adsorbed (red bar) as compared to \( L^{s,zw} \) adsorption above \( T_m \) (brown bar). This implies that the anchoring of \( L^{s,zw} \) to the polymer is facilitated via the oleyl linkage and a temperature above \( T_m \) was required to promote interaction between the oleyl units and the lipid membrane, as previously observed for the cholesterol-assisted anchoring with PMA\(_{c}\). Similar to \( L^{u,zw} \), no difference in \( L^{s,zw} \) adsorption onto the different PMA\(_{oa}\) precursor layers was found (\( \Delta F = -200 \pm 20 \) Hz). In fact, this value is comparable to \( L^{s,zw} \) adsorbed onto PMA\(_{c}\); thus, PMA\(_{oa}\) is an equally suitable precursor layer for \( L^{s,zw} \) incorporation into polymer films. Further, this observation demonstrates that the presence of anchor molecules (cholesteryl or oleyl moieties), even in small amounts, significantly enhances the adsorption of liposomes onto a PMA-coated surface.
Figure 4.7. a) QCM-D frequency changes for the binding of unsaturated (L^u,zw) or saturated (L^s,zw) zwitterionic liposomes to different polymer precursor layers (PMA_c, PMA_{oa-2%}, PMA_{oa-3.4%}, or PMA_{oa-8%}) adsorbed onto PLL-precoated silica QCM crystals. For L^s,zw, the adsorption step was performed below (T = 24 °C) and above (T = 37 °C) T_m. b) QCM-D frequency changes for the binding of different polymer capping layers (PMA_c, PMA_{oa-2%}, PMA_{oa-3.4%}, or PMA_{oa-8%}) to unsaturated (L^u,zw) or saturated (L^s,zw) zwitterionic liposomes adsorbed onto PLL-precoated silica QCM crystals. For L^s,zw, the adsorption of the capping layers was performed below (T = 24 °C) and above (T = 37 °C) T_m.
The adsorption of PMA\textsubscript{oa} with different oleyl amounts as a polymer capping layer to polymer/liposome assemblies is shown in Figure 4.7b. With increasing oleyl content, a proportional increase in the amount of adsorbed PMA\textsubscript{oa} onto L\textsuperscript{u,zw} was observed ($\Delta F$ PMA\textsubscript{oa-2\%} = -26 ± 6 Hz; $\Delta F$ PMA\textsubscript{oa-3.4\%} = -53 ± 8 Hz; $\Delta F$ PMA\textsubscript{oa-8\%} = -93 ± 13 Hz). This indicates that the amount of PMA\textsubscript{oa} adsorbed as a capping layer can be tuned via the oleyl content on PMA.

As expected, L\textsubscript{s,zw} desorption was observed when the capping layers were introduced below the $T_m$ of the saturated liposomes for all of the polymers examined. This is likely due to the dominant electrostatic interaction between PMA\textsubscript{c} or PMA\textsubscript{oa} and the PLL precursor layer. On the other hand, while PMA\textsubscript{c} was adsorbed onto the polymer/liposome assembly above $T_m$, PMA\textsubscript{oa} with different oleyl amounts were not found to be suitable capping layers for L\textsubscript{s,zw}. Both PMA\textsubscript{oa-2\%} and PMA\textsubscript{oa-3.4\%} resulted in ~30 - 50% of L\textsubscript{s,zw} desorption, and even PMA\textsubscript{oa-8\%} caused ~5% liposomal loss. Therefore, while PMA\textsubscript{oa-8\%} is a suitable polymer precursor layer for all of the liposomes examined and as a capping layer for unsaturated liposomes, it is not suitable as a polymer capping layer for saturated liposomes. Although the number of anchor molecules contained per polymer chain is similar to PMA\textsubscript{c} (i.e., 7 vs 8%), the adsorption behavior onto a polymer/liposome assembly was found to be different for the L\textsubscript{u,zw} and L\textsubscript{s,zw} cases. This suggests that the interaction of cholesterol with a lipid membrane is more suitable to anchor liposomes to polymer films than oleyl units. It is speculated that this is due to cholesterol being a natural lipid membrane constituent and, therefore, being more effective in anchoring into liposomes compared with oleyl moieties.

\subsection*{4.4.2.2 Assembly on Particle Substrates}

The assembly of the same system on 3 μm-diameter silica particles was examined. Fluorescently labeled liposomes were used to verify their adsorption onto a polymer film by varying two parameters, the polymer precursor and capping layers. The fluorescence intensity of the coated particles was monitored by flow cytometry and the fluorescence intensity after the L\textsuperscript{u,zw NBD} adsorption step onto the PMA\textsubscript{oa-8\%} layer was set to 100% (Figure 4.8a). PMA\textsubscript{oa}, for all molar percentages of oleyl units, was
found to allow the adsorption of a monolayer of $L^{u,zw}_\text{NBD}$, which is in agreement with the findings on planar surfaces (Figure 4.7a). However, the adsorption behavior of $L^{s,zw}_\text{NBD}$ to a PMA$_{oa}$ polymer precursor layer on particle substrates was different from those found on planar surfaces. Higher liposomal adsorption was observed for PMA$_{oa}$ with increasing oleyl contents. Above $T_m$, PMA$_{oa-2\%}$ resulted in ~60% adsorption of $L^{s,zw}_\text{NBD}$, while PMA$_{oa-8\%}$, similar to PMA$_c$, resulted in 100% adsorption of the saturated liposomes onto the polymer-coated particles. This trend was also observed when $L^{s,zw}_\text{NBD}$ adsorption was performed below $T_m$, but the amount of adsorbed liposomes was lower. This might, in part, be explained by the different washing conditions used for planar surfaces and particle substrates. Deposition onto particles involves centrifugation, followed by resuspension of the particles into the buffer solution.

Figure 4.8b shows the normalized fluorescence intensity of particles upon binding of the different polymer capping layers (PMA$_c$, PMA$_{oa-2\%}$, PMA$_{oa-3.4\%}$, or PMA$_{oa-8\%}$) to a layer of zwitterionic liposomes adsorbed onto PLL-precoated silica particles. The fluorescence intensity after the PMA$_c$ adsorption step onto $L^{u,zw}_\text{NBD}$ was set to 100%. The different PMA polymers adsorbed onto $L^{u,zw}_\text{NBD}$ equally well, as there was no displacement of liposomes observed. However, a loss of ~50% of adsorbed $L^{s,zw}_\text{NBD}$ was observed upon adsorption of the PMA$_{oa}$ below and above $T_m$, while PMA$_c$ was able to retain ~90% of the liposomes when assembled above $T_m$. In summary, both studies on planar surfaces and particles suggest that PMA$_c$ is the more suitable capping layer for the saturated liposomes, while both PMA$_c$ and PMA$_{oa}$ are equally suitable for the unsaturated liposomes.
Figure 4.8. a) Normalized fluorescence intensity measured for the binding of unsaturated (L^{u,zw}_{NBD}) or saturated (L^{s,zw}_{NBD}) zwitterionic liposomes to different polymer precursor layers (PMA\textsubscript{c}, PMA\textsubscript{oa}(2\%), PMA\textsubscript{oa}(3.4\%), or PMA\textsubscript{oa}(8\%)) adsorbed onto PLL-precoated silica particles. For L^{s,zw}_{NBD}, the adsorption step was performed below (T = 24 °C) and above (T = 37 °C) T\textsubscript{m}. b) Normalized fluorescence intensity measured for the binding of different polymer capping layers (PMA\textsubscript{c}, PMA\textsubscript{oa}(2\%), PMA\textsubscript{oa}(3.4\%), or PMA\textsubscript{oa}(8\%)) to a layer of unsaturated (L^{u,zw}_{NBD}) or saturated (L^{s,zw}_{NBD}) zwitterionic liposomes adsorbed onto PLL-precoated silica particles. For L^{s,zw}_{NBD}, the adsorption of the capping layers was performed below (T = 24 °C) and above (T = 37 °C) T\textsubscript{m}. 
4.4.3 Liposome Multilayer Assembly

In order to maximize the number of subcompartments in the assembly, the deposition of multilayer of liposomes is performed by the alternating adsorption of liposomes (L$^{u-}$, L$^{5-}$, L$^{u,2W}$, or L$^{5,2W}$) and polymer separation layers (PLL, PLL$_c$, or PMA$_c$) onto particle templates. Different polymer separation layers to anchor various types of liposomes to a polymer film during multiple deposition steps are identified.

4.4.3.1 Multilayers of Negatively Charged Liposomes

The first approach to assemble liposome multilayers was performed by alternately adsorbing positively charged polymer separation layers (PLL$_c$ or PLL) and negatively charged unsaturated or saturated liposomes (L$^{u-}$ or L$^{5-}$), thus expecting film growth driven by electrostatic interactions. The intensity of particles coated with fluorescently labeled liposomes was monitored by flow cytometry and the fluorescence intensity after the first liposome adsorption step was set to 100% and referred to as a single layer of liposomes.

To start the multilayer assembly, a polymer precursor layer, PLL$_c$ or PLL, was adsorbed onto 3 µm-diameter silica particles. Both polymers have previously been proven to be suitable to adsorb a first layer of liposomes (Figure 4.3). PLL$_c$, as a separation layer between the liposomes (L$^{u-}$ or L$^{5-}$), supported the consistent addition of a single layer of liposomes (~95%) in each adsorption step (Figure 4.9a, ★ and ▲). However, the number of alternating liposomes and PLL$_c$ deposition steps was found to be limited; any additional exposure of the particles to a liposome solution did not result in further liposome adsorption but caused aggregation of the particles. The aggregation is likely caused by the multiple layers of liposomes affecting the colloidal stability of the particles. Moreover, the aggregation during the assembly of the saturated liposomes is possibly due to the temperature cycling between 37 °C for the liposome deposition steps and room temperature during the washing/centrifuging steps. On the other hand, using PLL as a separation layer only allowed for multiple liposome adsorption steps for L$^{5-}$ but not for L$^{u-}$ (Figure 4.9a, □ and ■). Taken together, these findings suggest that the adsorption of L$^{u-}$ strongly
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relies on the presence of the cholesterol moieties, while the deposition of L^{5-} is predominantly electrostatically driven.

Aiming to confirm this hypothesis, PMA_{c}, a negatively charged cholesterol-modified polymer was employed as a separation layer between the liposomes. PMA was not considered since it was previously demonstrated that unmodified PMA is not suitable to anchor liposomes into a polymer film (Figure 4.3). Using PMA_{c} as the separation layer enabled six deposition steps for L^{u-} before no further liposomal adsorption was observed (Figure 4.9b, ■). Multilayer assembly for the L^{5-} was not observed when PMA_{c} was used as a separation layer (Figure 4.9b, ★), confirming the unfavorable combination in assembling L^{5-} multilayers. Since only the cholesterol moieties are expected to anchor the liposomes to the polymer, these results support the hypothesis that L^{5-} requires electrostatic interaction with the polymer separation layer to enable liposome multilayer formation.

Interestingly, the use of PMA_{c} and PLL as a separation layer between the liposomes showed a synergistic loading effect (Figure 4.9c). While L^{u-} (Figure 4.9c, ■) and L^{5-} (Figure 4.9c, ★) were adsorbed in a similar amount during the first two deposition steps, the L^{u-} adsorption during the third adsorption step showed a ~400% increase in fluorescence, suggesting a high liposome loading. This assembly combination allowed for the anchoring of an equivalent amount of seven layers of liposomes in just three adsorption steps. This “boost loading” of liposomes, particularly useful when a high loading of the same cargo is required, was also observed for the subsequent L^{u-} adsorption steps. The same effect was observed for L^{5-} but in a less pronounced manner. These results suggest that the combination of the two polymers PMA_{c} and PLL as separation layers is particularly well-suited for the assembly of multilayers of negatively charged liposomes due to the combined effect of electrostatically-driven adsorption and cholesterol-driven anchoring. A maximum of the equivalent of 20 single liposome layers could be assembled within seven deposition steps (Figure 4.9c, ■).
Figure 4.9. Multilayers of negatively charged liposomes. The fluorescence intensity of silica particles was measured by flow cytometry when PLLc or PLL (a), PMAc (b) or PMAc/PLL (c) were used as polymer separation layers between the liposome deposition steps. Boost-loading of unsaturated and saturated negatively charged liposomes, L^u− and L^s−, was observed when PMAc/PLL was employed as the separation layer (c).
4.4.3.2 Multilayers of Zwitterionic Liposomes

Multilayers of zwitterionic liposomes were assembled using the same set of polymer separation layers as previously described. Independent of the type of liposomes, PLL\(_c\), PLL, and PMA\(_c\)/PLL as a separation layer did not allow for liposome multilayer assembly (Figure 4.10a and 4.10c). However, as depicted in Figure 4.10b, a constant increase in liposomal payload was observed when only PMA\(_c\) was used as a separation layer (Figure 4.10b, ★ and ☲). Using PMA\(_c\) and PLL\(_c\) as alternating separation layers only allowed additional liposome deposition after a PMA\(_c\) layer (Figure 4.10b, ■ and □), suggesting that the assembly of zwitterionic liposomes is predominantly driven by the cholesterol anchoring. The multilayer assembly of Lu,zw or Ls,zw with PMA\(_c\) as a separation layer enables the “stepwise loading” of liposomes, an approach applicable for entrapping equivalent amounts of multicargo in a single capsosome (e.g., for enzymatic cascade reactions or controlled dosage for drug co-delivery approaches).
Figure 4.10. Multilayers of zwitterionic liposomes. The fluorescence intensity of silica particles was measured by flow cytometry when PLLc or PLL (a), PMAc (b) or PMAc/PLL (c) were used as polymer separation layers between the liposome deposition steps. Stepwise loading of unsaturated and saturated zwitterionic liposomes, L^{u,zw} and L^{s,zw}, was observed when PMAc was used as the separation layer (b).
4.5 Conclusions

This chapter provides a detailed investigation of polymer/liposome coassembly on planar surfaces and particle substrates. PLL, PMA, and PMAoa containing different percentages of oleyl methacrylate were successfully synthesized and the ability of these polymers to anchor liposomes to polymer films was assessed. Different types of liposomes (unsaturated or saturated, zwitterionic or negatively charged) can be stably anchored noncovalently to the polymer films by sandwiching them between a tailor made PLL precursor layer and a PMA capping layer. The ability of PMAoa to anchor liposomes to polymer films was compared to PMA. Both studies on planar surfaces and particles suggest that PMA was found to be the superior capping layer, in particular, for saturated liposomes. The alternating deposition of liposomes and polymer separation layers enables the deposition of multilayer of liposomes in the polymer/liposome assembly. PMA and PLL as separation layers facilitate the multilayer assembly of unsaturated or saturated negatively charged liposomes and a “boost loading” of 20 single liposome layers was obtained within seven liposome deposition steps. On the other hand, the multilayer assembly of unsaturated or saturated zwitterionic liposomes with PMA as a separation layer enables the “stepwise loading” of liposomes and 16 single liposome layers were assembled within eight liposome deposition steps. The information gained from this investigation point to the optimized polymer/liposome assembly via cholesterol-driven anchoring concept and can be used to optimize the capsosome assembly, which requires stable incorporation of intact liposomal subcompartments into polymer capsules.
4.6 References


Chapter 5

Capsosome Assembly

5.1 Aim

This chapter describes the assembly of (bio)degradable capsosomes, polymer carrier capsules containing controlled amount of intact liposomal subcompartments, based on the optimized polymer/liposome assembly as previously described in Chapter 4. Two alternative crosslinking pathways, namely oxidative and non-oxidative crosslinking, are explored to form the membrane of the polymer carrier capsules. Capsosomes with single- or multilayer of liposomes are assembled and their appearance and structural integrity are compared. Furthermore, the ability to control the spatial position of the liposomal subcompartments in the capsosomes is investigated.

5.2 Introduction

The creation of capsosomes is based on the sequential assembly of liposomes and polymers onto a particle template. In Chapter 4, noncovalent linkage concept for liposomes based on cholesterol-functionalized polymers was introduced with the aim to optimize the loading of intact liposomes into polymer multilayer films. Cholesterol-modified poly(L-lysine) ($\text{PLL}_c$) as a polymer precursor layer and poly(methacrylic acid)-co-(cholesteryl methacrylate) ($\text{PMA}_c$) as a polymer capping layer were found to be the most suitable combination for the stable incorporation of intact liposomes into the polymer films. The polymer/liposome assembly is then followed by the subsequent deposition of the polymer membrane of the carrier capsule (Scheme 5.1).

The choice of the building blocks of the polymer carrier capsules governs the structural properties, including the long-term stability of the capsosomes, and thus the encapsulated species. (Bio)degradable polymer membranes enable the body to deconstruct the carrier vehicles into their building blocks after their task is fulfilled. Among a variety of polymer systems that are suitable for the assembly of (bio)degradable membranes,\textsuperscript{1-5} poly(N-vinyl pyrrolidone) (PVP) and thiol-functionalized poly(methacrylic acid) (PMA$_{SH}$) were chosen due to the high colloidal stability (Scheme 5.2a). Crosslinking the thiols of the PMA$_{SH}$ and the release of PVP
at physiological conditions yield colloidally stable, (bio)degradable, single-component PMA capsules (Scheme 5.2b). Equally important as the choice of the individual building blocks is an understanding of the structural characteristics of the polymer carrier capsules containing liposomal cargo. In particular, the polymer membrane assembly and stability in physiological media are key factors to be investigated.

In this chapter, the optimal film assembly conditions on planar surfaces are subsequently transferred onto colloidal substrates to form (bio)degradable capsosomes. Different approaches in crosslinking of the thiols in the films are explored, the structural integrity of the capsosomes is presented, and the control over spatial positioning of the liposomal subunits is demonstrated.

**Scheme 5.1.** Schematic illustration of capsosome assembly. A silica particle (i) is coated with a cholesterol-functionalized polymer precursor layer (ii), liposomes (iii), and a cholesterol-functionalized polymer capping layer (iv), followed by subsequent polymer layering and stabilization of the multilayered film by crosslinking (v), and removal of the silica template core (vi). Liposomes are adsorbed onto the polymer surface noncovalently with cholesterol-modified polymers (iva). Cholesterol is spontaneously incorporated into the lipid membrane (ivb).
Scheme 5.2. a) Chemical structures of poly(N-vinyl pyrrolidone) (PVP) (i) and thiol-functionalized poly(methacrylic acid) (PMA$_{SH}$) (ii). b) Assembly of PMA capsule: (i) PMA$_{SH}$/PVP multilayer film is assembled on a sacrificial particle template and (ii) stabilization of PMA$_{SH}$ layers through disulfide linkages and core removal yield single-component PMA capsule at physiological conditions.
5.3 Experimental Section

5.3.1 Materials
Poly(methacrylic acid) (PMA, $M_w$ 15 kDa) was purchased from Polysciences (Pennsylvania, USA). Pyridine dithioethylethamine hydrochloride (PDA-HCl) was obtained from SpeedChemical Corp. (Shanghai, China). 1-Ethyl-3-(3-dimethylamino propyl)carbodiimide hydrochloride (EDC) was purchased from Thermo Fisher (Massachusetts, USA). DL-dithiothreitol (DTT), 2,2'-azoisobutyronitrile (AIBN), dimethylacetamide (DMAC), diethyl ether, poly(N-vinyl pyrrolidone) (PVP, $M_w$ 10 kDa), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), sodium chloride (NaCl), sodium acetate (NaOAc), 2-(N-morpholino)ethanesulfonic acid (MES), 3-(N-morpholino)propanesulfonic acid (MOPS), chloramine T (CaT), 2,2'-dithiodipyridine (DTDP), hydrofluoric acid (HF), and ammonium fluoride (NH$_4$F) were purchased from Sigma-Aldrich (Sydney, Australia). Silica particles (3.25 μm-diameter) were purchased from Microparticles GmbH (Berlin, Germany). Poly(hydroxypropyl methacrylamide) (HPMA), 7 2-(2-pyridyldisulfide)ethylmethacrylate (PDSM), 8 RAFT agent 4-(cyanopentanoic acid)-4-dithiobenzoate (CPADB), 9 and poly(N-vinyl pyrrolidone)-block-(cholesteryl acrylate) (PVP$_c$, $M_w$ 11 kDa) 10 was synthesized according to previously published protocols.

Four types of buffer were used for all of the experiments: (i) HEPES buffer consisting of 10 mM HEPES and 150 mM NaCl (pH 7.4); (ii) NaOAc buffer consisting of 20 mM NaOAc (pH 4.0); (iii) MES buffer consisting of 50 mM MES (pH 6.0); and (iv) MOPS buffer consisting of 20 mM MOPS (pH 8.0). The buffer solutions were made with ultrapure water (Milli-Q gradient A 10 system, resistivity = 18 MΩ cm, TOC < 4 ppb, Millipore Corporation, Massachusetts, USA).
5.3.2 Polymer Synthesis

5.3.2.1 Cholesterol-Modified Poly(L-lysine) (PLLc, \( M_w \) 40 - 60 kDa)
Refer to Chapter 4, Section 4.3.3.1.

5.3.2.2 Poly(methacrylic acid)-co-(cholesteryl methacrylate) (PMAc, \( M_w \) 11.5 kDa)
Refer to Chapter 4, Section 4.3.3.2.

5.3.2.3 Thiol-Functionalized Poly(methacrylic acid) (PMA\(_{PD(SH)}\), \( M_w \) 15 kDa)
PMA samples with 5 or 14 mol\% of thiol modification were synthesized through functionalization of PMA with PDA. 250 mg of 30 wt % of PMA was diluted into 5 mL of phosphate buffer (10 mM, pH 7.2). The PMA was reacted with EDC at 3-fold molar excess of the target modification for 15 min. Subsequently, PDA-HCl (target modifications of 5 or 14 mol\%) was added to the mixture and the reaction was allowed to proceed overnight. The reaction mixture was purified via dialysis for 3 days against water and the polymer was isolated via freeze-drying to obtain white powder of PMA\(_{PD}\). The thiol content of the resulting polymer was characterized by measuring the absorbance of PDA groups (\( \lambda_{\text{max}} = 343 \text{ nm} \)) and then derived by correlation with a calibration curve of PDA. The reduced form, PMA\(_{SH}\), was prepared by incubating PMA\(_{PD}\) in a solution of 0.5 M DTT (100 mg mL\(^{-1}\)) in MOPS buffer for at least 30 min at 37 °C prior to dilution in NaOAc buffer (2 mg mL\(^{-1}\)). PMA\(_{PD}\) with 5 mol\% thiol groups were employed as the crosslinker, while PMA\(_{SH}\) with 14 mol\% thiol groups were used as the building blocks for the membrane of the polymer carrier capsules.

5.3.2.4 Poly(hydroxypropyl methacrylamide) with Pyridyl Disulfide-Activated Thiol Groups (HPMA\(_{PD}\), \( M_w \) 33 kDa)
HPMA (0.40 g, \( 2.8 \times 10^{-3} \text{ mol} \)), PDSM (47.5 mg, 0.186 mmol), RAFT agent CPADB (2.79 mg, 10 mmol), and initiator AIBN (0.55 mg, 3.35 mmol) were dissolved in 3 mL DMAC. The flask was sealed and purged with argon for 1 h. The flask was then immersed in a preheated oil bath at 70 °C. The polymerization was quenched after 7.5 h by immersing the flask in liquid nitrogen. After thawing, the polymer solution
was analyzed by $^1$H NMR spectroscopy in DMSO-d$_6$ to determine the monomer conversion. The polymerization solution was precipitated in diethyl ether (3x). The yielding polymer (pink powder) was dried in a vacuum oven for 24 h. The polymer was characterized by $^1$H NMR spectroscopy and SEC. The number average molecular weight and polydispersity index were 33,000 g mol$^{-1}$ and 1.26, respectively.

5.3.3 Liposome Formation

Saturated zwitterionic liposomes ($L^{S,zw}$) and the fluorescent counterpart ($L^{S,zw,NBD}$) were prepared as detailed in Chapter 4, Section 4.3.4.

5.3.4 Polymer/Liposome Multilayer Film on Planar Surfaces

QCM-D measurements (Q-sense E4, Sweden) were used to characterize the assembly of polymer/liposome multilayer film on planar surfaces. Silica-coated crystals (QSX300, Q-sense) were cleaned by immersion in a 2 wt % sodium dodecyl sulfate solution overnight, followed by rinsing with Milli-Q water. The crystals were then blow-dried with nitrogen and exposed to UV (Bioforce Nanosciences, U.S.A.) for 30 min before being mounted into the liquid-exchange chambers of the instrument. Unless otherwise mentioned, the mass and dissipation measurements were monitored at 24 ± 0.02 °C. When a stable baseline in HEPES buffer was achieved, PLLc (1 mg mL$^{-1}$, 15 min) was introduced into the measurement chamber and left to adsorb onto the crystal to form a polymer precursor layer. After the surface was saturated, the chamber was rinsed with buffer solution to remove the excess polymer. The resulting polymer-coated surface was then exposed to liposomes (2.5 mg mL$^{-1}$, 1 h) and left to incubate until the surface was saturated. The liposome solution was replaced with buffer solution and the film assembly was continued by adsorbing PMAc (1 mg mL$^{-1}$, 15 min). When the surface was saturated, the chambers were washed with buffer solution. After changing from HEPES to NaOAc buffer, the polymer layering was continued by the alternating adsorption of PVP (1 mg mL$^{-1}$, 15 min) and PMA (1 mg mL$^{-1}$, 15 min). Normalized frequencies using the third overtone are presented.
5.3.5 Capsosome Assembly

5.3.5.1 Capsosomes with Membrane-Associated Liposomal Subcompartments
A suspension of 3.25 μm-diameter SiO$_2$ particles (5 wt %) in HEPES buffer was incubated with the polymer precursor layer, PLL$_c$ (1 mg mL$^{-1}$, 15 min), and washed three centrifugation/redispersion cycles (1060 g, 30 s). Liposomes (2.5 mg mL$^{-1}$, 40 min) were allowed to interact with the polymer-coated particles, washed three times, and polymer separation layers were subsequently adsorbed (1 mg mL$^{-1}$, 15 min). The adsorption of liposomes and polymer separation layer(s) was repeated until the required number of layers was deposited, followed by the adsorption of a PMA$_c$ capping layer (1 mg mL$^{-1}$, 15 min). The buffer was changed to NaOAc and five bilayers of alternating PVP (1 mg mL$^{-1}$, 10 min) and PMA$_{SH}$ (1 mg mL$^{-1}$, 10 min) were sequentially deposited.

5.3.5.2 Capsosomes with “Free-Floating” Liposomal Subcompartments
A suspension of 3.25 μm-diameter SiO$_2$ particles (5 wt %) in HEPES buffer was incubated with PVP$_c$ (1 mg mL$^{-1}$, 15 min), washed with three centrifugation/redispersion cycles (1060 g, 30 s), followed by the adsorption of liposomes (2.5 mg mL$^{-1}$, 40 min). The coated particles were allowed to interact with the capping layer PVP$_c$ (1 mg mL$^{-1}$, 15 min) and the buffer was exchanged to NaOAc buffer. The assembly was continued by adding PMA (1 mg mL$^{-1}$, 10 min), followed by the sequential deposition of five bilayers of PVP (1 mg mL$^{-1}$, 10 min) and PMA$_{SH}$ (1 mg mL$^{-1}$, 10 min).

5.3.5.3 Crosslinking of the Membrane of Carrier Capsules
The thiols within the polymer layers were crosslinked with either: (a) CaT (2.5 mM, 1 min) in MES buffer, (b) PMA$_{PD}$ (1 mg mL$^{-1}$, 15 h) in NaOAc buffer, or (c) HPMA$_{PD}$ (1 mg mL$^{-1}$, 15 h) in NaOAc buffer. Capsosomes were obtained by dissolving the silica core particles using a 2 M HF/8 M NH$_4$F solution for 2 min, followed by multiple centrifugation (4500 g, 3 min)/NaOAc buffer washing cycles. Caution! HF and NH$_4$F are highly toxic. Extreme care should be taken when handling HF solution and only small quantities should be prepared.
5.3.6 Characterization Methods

5.3.6.1 Differential Interference Contrast (DIC) Microscopy
DIC images of capsosomes were taken with an Olympus IX71 digital wide-field microscope equipped with a DIC slider (U-DICT, Olympus), the corresponding filter sets, and a 60x oil immersion objective.

5.3.6.2 Confocal Laser Scanning Microscopy (CLSM)
Fluorescently labeled capsosomes were imaged with a Leica TCS SP2 AOBS confocal microscope equipped with an argon laser ($\lambda = 488$ nm) using a 63x oil immersion objective (Leica, Germany).

5.3.6.3 Transmission Electron Microscopy (TEM)
Five microliters of the capsosome sample was adsorbed for 2 min onto a carbon-coated Formvar film mounted on 300 mesh plasma-treated copper grids (ProSciTech, Australia). The grids were blotted and investigations were undertaken using an FEI Tecnai TF30 instrument (FEI-Company, Eindhoven, The Netherlands).
5.4 Results and Discussion

5.4.1 Polymer/Liposome Multilayer Film on Planar Surfaces

Having identified suitable polymer precursor and capping layers for the stable adsorption of different types of liposomes onto surfaces (Chapter 4), the possibility to subsequently assemble a polymer multilayer film was verified. The polymer system that has been used as a platform to create polymer multilayer films with versatile and tunable properties was chosen: PVP and PMA, a pair of polymers that interact via hydrogen bonding.\

Figure 5.1 shows the frequency changes for the PVP/PMA film assembly on planar substrates on a bare silica crystal or a crystal pre-coated with PLL<sub>c</sub>, L<sub>L,s,zw</sub>, and PMA<sub>c</sub>. Similar linear film build-up of PVP (ΔF = -14 ± 6 Hz) and PMA (ΔF = -8 ± 1 Hz) was observed in both cases. These data demonstrate that there is a negligible effect of the presence of liposomes and the cholesterol moieties on the subsequent polymer film assembly. This also further substantiates the utility of PMA<sub>c</sub> as a capping layer, which not only supports the incorporation of the liposomes, but also allows the subsequent deposition of the hydrogen-bonded polymer multilayer film.
5.4.2 Capsosome Assembly

Capsosome formation requires film assembly on colloidal substrates via the layer-by-layer (LbL) technique (Scheme 5.1). Here, the optimized film assembly conditions based on the findings on planar surfaces (Section 5.4.1) were used for the formation of (bio)degradable capsosomes. The film assembly on colloidal particles, the subsequent formation of hollow capsules, and the structural integrity of capsosomes under assembly and physiological conditions were investigated using fluorescence microscopy.

5.4.2.1 Capsosomes with Single Layer of Liposomal Subcompartments

With the aim to control the properties of the polymer carrier capsules containing intact liposomes, structural stability was explored as a function of the number of polymer layers assembled, the crosslinking approach, and the buffer conditions. The
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optimized film assembly (i.e., PLL\textsubscript{c} as a precursor and PMA\textsubscript{c} as a capping layer) was used to stably anchor $L^{5,2w}$ within a PMA\textsubscript{SH}/PVP carrier capsule. Assembly of the carrier capsules via the sequential adsorption of PVP and Alexa Fluor 633-labeled PMA\textsubscript{SH} (PMA\textsubscript{SH}-633) at pH 4 was confirmed by a progressive increase in the fluorescence of the particles (Figure 5.2a). One bilayer of PMA\textsubscript{SH}-633/PVP was sufficient to obtain stable capsosomes, although ~25% of them were distorted and/or aggregated. At least three polymer bilayers were required to enable the formation of intact, non-aggregated capsosomes. To obtain capsosomes that retain their integrity at physiological conditions (pH 7.4), the thiol groups of PMA\textsubscript{SH} need to be crosslinked into bridging disulfide bonds.\textsuperscript{3,6}

![Fluorescence microscopy images of capsosomes](image)

**Figure 5.2.** Fluorescence microscopy images of PLL\textsubscript{c}/L\textsuperscript{5,2w}/PMA\textsubscript{c}/PVP/(PMA\textsubscript{SH}/PVP)\textsubscript{x} capsosomes in NaOAc (pH 4) (a) and HEPES buffer (pH 7.4) (b), revealing a progressive increase in fluorescence intensity due to the subsequent addition of one to five bilayers of PMA\textsubscript{SH}-633/PVP (all scale bars are 5 µm).

In this study, two alternative crosslinking pathways were considered, namely oxidative (with the use of an oxidizing agent CaT)\textsuperscript{6} and “non-oxidative” crosslinking achieved via thiol-disulfide exchange. While the former method has proven to be effective to afford stable capsules, it can have deleterious effects to therapeutics and biomolecules that are susceptible to oxidation, and consequently can result in a loss of their functional activity; therefore, the use of an oxidation-free approach to stabilize the polymer carrier capsules is particularly important in the creation of
capsosomes. This led to the use of a polymeric crosslinking reagent, PMA with thiol groups activated toward thiol-disulfide exchange with pyridyl disulfide (PMA_{PD}). This concept neither affects fragile cargo within the polymer carrier capsules due to its polymeric nature, nor causes harm by the presence of other solutes involved in the crosslinking process. In both cases, upon increasing the pH from 4 to 7.4, which causes the release of PVP, the single-component disulfide-stabilized PMA carrier capsules kept their structural integrity, and the ionization of PMA caused a pronounced swelling of the capsosomes (Figure 5.2b).

Figure 5.3 summarizes the measured size of the capsosomes as a function of the number of PMA_{SH}/PVP bilayers at assembly conditions (NaOAc buffer, pH 4) and physiological conditions (HEPES buffer, pH 7.4) using either PMA_{PD} (Figure 5.3a) or CaT (Figure 5.3b) to crosslink the thiols in the film. In NaOAc buffer, the capsosome diameter was uniform for all the different assemblies and insensitive to the crosslinker (average 3.46 ± 0.05 μm). However, at physiological conditions (pH 7.4), the swelling of the PMA_{PD}-crosslinked capsosomes was observed to be proportional ($R^2 = 0.90$) to the number of polymer bilayers assembled. This corresponds to an increase in diameter from 27% to 75% for capsosomes assembled from one to five bilayers of PMA_{SH}/PVP, possibly due to a decrease in crosslinking density caused by limited infiltration of the polymeric crosslinker into the multilayered film. In comparison, the swelling of CaT-crosslinked capsosomes was observed to be ~27% and largely independent of the number of PMA_{SH}/PVP bilayers assembled, in agreement with the previous work.\(^6\) Although different swelling behavior was observed, both crosslinking approaches yielded stable capsosomes, showing that the presence of both liposomes and cholesterol groups on the polymer did not interfere with the subsequent polymer film assembly.
Figure 5.3. Diameters of PLLc/L5zw/PMAc/PVP/(PMAc/PVP)x capsosomes as a function of the number of assembled PMAc/PVP bilayers crosslinked with PMAPD (a) or CaT (b) in NaOAc buffer pH 4 (yellow bars) or HEPES buffer pH 7.4 (brown bars).

The incorporation of large soft objects such as 50 nm intact liposomes within a polymer film may drastically change the properties of the polymer carrier capsules including the crosslinking density. To characterize the effect of the liposomes on the structural integrity of the polymer carrier capsules, the swelling behaviors of polymeric-crosslinked and CaT-derived capsosomes were also compared to pristine PMA capsules. In addition to PMAPD, a nonionic polymer – HPMAPD, is employed as a crosslinker due to potentially enhanced infiltration of this polymer into the multilayers. Nonoxidative crosslinkers (PMAPD or HPMAPD) or oxidative crosslinker (CaT) were infiltrated into the preassembled film, either before or after removal of the particle templates, referred to as pre- and post-crosslinking, respectively (Scheme 5.3).
Scheme 5.3. a) Schematic illustration of crosslinking of the membrane of the polymer carrier capsules for capsosome assembly. The thiol groups in the polymer films are crosslinked prior to core removal (pre-XL), while post-XL refers to converting thiols into bridging disulfide linkages after dissolution of the template cores to yield capsosomes. b) Chemical structures of polymeric crosslinkers: (i) poly(methacrylic acid) with 5 mol% pyridyl disulfide-activated thiol group (PMA<sub>PD</sub>) and (ii) poly(hydroxypropyl methacrylamide) with 13 mol% pyridyl disulfide-activated thiol groups (HPMA<sub>PD</sub>).
All tested crosslinking approaches yielded colloidaly stable 3 μm-diameter Alexa Fluor 488-labeled PMA capsules (Figure 5.4a) and capsosomes (Figure 5.4b) in both assembly (pH 4, i) and physiological (pH 7.4, ii) conditions. In the absence of liposomes, the 3 μm-diameter PMA capsules were well-crosslinked with the use of PMA\textsubscript{PD} or HPMA\textsubscript{PD}, evidenced by their similar swelling behavior to the CaT-crosslinked capsules (Figure 5.4c, white and red bars).

**Figure 5.4.** Fluorescence microscopy images of AF488-labeled PMA capsules (a) and capsosomes (b), pre-crosslinked with PMA\textsubscript{PD} at pH 4 (i) and pH 7.4 (ii). All scale bars are 5 μm. c) Diameters of PMA capsules and capsosomes, depending on the different crosslinking methods at both assembly (pH 4) and physiological (pH 7.4) conditions.
In the case of HPMA\textsubscript{PD}, almost no swelling was observed upon increasing the pH from 4 to 7.4 indicating that the PMA capsules have a high crosslinking density, which may have a significant impact on their permeability characteristics. For capsosomes, the use of PMA\textsubscript{PD} gave rise to somewhat lower crosslinked samples with a higher radius as compared to parent PMA capsules. In contrast, HPMA\textsubscript{PD}-afforded capsosomes showed similar swelling characteristics to those obtained with the use of CaT, suggesting a high degree of crosslinking. The use of polymeric crosslinkers is expected to preserve the activity of encapsulated enzymatic cargo within the liposomal subcompartments to yield functional capsosomes.

5.4.2.2 Capsosomes with Multiple Layers of Liposomal Subcompartments

Chapter 4, Section 4.4.3.2 demonstrates the multilayer assembly of saturated zwitterionic liposomes with PMA\textsubscript{c} as a separation layer, which enables the deposition of 16 single liposome layers within eight liposome deposition steps. Herein, capsosomes with multiple layers of liposomal subcompartments are obtained by capping the liposome multilayer assembly on particle templates with PMA\textsubscript{c}, followed by subsequent deposition of PVP and PMA\textsubscript{SH} to form the membrane of the carrier vehicle, crosslinking of the thiols in the polymer film, and removal of the template core (Scheme 5.4). Capsosomes prepared with one or eight L\textsubscript{5,2w} deposition steps, C\textsubscript{L1} or C\textsubscript{L8}, respectively, were visualized using optical and electron microscopy techniques to confirm and compare their appearance and structural integrity (Figure 5.5). DIC images taken under physiological conditions demonstrated that both types of capsosomes were intact and non-agglomerated and preserved the spherical shape of the template silica particles (Figure 5.5a and 5.5b). Moreover, CLSM images of capsosomes assembled using fluorescently labeled liposomes confirmed the presence of additional liposome layers for C\textsubscript{L8} by the observation of a thicker shell and consequently the larger diameter of 5.01 ± 0.46 μm (Figure 5.5d) compared to 3.55 ± 0.30 μm for C\textsubscript{L1} (Figure 5.5c). This difference is expected assuming that intact 50 nm liposomes were loaded in eight adsorption steps (~16 layers of liposome), resulting in the addition of ~1.6 μm in diameter for C\textsubscript{L8}, in comparison to C\textsubscript{L1}. TEM images further supported the presence of a high liposomal loading for C\textsubscript{L8}; while C\textsubscript{L1} showed the expected folding of the polymer carrier capsule.
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(Figure 5.5e), C_{L8} remained spherically intact and appeared particle-like (Figure 5.5f). This observation demonstrates that one layer of liposomes is not sufficient to stabilize the polymer membrane, that is, to keep the capsosomes in their spherical shape under vacuum, while multiple layers of liposomes sustain the capsosomes’ shape even under vacuum.

Scheme 5.4. Schematic illustration of the assembly of capsosome with multiple layers of liposomal subcompartments. A silica core is coated with a polymer precursor layer (i) and liposomes (ii), followed by the alternating adsorption of separation layer(s) and liposomes until the required number of layers is deposited (iii and iv). A polymer capping layer (v) is adsorbed prior to the deposition of five bilayers of PVP and PMA_{SH} (vi). Crosslinking of the thiols and dissolution of the core (vii) result in a capsosome with multiple layers of intact liposomes (C_{Lx}).
5.4.3 Spatial Positioning of Liposomal Subcompartments in Capsosomes

Spatial positioning of the liposomal subunits within the polymer carrier capsules can be controlled to yield capsosomes with membrane-associated or “free-floating” liposomal subcompartments (Scheme 5.5). As depicted in Figure 5.5, sandwiching liposomes between PLLc precursor layer and PMAc capping layer yields capsosomes with membrane-associated liposomal subunits. For the assembly of capsosomes with “free-floating” liposomal subunits, PVPc was identified as the suitable polymer precursor and capping layers.10 PVPc is an uncharged block copolymer consisting of a long PVP segment and a short block of cholesteryl acrylate, which therefore minimizes the entanglement of the copolymer with the membrane of the carrier capsule. After capping the liposomes with PVPc, a polymer sacrificial layer, PMA, is subsequently adsorbed. The polymer carrier capsules are created by the sequential deposition of five bilayers of PVP and PMA_{SH}. The thiol groups in the polymer multilayer film of these core-shell particles are crosslinked into disulfide linkages prior to dissolution of the template particles. When these capsosomes are dispersed in pH 7.4, the hydrogen bonds between PVPc and PMA are disrupted, consequently
assisting in detachment of the liposomes from the polymer membrane to form capsosomes with with “free-floating” liposomal subunits.

Scheme 5.5. Schematic illustration of the assembly of capsosomes with membrane-associated (a-f) or “free-floating” (a’-f’) subunits. A silica particle is coated with a polymer precursor layer (a, a’) and liposomes (b, b’), followed by the deposition of a polymer capping layer, a polymer separation layer, and five bilayers of PVP/PMA$_{SH}$ (c, c’). Stabilization of the multilayered film by crosslinking of the thiols and core dissolution (d, d’) result in capsosomes with the subunits either in the membrane (e) or “free-floating” (e’) in the assembly when dispersed at physiological conditions. CLSM images of capsosomes with membrane-associated (f) or “free-floating” (f’) subunits at physiological conditions (pH 7.4). Scale bars are 5 µm.

While the loading efficiency of the subunits is not expected to be affected, the functional activity of capsosomes could benefit from spatial control over the positioning of the subcompartments. The former approach allows the assembly of multilayers of liposomal subcompartments; that is, a strata-like film consisting of alternating liposome and polymer layers. This design has potential benefits in, for example, the delivery of high dosages of therapeutic agents, coadministration of complementary therapeutics with the ability to control the ratio of encapsulated cargo, or coloading of enzymes for the performance of one-pot enzymatic cascade reactions or multiple parallel reactions in the same vessel. The latter design contains liposomal subunits that are encapsulated in the void of the carrier capsules and is suitable for the performance of multistep catalytic cascade processes that require rapid reagent mixing within a confined environment.
5.5 Conclusions

This chapter describes the assembly of (bio)degradable capsosomes – disulfide-stabilized polymer carrier capsules subcompartmentalized with controlled amount of intact liposomal subunits. Experiments on planar and colloidal substrates showed that $\text{PMA}_{\text{SH}}/\text{PVP}$ multilayer films can be subsequently assembled on top of the polymer/liposome assembly. At least three polymer bilayers were required to form intact, colloidally stable capsosomes. Two approaches to achieve thiol-to-disulfide conversion were examined with the use of an oxidizing agent (CaT) or via thiol-disulfide exchange with polymeric crosslinkers ($\text{PMA}_{\text{PD}}$ or $\text{HPMA}_{\text{PD}}$). While each approach yielded stable capsosomes at both assembly (pH 4) and physiological conditions (pH 7.4), the use of oxidation-free polymeric crosslinkers is expected to preserve the enzymatic activity of the proteins within the liposomal subcompartments. The number of subcompartments within a capsosome can be controlled by the alternate layering of liposomes and polymer separation layers. Capsosomes with eight liposome deposition steps were obtained and their structural integrity was compared to capsosomes with single liposome deposition steps. Furthermore, control over spatial positioning of the liposomal subunits within the PMA carrier capsules was demonstrated through the choice of polymer precursor and capping layers, yielding capsosomes with membrane-associated or “free-floating” subunits. These results highlight the pivotal features on the way to developing capsosomes into a functional biomedical platform and as mimics for cells.
5.6 References

Chapter 6

Liposomal Subcompartments in Capsosomes: Cargo Retention and Stability

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6.1 Aim

The aim of this chapter is to examine the encapsulation efficiency of (enzymatic) cargo within the liposomal subcompartments of capsosomes. The size-dependent retention of the cargo encapsulated within the liposomal subunits during capsosome assembly and its dependence on environmental pH changes are detailed. Furthermore, long-term stability of the cargo encapsulated within the capsosomes is investigated.

6.2 Introduction

Successful assembly of (bio)degradable capsosomes was described in Chapter 5; cholesterol-modified poly(L-lysine) (PLLc) as a polymer precursor layer and poly(methacrylic acid)-co-(cholesteryl methacrylate) (PMAc) as a polymer capping layer were used to stably anchor liposomes within a carrier capsule assembled via the layer-by-layer (LbL) of poly(N-vinyl pyrrolidone) (PVP) and thiol-functionalized poly(methacrylic acid) (PMAcSH). Apart from gaining control over the number of assembled liposomal subcompartments, understanding the capsosome’s ability to retain (enzymatic) cargo within the liposomal subunits is crucial to establish this subcompartmentalized system as microreactors and therapeutic carriers.

Coencapsulation of different enzymes and/or small molecules requires an understanding of the size-dependent retention of different cargo in capsosomes, especially during their assembly. Although several studies have been reported on cargo encapsulation and the pH properties in liposomes, predominantly in solution,\textsuperscript{1-3} there are different aspects that have to be taken into account when capsosomes are considered. In particular, effects attributed to the immobilization of liposomes to polymer-coated silica particle surfaces, that is, their anchoring to different cholesterol-modified polymers, as well as environmental pH changes during the capsosome assembly, have to be considered.

In this study, the size-dependent retention of the cargo within the liposomes during the capsosome assembly process as a function of the environmental pH is
characterized. Fluorescently labeled cargo of different sizes are examined: luciferase fluorescein isothiocyanate (Luc<sub>FITC</sub>, $M_w$ 79 kDa), 10 kDa dextran fluorescein isothiocyanate (Dextran<sub>10-FITC</sub>), 4 kDa dextran fluorescein isothiocyanate (Dextran<sub>4-FITC</sub>), carboxyrhodamine (CR, $M_w$ 550 Da), and carboxyfluorescein (CF, $M_w$ 400 Da). The pH-sensitive dye FITC is used to monitor the cargo retention and the pH changes within the liposomes simultaneously, while the pH-insensitive dye rhodamine is solely used to characterize cargo retention. The size range covers large molecules such as enzymes down to models for low molecular weight drugs.

Scheme 6.1. Encapsulation of (enzymatic) cargo within the liposomal subcompartments of capsosomes.
6.3 Experimental Section

6.3.1 Materials

Zwitterionic lipids, 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC, phase transition temperature -20 °C), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC, phase transition temperature 23 °C), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC, phase transition temperature 41 °C), were purchased from Avanti Polar Lipids (Alabama, USA). β-lactamase, luciferase (from Vibrio fischeri), fluorescein isothiocyanate (FITC), Dextran<sub>10-FITC</sub> (<i>M<sub>w</sub></i> 10 kDa), Dextran<sub>4-FITC</sub> (<i>M<sub>w</sub></i> 4 kDa), carboxyrhodamine (CR, <i>M<sub>w</sub></i> 550 Da), carboxyfluorescein (CF, <i>M<sub>w</sub></i> 400 Da), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), sodium chloride (NaCl), and sodium acetate (NaOAc) were purchased from Sigma-Aldrich (Sydney, Australia).

Two types of buffer were used for all of the experiments: (i) HEPES buffer consisting of 10 mM HEPES and 150 mM NaCl (pH 7.4) and (ii) NaOAc buffer consisting of 20 mM NaOAc (pH 4.0). The buffer solutions were made with ultrapure water (Milli-Q gradient A 10 system, resistivity ≈ 18 MΩ cm, TOC < 4 ppb, Millipore Corporation, Massachusetts, USA).

6.3.2 Liposome Formation

Fluorescently labeled saturated zwitterionic liposomes (<i>L<sup>5,SW</sup> NBD</i>) were prepared as detailed in Chapter 4, Section 4.3.4.

For the cargo size-dependent retention study, unilamellar liposomes were prepared as follows: chloroform of the lipid solution (2.5 mg of DOPC (<i>L<sup>1,SW</sup></i>) or DMPC/DPPC = 4:1 wt % (<i>L<sup>5,SW</sup></i>)) was evaporated under nitrogen for 1 h, followed by hydration with 200 µL of 3 mg mL<sup>-1</sup> Luc<sub>FITC</sub> in HEPES buffer. The concentration of Dextran<sub>10-FITC</sub>, Dextran<sub>4-FITC</sub>, or CF encapsulated within the liposomes was 1 mM, while CR was encapsulated with a concentration of 60 mM. Each solution was diluted into 1 mL of HEPES buffer and extruded through 50 nm filters (31 times) to obtain liposomes of monodisperse size.
For the long-term stability study, enzyme-loaded saturated zwitterionic liposomes were prepared by evaporation of the chloroform of the lipid solution (2.5 mg of DMPC/DPPC = 4:1 wt % (L$_{5,2w}$)) under nitrogen for 1 h, followed by hydration with 200 µL of 0.3 mg mL$^{-1}$ Alexa Fluor 488-labeled luciferase (luciferase$_{488}$) or β-lactamase (β-lactamase$_{488}$) in HEPES buffer, dilution into 1 mL of HEPES buffer, and extrusion through 50 nm filters (31 times).

### 6.3.3 Capsosome Assembly

Refer to Chapter 5, Section 5.3.5.1 and Section 5.3.5.3. All of the capsosomes assembled in this study were crosslinked with PMA$_{PD}$ (1 mg mL$^{-1}$, 15 h) in NaOAc buffer.

### 6.3.4 Characterization Methods

#### 6.3.4.1 Flow Cytometry

A Cyflow Space (Partec GmbH, Germany) flow cytometer using an excitation wavelength of 488 nm was used for all of the flow cytometry experiments. At least 20,000 particles or capsosomes were analyzed in each experiment.
6.4 Results and Discussion

6.4.1 Cargo Size-Dependent Retention within the Liposomal Subunits

To simultaneously monitor the pH changes within the liposomes and to identify the lower limit of the size of cargo that can be encapsulated, cargo of different molecular weights coupled to the pH-sensitive fluorophore FITC were entrapped within the liposomal subcompartments. The fluorescence intensity of silica particles was measured upon assembly of a PLLc precursor layer, the cargo-loaded liposomes, and a PMAc capping layer under physiological conditions (HEPES buffer, pH 7.4). The assemblies were then exposed to buffer solutions with pH between 4 and 7.4. This pH range was chosen because the assembly and crosslinking of the polymer membrane requires buffer solutions at pH 4 and 6.

Figure 6.1a depicts an example of such an experiment using Dextran10-FITC encapsulated within zwitterionic liposomes (L12,zw, ■ and L5,zw, □). After capping the liposomes with PMAc in pH 7.4, the buffer solution was changed from HEPES (pH 7.4) to NaOAc (pH 4), which caused a decrease in fluorescence intensity, suggesting that the pH within the liposomes was lowered. The environmental change within the liposomes was further supported by a stepwise increase in intensity upon returning the environmental pH back from 4 to 7.4. Nevertheless, when returned to pH 7.4, the measured intensity was the same as before the pH changes, suggesting that there was no measurable loss of cargo from the liposomes.
Figure 6.1. Cargo size-dependent retention within the liposomal subunits of capsosomes. a) Normalized fluorescence intensity of silica particles measured by flow cytometry, due to the encapsulation of Dextran_{10,FITC} (M_w 10 kDa) within the zwitterionic liposomes, during capsosome assembly followed by exposure to buffer solutions with pH between 4 and 7.4. b) Summary of cargo size-dependent retention for unsaturated (L_{u,zw} (i)) and saturated (L_{s,zw} (ii)) zwitterionic liposomes encapsulating Luc_{FITC} (M_w 79 kDa), Dextran_{4,FITC} (M_w 4 kDa), carboxyrhodamine (CR, M_w 550 Da), or carboxyfluorescein (CF, M_w 400 Da) during capsosome assembly.
Figure 6.1b summarizes the cargo retention of Luc<sub>FITC</sub>, Dextran<sub>4-FITC</sub>, CR, and CF entrapped within L<sub>u,zw</sub> (Figure 6.1bi) and L<sub>s,zw</sub> (Figure 6.1bii) after liposome deposition onto PLL<sub>c</sub>-precoated silica particles, and after capping with PMA<sub>c</sub> and exposure to different pH. The data suggest that only L<sub>u,zw</sub> could retain small-sized cargo (i.e., CF, <500 Da) during the initial liposome adsorption step. The necessity of raising the temperature above the phase transition temperature (<i>T</i><sub>m</sub>) of the liposomes during the assembly of the L<sub>s,zw</sub> (Chapter 4, Section 4.4.1.2) likely caused the loss of the encapsulated CF. The cargo (with the exception of Luc<sub>FITC</sub>) entrapped in L<sub>u,zw</sub> remained unaffected by capping with PMA<sub>c</sub>, while ~30 - 40% of the cargo encapsulated within L<sub>s,zw</sub> was lost, again most likely due to the required cycling above <i>T</i><sub>m</sub> for the PMA<sub>c</sub> adsorption. On the other hand, upon pH cycling from 7.4 to 4 and back to 7.4, a ~20 and 35% cargo loss for Dextran<sub>4-FITC</sub> and CR was monitored, respectively, from L<sub>u,zw</sub>, while the cargo within L<sub>s,zw</sub> was largely retained despite the pH cycling. Additionally, even though unsaturated liposomes were able to encapsulate CF during the initial stages of the assembly (at physiological conditions), there was no restoration of the fluorescence intensity observed when the pH was raised from 4 back to 7.4, suggesting the entire release of the cargo from the liposomes occurred due to exposure to the acidic environment. This retention characteristic suggests that, when exposure to an acidic environment is required for the capsosome assembly, the cutoff size for the cargo encapsulated within the liposomes is between the molecular size of CF and CR (i.e., between 400 and 550 Da). It was previously reported that the presence of weak acids, in this case acetic acid, or fetal bovine serum can induce the release of encapsulated CF from liposomes.

While the change in permeability of the lipid membrane due to the increased temperature or the presence of weak acids has to be considered for the assembly of capsosomes loaded with small cargo, this feature might be beneficial for biomedical applications, that is, for the performance of continuous enzymatic reactions or for the slow release of small drugs in the acidic intracellular compartments.
6.4.2 Stability of Cargo Encapsulated within the Liposomal Subunits

Successful encapsulated catalysis in capsosomes toward enzyme therapy is governed by the stability of the encapsulated enzymatic cargo within the subcompartments. Herein, the ability of capsosomes to retain enzymatic cargo within the liposomal subcompartments was examined as a function of the number of polymer layers assembled. Two different enzymes were encapsulated into the liposomal subcompartments: luciferase ($M_w$ 79 kDa) and β-lactamase ($M_w$ 31 kDa), with isoelectric points of 6.5 and 6.1, respectively. Therefore, the size of the enzyme is the main parameter that is considered in this investigation. Figure 6.2 shows the normalized fluorescence intensity of capsosomes with luciferase$_{488}$-loaded or β-lactamase$_{488}$-loaded liposomes as a function of the number of assembled PMA$_{SH}$/PVP bilayers, as measured by flow cytometry. At assembly (yellow bars) and physiological (brown bars) conditions, the quantity of cargo entrapped inside the polymer carrier capsules remained constant and was independent of the number of encasing PMA$_{SH}$/PVP bilayers. This further confirmed the successful anchoring of the liposomes within the polymer multilayer film via noncovalent cholesterol linkage. More importantly, the lack of any measurable loss of cargo showed that the enzymes along with the liposomal subcompartments remained entrapped during the subsequent polymer layering, which involved multiple washing/re-dispersion steps, and template core removal.
In order to verify the encapsulation of cargo within the liposomal subcompartments, a surfactant, Triton X (TX), was used to lyse the liposomes and release the encapsulated enzymes. Changes in fluorescence intensity with respect to the number of PMA<sub>SH</sub>/PVP bilayers assembled were measured upon lysis of the luciferase<sub>488</sub>-loaded liposomes within the capsosomes in HEPES buffer. The quantity of free enzyme that remained trapped inside the PMA capsules was found to proportionally increase \((R^2 = 0.93)\) with the number of assembled polymer bilayers (Figure 6.3). That is, in the absence of liposomes, PMA capsules exhibit a bilayer number dependent encapsulation efficiency that is different to the retention capabilities of capsosomes. These data indicate that: (i) the enzyme was encapsulated within the liposomal subcompartments; (ii) the liposomes remained intact and retained their enzymatic cargo during the polymer film assembly and after dissolution of the core; and (iii) cargo release could be triggered by lysis of the liposomes.
6.4.3 Long-Term Stability of Capsosomes

To understand the long-term stability of capsosomes and their ability to retain cargo, the fluorescence intensity of enzymes within the liposomes or the liposomes themselves was measured by flow cytometry directly after core dissolution (day 0), and after 7 and 14 days. Figure 6.4 compares the fluorescence intensity of encapsulated L5,2W NBD, luciferase488-loaded or β-lactamase488-loaded liposomes within polymeric crosslinked capsules in HEPES buffer. The fluorescence data for capsosomes loaded with L5,2W NBD (without encapsulated cargo) showed no measurable loss of the liposomes as a function of time, further supporting the successful noncovalent anchoring of liposomes by means of cholesterol-modified polymers. In comparison, the quantity of enzymatic cargo localized in the capsosomes progressively decreased: ~97% and ~88% for luciferase, and ~42% and ~33% for β-lactamase at physiological pH, after 7 and 14 days, respectively. This suggests that with time the enzymatic cargo leaked from the liposomal subcompartments, and then a size dependent diffusion of the enzymes across the polymer membrane was observed.
Figure 6.4. Normalized fluorescence intensity of $L^{s,zw}_{NBD}$, luciferase$_{488}$-loaded, and β-lactamase$_{488}$-loaded PLL$_c$/$L^{s,zw}/PMA_c/PVP/(PMA_{SH}/PVP)_5$ capsosomes measured by flow cytometry directly after core dissolution (day 0), after 7 days, and after 14 days in HEPES buffer.
6.5 Conclusions

This chapter examines the retention of encapsulated cargo within the liposomal subcompartments in capsosomes. It was determined that cargo trapped within L\textsuperscript{136,20w} was predominantly lost during pH cycling, while cargo encapsulated within L\textsuperscript{5,20w} was mainly lost during capping with PMA\textsubscript{c}. In the case when acidic pH conditions are required during the capsosome assembly, the cutoff size of (bio)molecules that can be encapsulated with more than 50% retention efficiency within the liposomal subcompartment has been identified to be \(~500\) Da. Luciferase and β-lactamase, two model enzymes with similar isoelectric points but different sizes, were successfully retained within the liposomes during the LbL process, silica core removal, and change of the assembly conditions (pH 4) to physiological conditions (pH 7.4). The liposomal subcompartments remained intact, their encapsulated enzymatic cargo were retained within the subunits, and their retention was independent of the thickness of the capsule membrane over one to five bilayers. This offers new opportunities to create capsosomes with controlled permeability to biomolecules and therefore enables selective communication with the external environment. Upon long-term storage, the capsosomes exhibited excellent retention of liposomes and a size-dependent retention of the enzymatic cargo. As the liposomal subunits have proven to provide an effective tool to encapsulate a range of biomolecules, engineered capsosomes represent promising systems to perform enzymatic cascade reactions within confined environments, opening new avenues for constructing artificial cells and organelles.
6.6 References

Chapter 7

Degradation of Liposomal Subcompartments in PEGylated Capsosomes

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7.1 Aim

This chapter demonstrates the surface immobilization of poly(ethylene glycol) (PEG) onto the membrane of capsosomes and the effect of PEGylation on the permeability of the polymer carrier capsules. The influence of PEGylation is investigated by assessing the stability of the liposomal subcompartments in the presence of phospholipases in different media. The liposomal degradation behavior of capsosomes with subcompartments in different spatial positions (membrane associated or “free-floating”) is compared.

7.2 Introduction

A number of studies on the fabrication of capsosomes have been described in Chapter 4, 5, and 6, including: (i) a unique approach to noncovalently anchor liposomes to polymer layers using cholesterol-modified polymers; (ii) deposition of multilayers of liposomal subcompartments; (iii) incorporation of liposomes into (bio)degradable disulfide-stabilized poly(methacrylic acid) (PMA) carrier capsules; (iv) control over positioning of the liposomal subunits in the carrier capsules, which yields capsosomes containing membrane-associated or “free-floating” subunits; (v) encapsulation of cargo within the liposomal subunits; and (vi) size-dependent retention of the cargo encapsulated within saturated or unsaturated liposomes.

(Bio)degradable disulfide-stabilized PMA hydrogel capsules,\textsuperscript{1-5} assembled via the layer-by-layer (LbL) of poly(N-vinyl pyrrolidone) (PVP) and thiol-functionalized poly(methacrylic acid) (PMA\textsubscript{SH}) that are used to enclose the liposomal subunits, are attractive microcarriers for biomedical applications. These capsules have been used to encapsulate DNA,\textsuperscript{6,7} oligopeptides,\textsuperscript{3,8} intact proteins,\textsuperscript{1} and lipophilic drugs.\textsuperscript{9,10} However, to date, the surface functionalization of these PMA hydrogel capsules with, for example, poly(ethylene glycol) (PEG), has not yet been demonstrated.

PEG is a biocompatible polymer that is widely used and explored to endow surfaces with protein resistant properties in biomedical applications. In drug delivery, the surface functionalization of drug carriers such as liposomes and polymer capsules
with this low-fouling polymer has been shown to restrict protein adsorption,\textsuperscript{11-14} which is the first step toward enhancing specific interactions with biomolecules or cells of interest for targeting. In addition, extended circulation times of these carriers in the bloodstream were demonstrated\textsuperscript{15} and reduced phagocytic uptake was shown.\textsuperscript{16} PEG can be attached to surfaces via covalent attachment or by the adsorption of PEG-grafted copolymers, which have been reported to PEGylate multilayered polymer capsules composed of poly(L-lysine) (PLL),\textsuperscript{13} poly(allylamine hydrochloride)/poly(styrene sulfonate) (PAH/PSS),\textsuperscript{16} or poly(diallyldimethyl ammonium) (PDADMAC)/PSS.\textsuperscript{17} In this study, the latter approach is singled out because the immobilization of the PEG copolymers can be performed with a similar mechanism as in assembly of the films, and the copolymer can be characterized prior to adsorption, therefore enabling control and reproducibility over the interfacial PEG chain density.

For capsosomes, a crucial feature for the success of continuous enzymatic reactions is governed by the stability and integrity of the liposomal subcompartments within the carrier capsules. This aspect, especially under biomedically relevant conditions, i.e., in the presence of serum and degradative enzymes such as phospholipase A\textsubscript{2} (PLA\textsubscript{2}), is yet to be examined. To promote the stability of the subunits under these conditions and therefore prevent lysis of these structures and premature release of the encapsulated cargo, selective permeability of these biomolecules across the capsosome membrane is required. In general, the permeability of capsules (including disulfide-stabilized PMA hydrogel capsules) can be controlled by the number of deposited layers.\textsuperscript{18} Recent studies showed that the permeability of PMA capsules can also be tuned by varying the thiol content of PMA\textsubscript{SH} chains as well as the type of crosslinkers used to stabilize the multilayered film.\textsuperscript{5} While PEGylation of polymer capsules has been proven to restrict surface adsorption of proteins and block phagocytosis, it has not been considered to tune the permeability of microcarriers.
Scheme 7.1. Schematic illustration of the assembly of PEGylated capsosomes with membrane-associated (a–e) or “free-floating” (a’–e’) subunits. A silica particle is coated with a polymer precursor layer and liposomes (a, a’), followed by the deposition of a polymer capping layer and four bilayers of PVP/PMA₅H (b, b’). PEG surface functionalization (c, c’) is performed via adsorption where the PMA₅H-PEG copolymer interacts with the PVP layer via hydrogen bonding (i), followed by stabilization of the multilayered film and the formation of covalent attachment of the PMA-PEG copolymers to the film by crosslinking of the thiols with 2,2’-dithiodipyridine (DTDP) (ii). Core dissolution (d, d’) results in PEGylated capsosomes with the subunits either in the membrane (e) or “free-floating” (e’) in the assembly when dispersed at physiological conditions.
This chapter therefore: (i) describes conjugation of PEG molecules to a thiol-functionalized PMA chain via thiol-maleimide coupling chemistry to obtain a PMA–PEG copolymer; (ii) evaluates the immobilization efficiency of this copolymer on the outer surface of two different types of capsosomes (capsosomes with membrane-associated or “free-floating” liposomal subunits (Scheme 7.1)); (iii) investigates the adsorption of bovine serum albumin (BSA) onto these capsosomes; (iv) assesses the effect of PEGylation on the permeability of the polymer carrier capsules by performing time-course experiments on the enzymatic degradation of the liposomal subcompartments in capsosomes by PLA$_2$ (Scheme 7.2) in phosphate buffered saline (PBS), in BSA-containing PBS, and in a cell medium; and (v) compares the liposomal degradation behavior of capsosomes with subcompartments in different spatial positions (membrane-associated or “free-floating”).

Scheme 7.2. Schematic illustration of the degradation of liposomal subcompartments in a capsosome by PLA$_2$. The enzyme catalyzes the hydrolysis of lipids from the second carbon group of glycerol and subsequently releases fatty acids.
7.3 Experimental Section

7.3.1 Materials
Maleimide polyethylene glycol succinimidyl ester (MAL-PEG-NHS, $M_w$ 5 kDa) was purchased from Jenkem Technology Co., Ltd (Beijing, China). Alexa Fluor 488 (AF488) cadaverine, Alexa Fluor 633 (AF633) carboxylic acid succinimidyl ester, and RPMI media 1640 with L-glutamine were purchased from Invitrogen (California, USA). Tris(2-carboxyethyl)phosphine (TCEP), DL-dithiothreitol (DTT), poly(N-vinyl pyrrolidone) (PVP, $M_w$ 10 kDa), poly(L-lysine) (PLL, $M_w$ 40–60 kDa), 4-(2-hydroxyethyl)piperazine-1-ethane-sulfonic acid (HEPES), sodium chloride (NaCl), sodium acetate (NaOAc), 3-(N-morpholino)propanesulfonic acid (MOPS), phosphate buffered saline (PBS), calcium chloride ($\text{CaCl}_2$), 2,2’-dithiodipyridine (DTDP), hydrofluoric acid (HF), ammonium fluoride (NH$_4$F), bovine serum albumin (BSA), phospholipase A$_2$ (PLA$_2$) from bovine pancreas, penicillin, and streptomycin were obtained from Sigma-Aldrich (Sydney, Australia). Fetal Bovine Serum (FBS) was obtained from JRH Biosciences (Kansas, USA).

Four types of buffer were used for all of the experiments: (i) HEPES buffer consisting of 10 mM HEPES and 150 mM NaCl (pH 7.4); (ii) NaOAc buffer consisting of 20 mM NaOAc (pH 4.0); (iii) MOPS buffer consisting of 20 mM MOPS (pH 8.0); and (iv) PBS buffer consisting of 10 mM phosphate buffer, 2.7 mM potassium chloride, 137 mM NaCl, and 10 mM CaCl$_2$ (pH 7.4). The buffer solutions were made with ultrapure water (Milli-Q gradient A 10 system, resistivity $\approx$ 18 M$\Omega$ cm, TOC < 4 ppb, Millipore Corporation, Massachusetts, USA).

7.3.2 Polymer Synthesis

7.3.2.1 Thiol-Functionalized Poly(methacrylic acid) (PMA$_{PD(SH)}$, $M_w$ 15 kDa)
Refer to Chapter 5, Section 5.3.2.3.
7.3.2.2 Poly(methacrylic acid)-Poly(ethylene glycol) Copolymer (PMA_{PD(SH)}-PEG_{488}, M_w 48.7 kDa)

A solution of 40 mg mL\(^{-1}\) MAL-PEG-NHS (typically 10 mg) in carbonate buffer (100 mM, pH 8.0) was incubated with 80 μL of AF488 cadaverine (1 mg mL\(^{-1}\) in water). The reaction was allowed to proceed overnight, followed by purification via size exclusion chromatography (SEC) (2x) and freeze-drying to obtain MAL-PEG\(_{488}\). A solution of 5.55 mg mL\(^{-1}\) PMA\(_{PD}\) (typically 1 mg) in HEPES-EDTA buffer (140 mM HEPES, 2 mM EDTA, pH 7.5) was incubated with 0.5 mg of TCEP for 5 min, followed by the addition of 4 mg of MAL-PEG\(_{488}\) (50 mg mL\(^{-1}\) in HEPES-EDTA buffer). The reaction was allowed to proceed overnight, followed by purification via SEC (2x) and freeze-drying to give the PMA\(_{PD}\)-PEG\(_{488}\). The concentration of PEG was derived by correlation with a calibration curve of PEG\(_{488}\) (Figure 7.1) and subsequently the PMA:PEG ratio was calculated. The substitution degree of PEG per PMA chain was evaluated and determined to be 6 mol%. The reduced form, PMA\(_{SH}\)-PEG\(_{488}\), was prepared by incubating PMA\(_{PD}\)-PEG\(_{488}\) in a solution of 0.5 M DTT (100 mg mL\(^{-1}\)) in MOPS buffer for at least 30 min at 37 °C prior to dilution in NaOAc buffer (2 mg mL\(^{-1}\)).

![Figure 7.1. Fluorescence calibration curve for PEG\(_{488}\)](image-url)
7.3.3 Liposome Formation
Saturated zwitterionic liposomes (L\textsuperscript{5,2w}) and the fluorescent counterpart (L\textsuperscript{5,2w}NBD) were prepared as detailed in Chapter 4, Section 4.3.4.

7.3.4 Capsosome Assembly

7.3.4.1 Capsosomes with Membrane-Associated Liposomal Subcompartments
Refer to Chapter 5, Section 5.3.5.1.

7.3.4.2 Capsosomes with “Free-Floating” Liposomal Subcompartments
Refer to Chapter 5, Section 5.3.5.2.

7.3.4.3 PEGylated Capsosomes
A layer of PVP (1 mg mL\textsuperscript{-1}, 10 min) was adsorbed onto the coated particles, followed by: (a) the adsorption/interaction of PMA\textsubscript{SH}-PEG\textsubscript{488} (1 mg mL\textsuperscript{-1}, 15 min) onto the PVP layer via hydrogen bonding or (b) reaction of PMA\textsubscript{PD}-PEG\textsubscript{488} (1 mg mL\textsuperscript{-1}, 15 h) with the thiol groups on the PMA chain via thiol–disulfide exchange. In all experiments, the thiols within the polymer layers were crosslinked with DTDP (0.5 mg mL\textsuperscript{-1}, 15 h) in NaOAc buffer. Capsosomes were obtained by dissolving the silica particles using a 2 M HF/8 M NH\textsubscript{4}F solution for 2 min, followed by multiple centrifugation (4500 g, 3 min)/NaOAc buffer washing cycles. Caution! HF and NH\textsubscript{4}F are highly toxic. Extreme care should be taken when handling HF solution and only small quantities should be prepared.

7.3.5 Quantification of PEG Chain Density per Capsosome
To allow quantification of PEG, the system was assembled with non-fluorescently labeled L\textsuperscript{5,2w} and fluorescently labeled PMA-PEG\textsubscript{488}. The number of PEG moieties immobilized on the membrane of the capsosomes was quantified by fluorescence measurements and derived by correlation with a calibration curve of PEG\textsubscript{488} (Figure 7.1). Subsequently, this value was divided by the number of capsosomes counted via flow cytometry to obtain the amount of PEG immobilized per capsosome. The
interfacial chain density of PEG was evaluated by dividing the amount of PEG per capsosome by the surface area of the particles.

7.3.6 BSA Adsorption
Suspensions of $6 \times 10^8$ capsosomes per mL in PBS, terminated with three different outer polymer layers (PLL, PMA, or PMA-PEG), were incubated with AF633-labeled BSA ($\text{BSA}_{633}$, 1 mg mL$^{-1}$) at room temperature for 2 h. These samples were then washed with three centrifugation/redispersion cycles in PBS (4500 g, 3 min) prior to measuring the fluorescence intensity of the labeled capsosomes via flow cytometry.

7.3.7 Degradation of the Liposomal Subcompartments
Suspensions of $6 \times 10^8$ capsosomes per mL were incubated at 37 °C in: (a) PBS buffer containing 0.05 mg mL$^{-1}$ PLA$_2$, (b) PBS buffer containing 0.05 mg mL$^{-1}$ PLA$_2$ and 0.5 mg mL$^{-1}$ BSA, or (c) in a cell medium (RPMI-1640 with L-glutamine, supplemented with 10% FBS, 500 U mL$^{-1}$ penicillin, and 50 mg mL$^{-1}$ streptomycin) containing 0.05 mg mL$^{-1}$ PLA$_2$. The fluorescence intensity of capsosomes containing the labeled liposomes was monitored over time via flow cytometry.

7.3.8 Characterization Methods
7.3.8.1 Fluorescence Spectroscopy
Quantification of PEG chain density per capsosome was performed with a Fluorolog-3 Model FL3-22 spectrofluorometer (Jobin Yvon Inc., USA) equipped with a HgXe lamp, using an excitation wavelength of 495 nm and an emission wavelength of 519 nm.

7.3.8.2 Flow Cytometry
A Cyflow Space (Partec GmbH, Germany) flow cytometer using excitation wavelengths of 488 and 633 nm was used to measure the intensity of $\text{L}^{5,2w_{\text{NBD}}}$-coated particles and capsosomes, and capsosomes with adsorbed BSA, respectively. At least 20,000 particles or capsosomes were analyzed in each experiment.
7.3.8.3 Confocal Laser Scanning Microscopy (CLSM)

Fluorescently labeled, PEGylated capsosomes were imaged with a Leica TCS SP2 AOBS confocal microscope equipped with an argon laser (λ = 488 nm) using a 63x oil immersion objective (Leica, Germany).

7.3.8.4 Differential Interference Contrast (DIC) Microscopy

DIC images of capsosomes were taken with an Olympus IX71 digital wide-field microscope equipped with a DIC slider (U-DICT, Olympus), the corresponding filter sets, and a 60x oil immersion objective.

7.4 Results and Discussion

7.4.1 Capsosome PEGylation

First, the immobilization of PEG moieties on the membrane of the PMA carrier capsules was explored. Two different types of capsosomes were studied: capsosomes containing either (i) membrane-associated subunits (Scheme 7.1e) or (ii) “free-floating” subunits (Scheme 7.1e’). PEGylation of the PMA carrier using PMA-PEG copolymers was achieved via passive adsorption of the reduced form of the copolymer, PMA\textsubscript{SH}-PEG, which interacts via hydrogen bonding with the PVP layer on the particle surface (Scheme 7.1i). Following PMA-PEG adsorption, the multilayered film was stabilized and the PMA-PEG copolymers were linked to the film through crosslinking of the thiols with DTDP. The particle cores were then removed to yield PEGylated capsosomes (PEG-capsosomes). The use of DTDP converts the PMA\textsubscript{SH} thiol groups into disulfides by thiol–disulfide exchange.\textsuperscript{5} This nonoxidative crosslinking approach exploits a similar principle to the polymeric crosslinker, PMA\textsubscript{PD}, previously used to crosslink the membrane of capsosomes (Chapter 5). However, the lower molecular weight of DTDP enables the facile infiltration into and throughout the preassembled polymer film, providing a higher degree of crosslinking and better encapsulation efficiency of small molecules.

PEG molecules were fluorescently labeled with AF488 prior to conjugation with PMA (PMA-PEG\textsubscript{488}), thereby allowing the fluorescence intensity of the coated particles or
capsosomes to be monitored via fluorescence spectroscopy and enabling the visualization of the presence of the PEG molecules on the surface via confocal laser scanning microscopy. Figure 7.2a summarizes the interfacial PEG chain density of the coated particles upon immobilization of the PMA-PEG copolymers. The stability of the adsorbed copolymers during crosslinking and core dissolution, and subsequent dispersion of the two types of capsosomes with subcompartments in different spatial positions (membrane-associated or “free-floating”) at pH 7.4 (physiological conditions) were assessed.

**Figure 7.2.** a) Interfacial PEG chain density on coated silica particles or capsosomes, demonstrating the stability of the immobilized PMA-PEG copolymers on the membrane of the carrier via the adsorption method. b) CLSM images of PEG₄₀₀-caposomes with membrane-associated Lₛₚzw subunits (i) or “free-floating” Lₛₚzw subunits (ii) in PBS (pH 7.4). The liposomes are not fluorescently labeled. Scale bars are 5 µm.
After binding of PMA$_{SH}$-PEG onto the carrier membrane, the PEG chain density of the coated particles for the assembly of capsosomes with membrane-associated subunits (black bars) was similar to those with the assembly for “free-floating” subunits (red bars). Subsequently, the adsorbed copolymers remained stable during the crosslinking step, core removal, and washing into PBS buffer. This demonstrated that the diffusion of the DTDP crosslinker through the polymer layers and the subsequent formation of disulfides by thiol-disulfide exchange, core dissolution, and the change of environmental pH did not alter the stability of the interaction between the adsorbed PMA-PEG copolymer and the PVP layer on the surface. For both types of capsosomes, the interfacial PEG chain density was determined to be \( \sim 0.15 \) chains per \( \text{nm}^2 \). CLSM images of capsosomes confirmed the presence of a PEG$_{488}$ corona on capsosomes with membrane-associated (Figure 7.2bi) or “free-floating” L$^{5,2L}$ subunits (Figure 7.2bii).

An alternative approach of PEGylation, similar to PMA$_{PD}$ polymeric crosslinking concept described in Chapter 5, was also applied. This involves the use of PMA$_{PD}$-PEG conjugates that can react via thiol-disulfide exchange with the thiol groups on the PMA$_{SH}$ chains constituting the membrane of the carrier capsule. However, this method was found to be less efficient than the adsorption method, i.e., this approach yielded a PEG density of \( \sim 0.08 \) chains per \( \text{nm}^2 \) (Figure 7.3).

### 7.4.2 BSA Adsorption onto PEGylated Capsosomes

To assess the fouling properties of the PEG-functionalized capsosomes, these carriers were incubated with BSA$_{633}$ and the amount of adsorbed protein was determined and compared to non-PEGylated capsosomes (PMA as the outermost layer) and PLL-coated capsosomes. PLL-coated capsosomes are expected to exhibit high protein binding$^{13}$ and this fluorescence reading was set to 100%. Figure 7.4 summarizes the normalized fluorescence intensity of these capsosomes upon incubation with BSA, as monitored by flow cytometry. In contrast, non-PEGylated PMA-terminated capsosomes showed 60% reduced adsorption of BSA$_{633}$, indicating that PMA itself is relatively low-fouling in comparison to PLL.$^{19}$ PEGylated
capsosomes exhibited a ~70% reduction in BSA\textsubscript{633} adsorption, showing that PEGylating the carrier capsules further reduces BSA adsorption.

Figure 7.3. a) PEG surface functionalization via reaction where the PMA\textsubscript{PD-PEG\textsubscript{488}} copolymer reacts with the thiol groups of the PMA\textsubscript{SH} chains via thiol-dilysulfide exchange. b) Interfacial PEG chain density on coated silica particles or capsosomes by the immobilization of PMA-PEG copolymers on the membrane of the carrier via reaction.
Degradation of Liposomal Subcompartments by Phospholipase

PLA$_2$ is an enzyme that catalyzes the hydrolysis of lipids from the second carbon group of glycerol and subsequently releases fatty acids. At physiological conditions, the presence of this enzyme may be detrimental to liposomal subunits in capsosomes since its enzymatic activity will result in destruction of the subunits and subsequent release of the encapsulated cargo.

Herein, a time-course degradation experiment of fluorescently labeled liposomes entrapped in capsosomes was performed by incubation with bovine pancreatic PLA$_2$ ($M_w$ 14 kDa, IEP 6.3). PLA$_2$ activity in serum was reported to be 10 units mL$^{-1}$, therefore this concentration was used to mimic in vivo conditions, which in this case refers to 0.05 mg mL$^{-1}$. A concentration of 1 mg mL$^{-1}$ was also used for comparison. Figure 7.5a shows a comparison between the degradation of the liposomal subunits by 1 mg mL$^{-1}$ and 0.05 mg mL$^{-1}$ PLA$_2$ in PBS. While the higher concentration of PLA$_2$ degraded the liposomes within 1 h, the data suggested that 50% of the subcompartments were degraded within 6 h, with complete degradation after 24 h for the biologically relevant PLA$_2$ concentration. The stability of liposomes in capsosomes is significantly improved when compared to these unilamellar liposomes adsorbed to PLL-coated silica particles, without the subsequent assembly of the PMA.
membrane. In the latter case, 0.05 mg mL\(^{-1}\) PLA\(_2\) completely degraded the liposomes in 5 min.

**Figure 7.5.** a) Degradation of fluorescently labeled membrane-associated liposomal subunits in capsosomes assembled with 5 PVP/PMA\(_{SH}\) bilayers when exposed to 1 mg mL\(^{-1}\) or 0.05 mg mL\(^{-1}\) PLA\(_2\), as monitored by flow cytometry. b) Degradation of fluorescently labeled membrane-associated liposomal subunits in capsosomes by 0.05 mg mL\(^{-1}\) PLA\(_2\), as monitored by flow cytometry. The carrier capsules were assembled with 5 or 6 PVP/PMA\(_{SH}\) bilayers.
Degradation of Liposomal Subcompartments in PEGylated Capsosomes

Due to the compartmentalized assembly, PLA₂ has to diffuse through the membrane of the carrier capsules to hydrolyze the lipids. The diffusion of proteins through polymer capsules has been previously described as a “relay-race” mechanism, which involves protein adsorption onto the hydrogel.²¹ To examine the permeability of PLA₂ through the crosslinked PMA membrane, capsosomes were assembled with a different numbers of bilayers (i.e., 5 or 6 bilayers of PVP/PMA₅₉). The PLA₂ degradation activity was investigated and is shown in Figure 7.5b. An additional polymer bilayer did not serve as a more effective barrier to the diffusion of PLA₂ across the membrane of the carrier capsule. Although liposomes in capsosomes have prolonged stability in comparison to naked liposomes, it would be beneficial for therapeutic cell mimics to have further extended stability of their active subunits.

To this end, PEG-functionalized capsosomes were considered with the aim to understand if the additional PEG layer alters the permeability properties of the crosslinked PMA layers. The diffusion of PLA₂ and subsequent degradation behavior of the liposomes in these capsosomes were investigated in different media; PBS, BSA-containing PBS, and cell media. Figure 7.6a depicts the degradation of the fluorescently labeled liposomal subcompartments in PEGylated and non-PEGylated capsosomes by 0.05 mg mL⁻¹ PLA₂ in PBS (pH 7.4). The subcompartments in PEGylated capsosomes were stable for the first 4 h and began degrading slowly after 5 h, with 60% of the liposomal subunits left after incubation of the capsosomes with the lipases for 48 h. Control samples in PBS showed only a small decrease in fluorescence of 10 - 20% in the same time frame. On the other hand, the liposomal subcompartments in non-PEGylated capsosomes were fully degraded within 48 h.
Figure 7.6. Degradation of fluorescently labeled membrane-associated liposomal subunits in PEGylated capsosomes by 0.05 mg mL$^{-1}$ PLA$_2$: (a) in PBS, (b) in PBS in the presence of 0.5 mg mL$^{-1}$ BSA, and (c) in cell media, as monitored by flow cytometry. d) DIC images of PEGylated capsosomes in PBS (i and ii) or in cell media (iii and iv), before and after exposure to PLA$_2$ for 48 h at 37 °C, respectively. Scale bars are 5 µm.

Figure 7.6b summarizes a similar degradation study in the presence of BSA. BSA, apart from being the most abundant protein in human blood, has been shown to exert positive modulation and enhance the maximum activity of phospholipases, which in this case refers to the performance of PLA$_2$ in hydrolyzing the lipids.$^{22,23}$ In the presence of BSA, the time to reach 50% degradation of the liposomes shifted from 6 h (Figure 7.6a) to 4 h (Figure 7.6b) in non-PEGylated capsosomes. On the other hand, the degradation trend using PEGylated capsosomes was observed to be similar to that in the absence of BSA, with a slight variation in the degree of degradation during the first hour, i.e., ~20% of the subunits were already degraded.
followed by a slow, gradual decrease, with the same amount of subunits (60%) remaining after 48 h.

In cell media, the presence of PLA$_2$ did not affect the degradation of liposomes (Figure 7.6c); liposomes were degraded at a similar rate irrespective of the presence of the lipases. This is likely due to the presence of abundant proteins in serum, which interact or insert into the lipids and destabilize the liposomes,$^{24}$ hence concealing the effect of the PLA$_2$. As observed, the degradation rate of liposomes in non-PEGylated capsosomes in cell media was faster in comparison to those incubated with PBS in the presence of BSA; that is, degradation of 50% of the subunits occurred within 2 h (compared with 4 h). When employing PEGylated capsosomes, on the other hand, the degradation behavior is similar in all three media and considerably slowed down. DIC images of the PEGylated capsosomes (Figure 7.6d) in PBS (top) and in cell media (bottom), before (left) and after (right) degradation of the liposomes by PLA$_2$ for 48 h at 37 °C, show that the structural integrity is preserved and not affected by the degradation. Also, no capsosome aggregation was observed in the presence of serum (Figure 7.7).

Figure 7.7. Light side scattering histogram of PEGylated capsosomes incubated in cell medium for 48 h, showing that the analyzed capsosomes are singlets, hence no aggregation is observed in the presence of serum.
The liposomal degradation kinetics in PEGylated capsosomes with membrane-associated subunits was also compared to capsosomes with “free-floating” subunits (Figure 7.8). During the first hour, 60% degradation of the “free-floating” subcompartments was observed, followed by a plateau through to 8 h. While the leveling off of the degradation kinetics was observed in both cases, the initial degradation rate was three times higher for “free-floating” subunits in comparison to the membrane associated liposomes. Since the interfacial PEG chain density for both types of capsosomes is similar (Figure 7.2a), this observation suggests that the permeability of the polymer membrane of the capsosomes (e.g., crosslinking density and degree of swelling) or the stability of the subunits themselves, or a combination of these effects, is different depending on the location of the liposomes in the assembly. In the former case, the presence of the liposomes and cholesterol embedded in the polymer shell might have altered the permeability, i.e., the higher density of the surface decreases the permeability of the membrane to biomolecules. In the latter case, the close proximity to the polymer layers might contribute to the stability of the liposomes, hence more efficient hydrolysis of the lipids is required. These results confirm the effect of PEG moieties in enhancing the effectiveness of the polymer membrane to act as a diffusion barrier with the aim to prevent the uncontrolled access of proteins to the capsosome interior.

Figure 7.8. Degradation of fluorescently labeled membrane-associated subunits (black) or “free-floating” subunits (red) in PEGylated capsosomes by 0.05 mg mL\(^{-1}\) PLA\(_2\) in PBS, as monitored by flow cytometry.
7.5 Conclusions

Surface functionalization of capsosomes with PMA-PEG copolymers can be achieved via hydrogen bonding interaction of PMA$_{SH}$-PEG with the PVP layer followed by subsequent crosslinking with DTDP. This approach afforded an interfacial PEG chain density of $\sim$0.15 chains per nm$^2$. Slightly lower amounts of BSA were adsorbed to PEGylated capsosomes than non-PEGylated capsosomes. The effect of PEG surface functionalization on the permeability of the carrier capsules was demonstrated. The degradation of the fluorescently labeled liposomal subcompartments due to diffusion of the degradative enzyme, PLA$_2$, across the polymer layers was examined. Degradation of 50% of the subunits in the non-PEGylated capsosomes occurred within 6, 4, and 2 h for capsosomes incubated with PLA$_2$ in PBS, in PBS with the presence of BSA, and in cell media, respectively, and full degradation was observed within 48 h. In contrast, PEGylated capsosomes exhibited a similar trend of liposomal stability in all of the three tested media, with 60% of the subunits remaining after 48 h. Furthermore, PEGylated capsosomes with “free-floating” subunits demonstrated a higher degree of permeability to PLA$_2$ compared to those with membrane-associated subunits. Given the facile and rapid surface functionalization method, and the subsequent higher liposomal stability in the compartmentalized assembly, these results further advance the development of capsosomes as a promising platform toward therapeutic cell mimicry.
7.6 References


Chapter 8

Encapsulated Enzymatic Catalysis in Capsosomes

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8.1 Aim

This chapter aims to demonstrate encapsulated enzymatic catalysis in capsosomes, which substantiate the functionality of enzymatic cargo within the liposomal subcompartments. Chemical and physical stimuli are explored as triggers to initiate (coupled) enzymatic activities in capsosomes. Furthermore, triggered controlled release of therapeutic molecules by enzymatic catalysis in capsosomes is demonstrated.

8.2 Introduction

Capsosomes, polymer capsules containing liposomal subcompartments, present new opportunities en route to the development of functional therapeutic artificial cells, with a key function predominantly focusing on enzymatic activities to degrade waste products or to support the synthesis of medically relevant biomolecules. With the polymer capsules providing structural scaffold with controlled permeability and liposomes encapsulating fragile hydrophilic and hydrophobic cargo, capsosomes can be utilized to allow successive encapsulated enzymatic catalysis within the carriers and simultaneous release of the products to the surrounding environment.

In Chapter 5, the encapsulation of enzymatic cargo into the liposomal subcompartments was verified and the long-term stability study of capsosomes exhibited excellent retention of the encapsulated cargo. In this chapter, the functional activity of the encapsulated enzymes is investigated by performing enzymatic catalysis in capsosomes. External chemical stimulus is used as a trigger to induce the enzymatic activities. In a different approach, temperature, a physical stimulus, can be used to initiate the enzymatic reactions without lysing the liposomal subunits and the possibility to repetitively use these capsosomes for multiple subsequent catalysis is investigated. The use of capsosomes for performing multiple coupled reactions, a key feature of biological cell mimics, is explored.
8.3 Experimental Section

8.3.1 Materials

Zwitterionic lipids, 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC, phase transition temperature 23 °C), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC, phase transition temperature 41 °C), were purchased from Avanti Polar Lipids (Alabama, USA). β-lactamase, glutathione reductase from baker’s yeast (Saccharomyces cerevisiae), cholesterol, poly(N-vinyl pyrrolidone) (PVP, $M_w$ 10 kDa), nitrocefin, Triton X (TX), oxidized L-glutathione (GSSG), reduced L-glutathione (GSH), 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman’s reagent), β-nicotinamide adenine dinucleotide 2′-phosphate (NADPH), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), sodium chloride (NaCl), sodium acetate (NaOAc), 2-(N-morpholino)ethanesulfonic acid (MES), phosphate buffered saline (PBS), hydrofluoric acid (HF), and ammonium fluoride (NH$_4$F) were purchased from Sigma-Aldrich (Sydney, Australia). Silica particles (3.25 μm-diameter) were obtained from Microparticles GmbH (Berlin, Germany). Poly(methacrylic acid) (PMA, $M_w$ 15 kDa) was purchased from Polysciences (Pennsylvania, USA). 1,8-bis(maleimido)diethylene glycol (BM(PEG)$_2$) was purchased from Thermo Fisher (Massachusetts, USA). Poly(N-vinyl pyrrolidone)-block-(cholesteryl acrylate) (PVP$_c$, $M_w$ 11 kDa)\(^1\) and Alexa Fluor 488-labeled PMA-KP9 (PMA-KP9$_{488}$) conjugates\(^2\) were prepared as described in previously published protocol.

Four types of buffer were used for all of the experiments: (i) HEPES buffer consisting of 10 mM HEPES and 150 mM NaCl (pH 7.4); (ii) NaOAc buffer consisting of 20 mM NaOAc (pH 4.0); (iii) MES buffer consisting of 50 mM MES (pH 6.0); and (iv) PBS buffer consisting of 10 mM phosphate buffer, 2.7 mM potassium chloride, and 137 mM NaCl (pH 7.4). The buffer solutions were made with ultrapure water (Milli-Q gradient A 10 system, resistivity $\approx$ 18 MΩ cm, TOC < 4 ppb, Millipore Corporation, Massachusetts, USA).
8.3.2 Polymer Synthesis

8.3.2.1 Cholesterol-Modified Poly(L-lysine) (PLLc, \(M_w 40 - 60 \text{ kDa}\))
Refer to Chapter 4, Section 4.3.3.1.

8.3.2.2 Poly(methacrylic acid)-co-(cholesteryl methacrylate) (PMAc, \(M_w 11.5 \text{ kDa}\))
Refer to Chapter 4, Section 4.3.3.2.

8.3.2.3 Thiol-Functionalized Poly(methacrylic acid) (PMA(SH), \(M_w 15 \text{ kDa}\))
Refer to Chapter 5, Section 5.3.2.3.

8.3.3 Liposome Formation
β-lactamase-loaded saturated zwitterionic liposomes were prepared as follows: chloroform of the lipid solution (2.5 mg of DMPC/DPPC = 4:1 wt % or DPPC) was evaporated under nitrogen for 1 h, followed by hydration with 200 µL of 3 mg mL\(^{-1}\) β-lactamase in HEPES buffer, dilution into 1 mL of HEPES buffer, and extrusion through 50 nm filters (31 times) to form \(L_{\text{DMPC/DPPC-β}}\) and \(L_{\text{DPPC-β}}\), respectively.

Glutathione reductase-loaded saturated zwitterionic liposomes were prepared as follows: DPPC (3.6 mg) and cholesterol (0.5 mg) were dissolved in chloroform and the mixture was dried by evaporation of the chloroform under nitrogen for 1 h. The dry lipid film was hydrated with 200 µL of 1.2 mg mL\(^{-1}\) glutathione reductase in HEPES buffer, followed by dilution into 1 mL of HEPES buffer, and extrusion through 200 nm filters (31 times) to obtain \(L_{\text{DPPC-GR}}\).

Fluorescently labeled saturated zwitterionic liposomes (L\(^{5,2W\text{NBD}}\)) were prepared as detailed in Chapter 4, Section 4.3.4.

8.3.4 Capsosome Assembly
Capsosomes containing β-lactamase-loaded liposomes were prepared as follows: a suspension of 3.25 µm-diameter SiO\(_2\) particles (5 wt %) in HEPES buffer was incubated with the polymer precursor layer, PLLc (1 mg mL\(^{-1}\), 15 min), and washed
three centrifugation/redispersion cycles (1060 g, 30 s). L_{DMPC/DPPC-β} or L_{DPPC-β}
(2.5 mg mL^{-1}, 40 min) were allowed to interact with the polymer-coated particles,
washed three times, and PMA_{c} separation layer was subsequently adsorbed
(1 mg mL^{-1}, 15 min). The adsorption of liposomes and polymer separation layer was
repeated 7 times, followed by the adsorption of a PMA_{c} capping layer (1 mg mL^{-1}, 15
min). The buffer was changed to NaOAc and five bilayers of alternating PVP
(1 mg mL^{-1}, 10 min) and PMA_{SH} (1 mg mL^{-1}, 10 min) were sequentially deposited. The
thiols within the polymer layers were crosslinked with either PMA_{PD} (1 mg mL^{-1}, 15
h) in NaOAc buffer or BM(PEG)_{2} (0.075 mg mL^{-1}, 15 h) in MES buffer. BM(PEG)_{2}
crosslinked capsosomes were only used for the temperature-triggered enzymatic
reaction. Capsosomes were formed by dissolving the silica core particles using a 2 M
HF/8 M NH_{4}F solution for 2 min, followed by multiple centrifugation (4500 g, 3
min)/NaOAc buffer washing cycles. Caution! HF and NH_{4}F are highly toxic. Extreme
care should be taken when handling HF solution and only small quantities should be
prepared.

Capsosomes coencapsulating glutathione reductase-loaded liposomes and PMA-KP9
conjugates were prepared as follows: a suspension of 3.25 μm-diameter SiO_{2}
particles (5 wt %) in HEPES buffer was incubated with PVP_{c} (1 mg mL^{-1}, 15 min),
washed with three centrifugation/redispersion cycles (1060 g, 30 s), and followed by
the adsorption of L_{DPPC-GR} (4 mg mL^{-1}, 40 min). PVP_{c} (1 mg mL^{-1}, 15 min) was allowed
to interact with the coated particles and the buffer was exchanged to NaOAc buffer.
Sacrificial layers PMA (1 mg mL^{-1}, 10 min) and PVP (1 mg mL^{-1}, 10 min) were
subsequently adsorbed. To this suspension, PMA-KP9_{488} was added (0.25 mg mL^{-1},
15 min), followed by the sequential deposition of three bilayers of PVP (1 mg mL^{-1},
10 min) and PMA_{SH} (1 mg mL^{-1}, 10 min). The thiols within the polymer layers were
crosslinked with BM(PEG)_{2} (0.075 mg mL^{-1}, 15 h) in MES buffer. Capsosomes were
obtained by dissolving the silica core using a 2 M HF/8 M NH_{4}F solution for 2 min,
followed by multiple centrifugation (4500 g, 3 min)/PBS buffer washing cycles.
Caution! HF and NH_{4}F are highly toxic. Extreme care should be taken when handling
HF solution and only small quantities should be prepared.
8.3.5 Hydrolysis of Nitrocefin by β-lactamase in Capsosomes
A suspension of $3 \times 10^7$ particles or capsosomes per mL in HEPES buffer, containing \( \text{L}_{\beta} \), was allowed to interact with 50 \( \mu \text{g mL}^{-1} \) nitrocefin. TX was subsequently added at a final concentration of 0.5% (v/v). An increase in the hydrolyzed product of nitrocefin was followed over time through monitoring absorbance readings at 492 nm using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific).

8.3.6 Temperature-Triggered β-lactamase Activity in Capsosomes
A suspension of $3 \times 10^7$ particles or capsosomes per mL in HEPES buffer, containing \( \text{L}_{\text{DPPC-β}} \), was allowed to interact with 50 \( \mu \text{g mL}^{-1} \) nitrocefin and this suspension was incubated at room temperature (24 °C) or 28 °C or 41 °C. Absorbance readings were monitored over time at 492 nm using a NanoDrop1000 spectrophotometer (Thermo Fisher Scientific) to follow the progression of nitrocefin hydrolysis. The capsosomes were centrifuged (4500 \( g \), 3 min), and the supernatant (the hydrolyzed product) was removed and replaced with fresh nitrocefin, followed by incubation at a given temperature and absorbance readings.

8.3.7 Temperature-Triggered Glutathione Reductase Activity in Capsosomes
A suspension of $1 \times 10^7$ capsosomes per mL in PBS, containing \( \text{L}_{\text{DPPC-GR}} \), was incubated with 2.5 mM GSSG, 2.5 mM NADPH, and 3 mM Ellman’s reagent at 24 °C or 37 °C. An increase of GSH production was followed over time by monitoring absorbance readings at 412 nm using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). Control samples were incubated without GSSG at 24 °C or 37 °C.

8.3.8 Quantification of KP9 Oligopeptides per Capsosome
The number of KP9 oligopeptides encapsulated in the capsosomes was quantified by fluorescence measurements and derived by correlation with a calibration curve of KP9 \(_{488} \) (Figure 8.1). Subsequently, this value was divided by the number of capsosomes counted via flow cytometry to obtain the amount of KP9 loaded per capsosome.
8.3.9 Release of Encapsulated KP9 Oligopeptide

A suspension of $1 \times 10^7$ capsosomes per mL in PBS, containing $\text{L}_{\text{DPPC-GR}}$ and KP9$_{488}$, was incubated with 2.5 mM GSSG and 2.5 mM NADPH at 24 °C or 37 °C. Negative control samples were incubated without GSSG at 24 °C or 37 °C and positive control samples were incubated with 5 mM GSH at 37 °C. The fluorescence intensity of the capsosomes was monitored over time via flow cytometry.

8.3.10 Characterization Methods

8.3.10.1 Fluorescence Spectroscopy

Quantification of KP9 oligopeptide in the capsosomes was performed with a Fluorolog-3 Model FL3-22 spectrofluorometer (Jobin Yvon Inc., USA) equipped with a HgXe lamp, using an excitation wavelength of 495 nm and an emission wavelength of 519 nm.

8.3.10.2 Flow Cytometry

A Cyflow Space (Partec GmbH, Germany) flow cytometer using an excitation wavelength of 488 nm was used to measure the fluorescence intensity of capsosomes encapsulating the KP9$_{488}$ oligopeptide. At least 20,000 capsules were analyzed in each experiment.
8.3.10.3 Confocal Laser Scanning Microscopy (CLSM)
KP9488-loaded capsosomes were imaged with a Leica TCS SP2 AOB5 confocal microscope equipped with an argon laser (λ = 488 nm) using a 63× oil immersion objective (Leica, Germany).

8.3.10.4 Differential Interference Contrast (DIC) and Fluorescence Microscopy
DIC and fluorescence microscopy images of capsosomes were taken with an Olympus IX71 digital wide-field microscope equipped with a DIC slider (U-DICT, Olympus), the corresponding filter sets, and a 60× oil immersion objective.

8.4 Results and Discussion

8.4.1 Triggered Enzymatic Catalysis in Capsosomes by Chemical Stimulus
To substantiate the encapsulation and functionality of the enzymes within the liposomal subcompartments, enzymatic assay in capsosomes was performed. β-lactamase was chosen as the model enzyme due to the possibility to visualize and quantify its activity by monitoring the conversion of the enzymatic substrate (nitrocefin, yellow) into its hydrolyzed product (red) in a colorimetric assay. To confirm the presence and activity of the β-lactamase confined within the liposomal compartments of the capsosomes, a surfactant, Triton X (TX) was used to lyse the liposomes and release the enzyme molecules, which made them accessible to their nitrocefin substrate (Scheme 8.1).
Scheme 8.1. Triggered β-lactamase catalysis in capsosomes by chemical stimulus. a) The enzyme β-lactamase is preloaded into liposomes, which are sandwiched between two cholesterol-modified polymers and then embedded inside a polymer carrier capsule to form capsosome. b) Upon addition of Triton X, the liposomes are lysed and the enzymes are released, thus causing the hydrolysis of nitrocefin.

Equal numbers of core-shell particles and capsosomes, containing β-lactamase-loaded DMPC/DPPC liposomes, were exposed to TX and they exhibited similar enzymatic activities (Figure 8.2, ● and ■). This result indicated that neither the amount of the β-lactamase within the capsosomes nor its activity was affected by removal of the silica core. In the absence of TX, the enzymatic activity was measured at a low level, indicating that intact liposomes provide an effective barrier for the diffusion of nitrocefin. This quantitative assay allows the estimation of the number of liposomal subcompartments within the capsosomes. From the experimental value of the enzymatic activity and taking into account the content of enzyme per liposome defined in the synthesis, it was calculated that each of these capsosomes, which are assembled with a single liposome layer, contains approximately 8,000 subcompartments. This value comprises approximately 70% of the theoretical maximum (i.e., the number of 50 nm spheres packed in a square lattice on the surface of a 3 µm-diameter colloidal particle), which confirms the success of the
developed noncovalent cholesterol linkage strategy to incorporate the liposomes into polymer capsules.

**Figure 8.2.** Absorbance measurements of the quantitative β-lactamase assay using core-shell particles (●) and capsosomes (■ in the presence of TX or □ in the absence of TX). A negative control (substrate only) is also shown (★).

Based on the estimation of the number of subcompartments deposited within a capsosome assembled with a single liposome layer, a 3 µm-diameter capsosome with 20 single liposome layers (Chapter 5, Section 5.4.2.2) therefore leads to an assembly of 160,000 liposomal subunits per carrier capsule. This amount exceeds what others have reported and is by far the highest level of subcompartmentalization system described for synthetic cell mimics.

To confirm the presence of multiple intact enzyme-loaded liposomal subcompartments within the PMA carrier capsules and that the amount of loaded cargo can be simply controlled by the number of deposited liposome layers, a similar enzymatic assay using β-lactamase was conducted for capsosomes with multiple layers of liposomal subunits. The enzymatic conversion rates of capsosomes
assembled with β-lactamase-loaded liposomes in one or eight deposition steps, \( C_{L1\beta} \) or \( C_{L8\beta} \), were compared. The enzymatic reaction only occurred when the β-lactamase-loaded liposomes were lysed using the surfactant TX (Figure 8.3a (i) after 2 h and (ii) after 9 h, left (\( C_{L1\beta} \)) and right (\( C_{L8\beta} \) tubes), while in the absence of TX, no reaction occurred (Figure 8.3a, middle tubes (\( C_{L8\beta} \))), verifying the presence of intact multiple layers of liposomes loaded with active enzymatic cargo within the capsosomes. While there was a clear difference in enzymatic conversion rate measured by UV-Vis spectroscopy over time, which confirmed the different amount of loaded active enzymatic cargo within \( C_{L1\beta} \) and \( C_{L8\beta} \) and in the corresponding core-shell particles (Figure 8.3b), the difference was lower than expected; after 9 h, \( C_{L8\beta} \) was 2.5x faster compared to \( C_{L1\beta} \). This discrepancy could be explained by a low encapsulation efficiency of the enzymes within the liposomes and/or the presence of empty liposomes, an observation which has recently been reported by Lohse et al.\(^3\)

![Figure 8.3. Enzymatic conversion rates of capsosomes with multiple layers of β-lactamase-loaded DMPC/DPPC liposomes. (a) Photographs taken after 2 h (i) and 9 h (ii) of capsosomes assembled with β-lactamase-loaded liposomes in one or eight deposition steps, \( C_{L1\beta} \) or \( C_{L8\beta} \), respectively, exposed to TX (left and right tubes) and \( C_{L8\beta} \) in the absence of TX (middle tube). (b) Absorbance measurements of the quantitative β-lactamase assay with TX using core-shell particles (□ and ■) and capsosomes (○ and ●) assembled with one or eight liposome deposition steps (open or closed symbols, respectively). Controls without the addition of TX are also shown (▷ and ◀).](image-url)
8.4.2 Triggered Enzymatic Catalysis in Capsosomes by Physical Stimulus

A main challenge in designing artificial cells that can provide long-term therapeutic solutions for chronic diseases lies in the assembly of a system that contains functional and reusable enzymatic cargo trapped within the compartments, which upon stimulating can be activated to convert molecules and simultaneously release the (therapeutic) products, provided there is a continuous supply of reactants. Previously, the surfactant Triton X was used as an external trigger to initiate enzymatic reactions in capsosomes (Section 8.4.1), a trigger which causes lysis of the liposomes and excludes the possibility to reuse the subunits. With the aim to repetitively use the enzymes trapped within the capsosomes, the enhanced permeability of the lipid membrane at the liquid-gel phase transition temperature ($T_m$) of the liposomes was exploited as a trigger to activate the enzymatic reaction by allowing the substrate to contact the enzymes (Scheme 8.2).

Scheme 8.2. Temperature-triggered β-lactamase catalysis in capsosomes. An increase in temperature to the phase transition temperature ($T_m$) of the liposomes results in a disordered liquid phase of the lipid membrane, allowing nitrocefin to cross the membrane to be hydrolyzed while retaining the β-lactamase inside the compartments.
Temperature-controlled permeability of lipid membranes can be tuned by employing liposomes with different lipid compositions and consequently different phase transition temperatures. The temperature trigger does not require the introduction of harmful solutes/factors that might be harmful to the enzymatic cargo, and while it is less specific than ion channel (e.g., gramicidin)-containing liposomes, it is a far simpler and robust approach. Although temperature-induced activation of liposomes has been previously used to initiate enzymatic reactions within liposome assemblies, it has not been considered in hierarchical polymer/liposome coassemblies. The close proximity to the polymer membrane, the incorporation of the cholesterol anchors into the lipid membrane, or the assembly process might potentially affect the properties of the lipid membrane. Herein, the phase transition temperature as a trigger to activate a colorimetric reaction was examined. Further, the retention and functionality of the enzymes within the liposomes over several reaction cycles were investigated.

To demonstrate that the enhanced permeability of the lipid membrane at \( T_m \) can be used as a trigger to initiate an enzymatic reaction without destruction of the liposomal subcompartments, capsosomes containing \( \beta \)-lactamase-loaded DPPC liposomes (\( T_m = 41 \, ^\circ\text{C} \)) were assembled. This lipid composition was chosen to trigger the enzymatic reaction solely at elevated temperature. Upon an increase of the temperature to the \( T_m \) of the liposomes, the substrate nitrocefin is able to cross the lipid membrane, and be hydrolyzed into its red product and released from the compartment again (Scheme 8.2). The capsosomes were incubated at room temperature (RT, 24 °C), at 28 °C and 41 °C, and the enzymatic conversion rate was followed over time (Figure 8.4a). The enzymatic conversion was only observed for capsosomes incubated at 41 °C, but not at RT or at 28 °C. Hence, although anchored to the polymer film, the saturated liposomes provide an effective diffusion barrier for nitrocefin below their phase transition temperature.
Encapsulated Enzymatic Catalysis in Capsosomes

Figure 8.4. Temperature-triggered enzymatic reaction in reusable capsosomes. (a) Absorbance readings of an enzymatic assay using capsosomes with β-lactamase-loaded DPPC liposomes incubated at room temperature (24 °C), 28 °C, or 41 °C. The enzymatic conversion was only observed when the capsosomes were incubated at the phase transition temperature ($T_m$) of the liposomal subunits. The retention of the functional enzymes inside the liposomal subcompartments was confirmed by repetitively performing the temperature-induced assay. (b) DIC image of capsosomes after being reused four times.

Further, to confirm that the temperature-triggered approach neither destructs the liposomal subcompartments nor causes the release of the enzymes, the enzymatic conversion was repeated by replacing the hydrolyzed nitrocefin with fresh substrate over several cycles. The enzymatic conversion occurred at a similar rate to that observed for three cycles (Figure 8.4a), indicating that: (i) β-lactamase was retained inside the liposomes; (ii) the reinitiation of the reaction is possible; and (iii) there is no loss of functional activity of the enzymes. The capsosomes, after repetitive exposure to elevated temperatures, preserved their structural integrity (Figure 8.4b).

In summary, the repetitive temperature-triggered activation of the enzymatic conversion provides unprecedented success in microencapsulated biocatalysis using a subcompartmentalized system and has the potential to control an enzymatic reaction, where the initiation/termination of several reaction steps is required.
8.4.3 Triggered Cargo Release by Encapsulated Enzymatic Catalysis in Capsosomes

Using membrane-associated capsosomes containing β-lactamase, a (one-step) successive temperature-triggered enzymatic catalytic reaction was demonstrated and the data confirmed that the temperature-triggered approach neither destroys the liposomal subcompartments nor causes release of the enzymes (Section 8.4.2). Many biological systems utilize multiple, coupled reactions, and mimicking cells in this regard still represents a significant challenge. Herein, to further the aim in addressing complexity in therapeutic cell mimicry, the ability of capsosomes to perform an encapsulated two-step enzymatic catalytic reaction is demonstrated, whereby reduction of a substrate is exploited to subsequently trigger cargo release. To achieve this, two different biomolecules, glutathione reductase (enzyme) and oligopeptides, are coencapsulated into capsosomes (Scheme 8.3) and the action of the enzyme on a substrate allows the subsequent release of the oligopeptides at a designated temperature.

This study: (i) describes the assembly of capsosomes containing glutathione reductase-loaded liposomes; (ii) confirms the functionality of the encapsulated enzyme to reduce a substrate (glutathione disulfide); (iii) describes the coencapsulation of disulfide-linked polymer-peptide conjugates and glutathione reductase-loaded liposomes within the capsosomes; and (iv) demonstrates the controlled release of the oligopeptides from the capsosomes by action of the enzyme.
**Scheme 8.3.** Schematic illustration of the assembly of capsosomes coencapsulating enzyme-loaded liposomes and polymer-peptide conjugates into the cavity of the polymer carrier capsules. A silica particle is coated with a PVP$_c$ precursor layer (a), followed by the deposition of glutathione reductase-loaded DPPC liposomes (L$_{DPPC-GR}$) (b) and a PVP$_c$ capping layer (c). Sacrificial layers, PMA and PVP, are then adsorbed onto the coated particle (d), followed by the adsorption of PMA-KP9 polymer-peptide conjugates (e). The assembly is continued with the LbL deposition of three bilayers of PVP$_c$ and PMA$_{SH}$ to form the shell of the carrier (f). The multilayer film is stabilized by crosslinking of the thiols within the polymer layers and core dissolution (g) results in a capsosome with “free-floating” glutathione-reductase liposomal subunits and PMA-KP9 conjugates at physiological pH.
Glutathione (GSH) is an important cellular antioxidant that prevents damage to cell components caused by free radicals. This predominant sulfhydryl reducing agent exists at a concentration of approximately 5 mM in cells due to the activity of glutathione reductase (GR), an enzyme that actively reduces glutathione disulfide (GSSG) to its sulfhydryl form (GSH).\textsuperscript{7} The presence of this enzyme is essential for maintaining intracellular levels of GSH and to mitigate oxidation to avoid dysfunction of biologically active molecules. The ratio of GSH to GSSG within cells is often used as a measure of cellular cytotoxicity. Several studies have shown that a decrease in glutathione reductase activity is age-related and a low GSH/GSSG ratio results in an increased level of oxidative stress and suboptimal immune responses.\textsuperscript{7,8} Supplementation of glutathione reductase to replenish the depleted enzymatic activity would allow for the continual reduction of glutathione disulfide.

In this study, glutathione reductase was encapsulated into DPPC liposomes, which were embedded into polymer carrier capsules to form capsosomes. Capsosomes with “free-floating” liposomal subunits (Chapter 5, Section 5.4.3) were used to perform the temperature-triggered enzymatic conversion of GSSG to GSH. The activity of glutathione reductase encapsulated in L\textsubscript{DPPC} was examined by the reduction of GSSG to GSH, as measured by Ellman’s assay (Scheme 8.4a). Equal numbers of capsosomes containing L\textsubscript{DPPC-GR} were incubated with GSSG, NADPH, and Ellman’s reagent at 24 °C or 37 °C in PBS (one mole of NADPH is required to reduce one mole of GSSG to produce two moles of GSH\textsuperscript{7}). In the presence of GSH, the disulfide bond of Ellman’s reagent is cleaved, releasing the 2-nitro-5-mercaptobenzoic acid (TNB) chromophore with an absorbance maximum at 412 nm.
Scheme 8.4. a) Temperature-triggered catalysis of encapsulated glutathione reductase in the “free-floating” liposomal subcompartments of capsosomes, which reduces glutathione disulfide (GSSG) to its sulfhydryl form (GSH). The production of GSH is measured using Ellman’s reagent as an indicator. b) Release of encapsulated oligopeptides triggered by enzymatic catalysis in capsosomes. Reduction of GSSG to GSH by the activity of encapsulated glutathione reductase in the liposomal subcompartments (1) facilitates release of the encapsulated peptide due to the cleavage of disulfide bonds linking the polymer carrier (PMA) and the peptide (KP9) (2).

Figure 8.5a shows the GSH production over time via the temperature-triggered enzymatic assay in capsosomes using glutathione reductase encapsulated within the “free-floating” liposomal subcompartments. The enzymatic conversion of GSSG to GSH was only observed for capsosomes incubated at 37 °C (▲) but not at 24 °C (△), confirming the temperature dependency of the capsosome-confined reaction. Below the phase transition temperature ($T_m$) of the liposomes, the reagents freely permeate the polymer component (PMA$_{SH}$ layers) but not the liposomes. Near the
phase transition temperature, substrate molecules pass through transient defects produced by disturbances to the packing order of the lipids. The production of GSH for capsosomes incubated at 37 °C was observed at 5 h and the absorbance readings continued to increase until ~30 h. Negative control samples (i.e., without the addition of GSSG) did not show any measurable increase in absorbance. A similar increase in absorbance readings observed for the core-shell particles (Figure 8.5a, ▲) substantiates that core removal does not cause a loss of glutathione reductase activity in capsosomes.

Figure 8.5. a) Temperature-triggered reduction of GSSG to GSH, catalyzed by glutathione reductase encapsulated within the “free-floating” liposomal subcompartments of capsosomes at $T = 24 \, ^\circ C$ or $37 \, ^\circ C$. An increase in absorbance at 412 nm is due to the reduction of Ellman’s reagent by GSH, which is only observed at $T = 37 \, ^\circ C$ (▲). Negative control samples are run in parallel without the addition of GSSG (○ and ●). Ellman’s assay using core-shell particles (▲) is also shown to confirm that core removal does not cause the loss of the glutathione reductase activity in the capsosomes. b) Ellman’s assay using glutathione reductase-loaded PMA hydrogel capsules, demonstrating the absence of enzymatic activity when glutathione reductase is not encapsulated within the liposomes.
In addition, there was no production of GSH observed when glutathione reductase was encapsulated into the cavity of the polymer carrier capsules without the use of liposomal subcompartments (Figure 8.5b). This demonstrates the benefit and necessity of liposomal encapsulation in protecting fragile biomolecules and maintaining their functional activity. A further advantage of this approach is the ability to trigger the enzymatic assay.

Controlled release of the peptides from capsosomes was subsequently demonstrated by the action of glutathione reductase by coencapsulating both polymer-peptide conjugates (PMA-KP9) and glutathione reductase-loaded liposomes (L\textsubscript{DPPC-GR}) in the cavity of the polymer carrier capsules. KP9 oligopeptide is a wild-type antigen used in vaccination\textsuperscript{10} and has been successfully encapsulated into disulfide-stabilized PMA capsules via their conjugation to a carrier polymer.\textsuperscript{2} Previous studies demonstrated that release of these therapeutic agents from the carrier capsules was triggered by the intracellular reductive environment and their immunostimulatory activity was confirmed by stimulation of specific T cells.\textsuperscript{2,11} The encapsulation of PMA-KP9\textsubscript{488} conjugates into capsosomes was achieved by their adsorption onto polymer/liposome-coated particles prior to the assembly of the membrane of the carrier capsules (Scheme 8.3). Figure 8.6a compares the encapsulation efficiency of PMA-KP9\textsubscript{488} conjugates in a 3 µm-diameter PMA capsule (i) to a capsosome (ii). A similar increase in fluorescence intensity of PMA-KP9\textsubscript{488} was measured using flow cytometry for both assemblies, indicating a negligible effect of the presence of liposomes on the adsorption of the conjugates. CLSM images confirmed the encapsulation of PMA-KP9\textsubscript{488} in the capsosomes (Figure 8.6b). In NaOAc buffer (pH 4), the presence of the fluorescently labeled conjugates is seen as a homogenous green corona on the membrane of the capsosomes (Figure 8.6bi). When these capsosomes were dispersed in PBS (pH 7.4), PVP and the sacrificial PMA were released from the multilayer film, resulting in single-component crosslinked PMA carrier capsule walls.\textsuperscript{1} Deprotonation of the PMA-KP9\textsubscript{488} and the PMA\textsubscript{SH} causes electrostatic repulsion between the conjugates and the negatively-charged hydrogel membrane, changing the spatial position of the encapsulated PMA-KP9\textsubscript{488}, which are now contained inside the cavity of the carrier capsules together with the L\textsubscript{DPPC-GR}. 
(Figure 8.6bii). By correlation with a calibration curve of KP9<sub>488</sub> (Figure 8.1), the number of KP9 oligopeptides encapsulated in the capsosomes was quantified as \(5 \times 10^5\) copies of oligopeptides per 3 µm-diameter capsosome.

**Figure 8.6.** a) Fluorescence intensity of PMA capsules encapsulating PMA-KP9<sub>488</sub> (i) or capsosomes encapsulating L<sub>S,zw</sub>NBD and PMA-KP9<sub>488</sub> (ii), as measured by flow cytometry. A similar increase in fluorescence intensity of PMA-KP9<sub>488</sub> indicates negligible effect of the presence of liposomes on the encapsulation of the polymer-peptide conjugates. b) CLSM images of capsosomes encapsulating L<sub>DPPC,GR</sub> and PMA-KP9<sub>488</sub>, dispersed at pH 4 (i) or pH 7.4 (ii). At physiological pH (pH 7.4), electrostatic repulsion between the conjugates and the negatively charged hydrogel membrane results in changes in the spatial position of the encapsulated polymer-peptide conjugates, which are now contained inside the cavity of the carrier capsules. Scale bars are 5 µm.
Release of encapsulated KP9 oligopeptides from PMA capsules has previously been demonstrated by incubating the loaded carrier capsules in 5 mM GSH. In this study, the GSH generated from the encapsulated enzyme glutathione reductase cleaves the disulfide linkage between the polymer and the peptides (Scheme 8.4b). Equal numbers of capsosomes encapsulating L\textsubscript{DPPC-GR} and PMA-KP9\textsubscript{488} were incubated with GSSG and NADPH in PBS at 24 °C or 37 °C and the release of KP9\textsubscript{488} was monitored over time by flow cytometry (Figure 8.7a). Approximately a 50% decrease of the green fluorescence intensity was observed within 24 h at 37 °C, followed by a plateau through to 72 h. In contrast, control samples showed a much smaller decrease in fluorescence (~10 - 20%) in the same time frame. These data are in agreement with the temperature-triggered activity of the glutathione reductase in capsosomes (Figure 8.5a); that is, the conversion of GSSG to GSH only occurred at 37 °C, thus the induced release of the oligopeptides only occurred at this temperature.

Figure 8.7b shows the corresponding flow cytometry dot plot of these capsosomes before (i) and after (ii) incubation at 37 °C in the glutathione reductase assay for 24 h. A decrease of fluorescence in the population was observed, while there was no change in the light scattering measurement. The latter confirmed that the presence of GSH did not compromise the structural integrity of the carrier capsules because the membrane of the capsosomes in this study is stabilized via a non-cleavable crosslinker, BM(PEG)\textsubscript{2}. Corresponding DIC and fluorescence microscopy images of these capsosomes before and after peptide release are shown in panels c and d of Figure 8.7, respectively. DIC images confirmed that the capsosomes remained intact after the enzyme-mediated peptide release (Figure 8.7c, i and 8.7d, i). Fluorescence microscopy images showed that upon release of the cargo, some of the peptides remained associated with the membrane of the capsosomes (Figure 8.7d, ii). This is likely due to non-specific adsorption and possible covalent interactions between the cysteine groups of the KP9\textsubscript{488} and maleimide ends of partially crosslinked BM(PEG)\textsubscript{2}.
Figure 8.7. a) Changes in normalized fluorescence intensity of capsosomes encapsulating L$_{\text{DPPC-GR}}$ and PMA-KP9$_{488}$ upon incubation with GSSG and NADPH, as monitored by flow cytometry as a function of time. A decrease in fluorescence intensity indicates release of the encapsulated KP9$_{488}$ due to cleavage of the disulfide linkage between the polymer carrier and the peptide by GSH produced by glutathione reductase. b) Flow cytometry dot plot of capsosomes encapsulating L$_{\text{DPPC-GR}}$ and PMA-KP9$_{488}$ incubated with GSSG and NADPH at 37 °C, before (i) and after (ii) release of the peptide (24 h). A decrease in green fluorescence is indicative of peptide release, while similar light scattering indicates a minimal change in the shape and the composition of the capsosomes. Corresponding DIC and fluorescence microscopy images of these capsosomes incubated at 37 °C, before (c, i and c, ii) and after (d, i and d, ii) the glutathione reductase-induced cleavage and subsequent release of KP9$_{488}$ (24 h) are shown. Scale bars are 5 µm.
Capsosomes were also incubated with 5 mM GSH at 37 °C to serve as a positive control (Figure 8.8). As expected, the flow cytometry dot plot of these capsosomes, with the corresponding DIC and fluorescence microscopy images, resemble those capsosomes subjected to the glutathione reductase assay (Figure 8.7b and 8.7d). Taken together, the results demonstrate that capsosomes encapsulating glutathione reductase may be used to reduce glutathione disulfide, which subsequently mediates the release of a small peptide.

**Figure 8.8.** Flow cytometry dot plot of capsosomes encapsulating L-DPPC-GR and PMA-KP9488 incubated with 5 mM GSH at 37 °C at 24 h. Scale bars are 5 µm.
8.5 Conclusions

This chapter demonstrates the functionality of capsosomes through triggered encapsulated enzymatic catalysis. A quantitative enzymatic reaction confirmed the presence of active enzymes within the capsosomes and the number of liposomal subcompartments incorporated within a 3 µm-diameter capsosome assembled with a single liposome layer was determined to be approximately 8,000. A maximum of 160,000 liposomal subunits (equivalent to 20 single liposome layers) can be assembled to form capsosomes on 3 µm-diameter templates, which allows encapsulation of large amounts of active biomolecules. β-lactamase activity confirmed the assembly of the multiple layers of intact liposomes within these capsosomes. Both chemical (surfactant) and physical (temperature) stimuli can be employed to initiate enzymatic reactions in capsosomes. While the former approach causes lysis of the liposomes and excludes the possibility to reuse the subunits, the latter approach employing phase transition temperature of the liposomes allows successive enzymatic catalytic reactions without any loss of the functional activity of the enzymes. Furthermore, the ability of capsosomes to perform two-step enzymatic catalysis was demonstrated. Capsosomes encapsulating glutathione reductase were able to generate glutathione, a potent antioxidant, while simultaneously releasing small molecule therapeutics. This study advances the development of the capsosome system into a biomimetic platform that can combine both enzyme therapy and controlled drug release in a single structure.
8.6 References


Capsosomes, polymer carrier capsules containing intact cargo-loaded liposomal subcompartments, developed in the current thesis represents a novel class of carrier system toward the creation of therapeutic cell mimics. Polymer capsules, prepared via layer-by-layer (LbL) adsorption of interacting polymers onto sacrificial template particles, have been developed as promising scaffolds for biomedical applications due to the facile control over size, shape, composition, and permeability of the capsule membrane by the choice of the deposited polymer and the number of layers adsorbed. On the other hand, liposomes are vesicular structures formed via the self-assembly of amphiphilic phospholipids. The combination of these two fundamentally different systems retains the key advantages of polymer capsules and liposomes while overcoming the inherent limitations of each individual component, i.e., (i) polymer capsules provide structural scaffold with controlled permeability and (ii) liposomes provide specialized subcompartments, allowing encapsulation of fragile cargo and successive triggered enzymatic catalysis within confined environments.

Fundamental aspects underpinning polymer/liposome coassembly on planar surfaces and particle substrates are detailed in Chapter 4. A stable and high loading of intact liposomal cargo into a polymer film was achieved by noncovalently sandwiching the liposomes (unsaturated or saturated, zwitterionic or negatively charged) between a tailor-made cholesterol modified poly(L-lysine) (PLLc) precursor layer and a poly(methacrylic acid)-co-(cholesteryl methacrylate) (PMAc) capping layer. The alternating deposition of liposomes and polymer separation layers enable the deposition of multilayers of liposomes in the polymer/liposome assembly, where...
a maximum of 16 zwitterionic liposome layers or 20 negatively charged liposome layers can be assembled onto the polymer film.

Following the polymer/liposome coassembly on particle templates, a polymer membrane was formed by the alternating deposition of poly(N-vinylpyrrolidone) (PVP) and thiol-functionalized poly(methacrylic acid) (PMA$_{\text{SH}}$). Stabilization of the multilayer film by crosslinking the thiols in the polymer film, followed by core removal, led to the construction of (bio)degradable capsosomes as described in Chapter 5. Spatial position of the liposomal subunits within the polymer carrier capsules can be controlled to obtain capsosomes with membrane-associated or “free-floating” subunits. Furthermore, two crosslinking approaches were examined, namely oxidative and non-oxidative, with the use of oxidizing agents and polymeric crosslinkers, respectively. While both approaches give rise to stable disulfide-crosslinked capsosomes at physiological conditions, the non-oxidative crosslinking approach is singled out because crosslinking strategy using oxidizing agent can have deleterious effect to therapeutics and biomolecules that are susceptible to oxidation.

Understanding the retention of (enzymatic) cargo within the liposomal subunits is highly important toward the development of capsosomes for conducting enzymatic cascade reactions within confined environments. A range of (bio)molecules, large molecules such as enzymes down to models for low molecular weight drugs, were successfully encapsulated into capsosomes and the size-dependent retention of the cargo encapsulated within the liposomal subcompartments was examined in Chapter 6. The cutoff size of (bio)molecules that can be encapsulated within the liposomal subunits has been identified to be 500 Da. Investigations on stability of the encapsulated cargo showed that the liposomal subunits remained intact and their encapsulated enzymatic cargo was retained within the subunits during the assembly of the membrane of the carrier capsules and upon core removal. Excellent retention of the liposomes and the enzymatic cargo within the polymer carrier capsules was observed up to 14 days.
A fundamental aspect that governs the success of continuous enzymatic reactions in capsosomes is the long-term stability of the liposomal subunits within the polymer carrier capsules at physiological conditions, as they are subject to degradation in the presence of lipases. In Chapter 7, the outer membrane of the capsosomes was surface functionalized by immobilizing graft copolymers of PMA and poly(ethylene glycol) (PEG). The effect of PEGylation on the permeability of the carrier capsules and the stability of the liposomal subcompartments in the presence of phospholipases in different media was assessed. Diffusion of lipases across the multilayered membrane of the carrier capsules was hindered when the capsules were coated with PEG molecules, which resulted in the prolonged stability of the liposomal subunits within the capsosomes, i.e., 60% of the subunits were remained after incubation with phospholipases for 48 h.

The functionality of capsosomes was demonstrated in Chapter 8 by encapsulating enzymes, e.g., β-lactamase or glutathione reductase, within the liposomal subcompartments. Triggered enzymatic catalysis by chemical and physical stimuli confirmed the activity of the encapsulated enzymes. While the former approach lysed the liposomal subunits, the latter strategy employing phase transition temperature of the liposomes to initiate the enzymatic reaction allowed the capsosomes to be repeatedly used for multiple subsequent conversions. The quantitative β-lactamase reaction allowed the estimation of the number of incorporated liposomes within the capsosomes. Approximately 8,000 liposomal subunits were assembled within a 3 µm-diameter capsosome with a single liposome layer and a maximum of 160,000 liposomal subunits can be encapsulated within a 3 µm-diameter capsosome, allowing encapsulation of large amounts of active biomolecules. To further the aim in addressing complexity in therapeutic cell mimicry, the ability of capsosomes to perform encapsulated two-step enzymatic catalysis was demonstrated; capsosomes encapsulating glutathione reductase were able to generate glutathione, a potent antioxidant, while simultaneously releasing small molecule therapeutics.
In summary, capsosomes, structurally stable polymer carrier capsules containing intact cargo-loaded liposomes, have been proven to be a promising carrier system toward the creation of therapeutic artificial cells. Optimized capsosome assembly, cargo encapsulation in the subcompartments, surface functionalization, and triggered encapsulated catalysis in capsosomes described in this thesis highlight crucial aspects for the successful application of capsosomes as cell mimics. These engineered capsosomes with tailored properties present new opportunities en route to the development of biomimetic structures toward enzymatic therapy applications, which focus on enzymatic activities to degrade waste products or to support the synthesis of medically relevant biomolecules. Future work will include the investigation into the performance of capsosomes in chemically diverse biological environments and studies of their interactions with cellular structures – these are critical to ensure long-term performance of these assemblies in biological systems. It is envisaged that future research based upon the findings presented in this thesis will lead to fabrication of robust, subcompartmentalized assembly approaching the long-term catalytic performance of living cells.
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