Layer-by-layer polyelectrolyte microcapsules with tunable release behaviour and versatile encapsulation ability

Luo, Rongcong

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ENCAPSULATION ABILITY**

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Abstract

Layer-by-layer (LbL) polyelectrolyte microcapsules with pre-loaded red blood cells (RBCs) ghosts and calcium carbonate (CaCO_3) microparticles as template cores were developed in this project. Taking advantage of the unique features of these templates, LbL polyelectrolyte microcapsules with a simplified preparation process, versatile encapsulation capability and tunable release ability are obtained.

RBCs were loaded with hydrophilic compounds (FITC-dextran or RITC-lysozyme) using hypotonic dialysis loading method. SEM and confocal fluorescent microscope confirmed the loading of compounds into the intact RBCs ghosts. The loaded RBCs ghosts were then coated with different layers of polyelectrolytes. Unlike the traditional solid polymeric templates, the pre-loaded RBCs ghosts can eliminate the core dissolution and substance post-loading steps. The release behaviors become tunable through adjusting the coated polyelectrolyte layer numbers. The release is principally controlled via solutes diffusion through the intact polyelectrolyte membrane.

Porous CaCO_3 microparticles were coated with PLGA layer containing hydrophobic compounds via PLGA *in situ* precipitation technique. The intact PLGA microcapsules were confirmed by confocal fluorescent microscope, AFM, SEM, TGA and ζ -potential studies. The PLGA microcapsules were further coated with multilayer polyelectrolytes to control the drug release behaviors, where dual-drug releasing carriers can also be obtained.

Chapter 1 Introduction

1.1 Background

The aim of this project is to develop layer-by-layer (LbL) polyelectrolyte microcapsules using pre-loaded red blood cells (RBCs) and calcium carbonate (CaCO_3) microparticles as template cores. Taking advantage of the unique features of these templates, LbL polyelectrolyte microcapsules with a simplified preparation process, versatile encapsulation capability and tunable release ability are developed in this project.

Although LbL polyelectrolyte microcapsules are young candidates as drug delivery vesicles, they have some distinctive advantages as compared to other drug carriers such as liposomes, polymeric micro-/nano-particles, micelles and emulsions [1-4]. LbL polyelectrolyte microcapsules have some beneficial features (1) the preparation process is completely aqueous-solution based (2) the size and shape of the final microcapsules can be easily controlled by the templates (3) the composition, permeability and stability of microcapsules shell can be finely tuned by changing the polyelectrolyte architectures. Consequently, LbL polyelectrolyte microcapsules have been used widely for drug encapsulation and delivery [1-4].

The formation of LbL microcapsules involves the stepwise coating of oppositely charged polyelectrolytes onto the surface of colloidal particles. After the designed numbers of polyelectrolyte coating are reached, the colloidal template cores are dissolved to yield hollow microcapsules. Template cores and

the coated polyelectrolyte layers are two central components for the LbL microcapsules [5-7].

Regarding the template cores, pre-loaded ones have several advantages. Pre-loaded template cores are not only employed as a sacrificial template support but also as a drug reservoir of the final LbL microcapsule. Vesicles-like RBCs and porous CaCO_3 microparticles are two attractive pre-loaded templates for the LbL polyelectrolyte microcapsules [5-7].

In the past, RBCs have already been used as natural cellular carriers to encapsulate and deliver therapeutic agents. The first efforts can be dated back to 1953 when Gárdos et al incorporated chemicals into RBCs via swelling. RBCs will swell and drugs can diffuse inside after being subjected to hypotonic buffer treatment. Such drug-loaded RBCs can be resealed in isotonic buffer and re-injected as a drug delivery system [8-10].

As template cores for LbL polyelectrolyte microcapsules, RBCs have some interesting characteristics such as their availability, mono-dispersity, spherical shape and stable surface charge. The first study of using RBCs as template cores for LbL polyelectrolyte microcapsules was carried out by Neu et al [11]. Thereafter, more studies followed. However, all these studies barely used native RBCs as sacrificial supports and post-loading of drugs into the hollow microcapsules was still necessary. In order to eliminate the core dissolution and achieve pre-loading of LbL polyelectrolyte microcapsules, a combination of drug-loaded RBCs and LbL polyelectrolyte coating would be an

effective way [12-14]. Such a combination has been developed in this project.

Porous CaCO_3 microparticles are another important kind of pre-loaded template for LbL microcapsules. Their porous interior and rough surface provide a good platform for substance immobilization [15-18]. CaCO_3 microparticles can be formed by simply mixing an equal volume of CaCl_2 solution and Na_2CO_3 solution under stirring (**Figure 1A**). Depending on the stirring speed, size of these particles can be ranging from 3 to 5 μm . Drugs can be loaded into the CaCO_3 microparticles via co-crystallization with CaCl_2 and Na_2CO_3 (**Figure 1B**) or infiltration after the porous particles have been formed (**Figure 1C**).

Figure 1. Schematic illustration showing the (A) formation process of porous CaCO_3 microparticles. (B) Drug encapsulation into CaCO_3 microparticles via

co-crystallization. (C) Drug encapsulation via infiltration after the CaCO_3 microparticles are formed.

Beside the simple formation conditions and versatile drugs encapsulating ability, CaCO_3 microparticles can be effectively decomposed by mild ethylene diamine tetraacetic acid (EDTA) or HCL solution, leaving no toxic residues inside the microcapsules. These features make them as promising templates for LbL polyelectrolyte microcapsules. Using CaCO_3 microparticles, many unique polyelectrolyte microcapsules such as matrix-type microcapsules, single component microcapsules and multi-compartment microcapsules have been formed [19-20].

Besides the template cores, the coated polyelectrolyte layers are another pivotal component for forming LbL polyelectrolyte microcapsules. One of the essential roles of the polyelectrolyte is to control the release kinetics of the incorporated drugs, which greatly depends on the permeability of the polyelectrolyte layers. Changing the polyelectrolyte composition and the number of coated layers are two major ways to tune the permeability [21-23].

Regarding the polyelectrolyte composition, it is generally observed that multilayers formed from strong polyelectrolyte are less permeable than those formed from weak polyelectrolyte. The high charge density and well-matched charge interaction between strong polyelectrolyte are believed to result into more compact and stable films. Regarding the layer numbers, increasing the layer numbers will increase the layer thickness and thus decrease

the permeability of the capsule shell. Increasing the layer numbers can also reduce defects of the films. When the layer numbers are few, cavities may exist. The further coating of polyelectrolyte can seal these cavities and reduce the release rate [24-26].

1.2 Problems Statement

Despite the various advantages of LbL polyelectrolyte microcapsules, there are still some unsolved problems limiting their application. Typical problems are: (1) Adverse side-effects associated with dissolution of solid polymeric template cores. (2) Inefficiency of drugs post-loading. (3) Inability of encapsulating hydrophobic drugs and controlling their release. (4) Increased aggregation when layer numbers increase. (5) Poor long term stability against environmental conditions changes. (6) Non-specific proteins binding to the microcapsules surface [1-3, 22-25].

This project will focus on developing solutions for the first three problems, since they determine the successful application of LbL polyelectrolyte microcapsules as drug delivery carriers.

The first problem is about the dissolution of template cores. Majorities of the LbL polyelectrolytes microcapsules are formed using solid polymeric microparticles as templates. The most frequently used templates are melamine-formaldehyde (MF) microparticles and polystyrene (PS) microparticles. There are some drawbacks using such templates. (1) Causing

rupture of the formed microcapsules. During the dissolution, the oligomers of dissolved polymers are too large to easily release out of the microcapsules. The high residence of oligomers in the interior generates high osmotic pressure and lead to swell and rupture of the microcapsules. (2) Incomplete removal of toxic residue like melamine inside the microcapsules. (3) Requirement of toxic solvents like tetrahydrofuran (THF) to dissolve the templates.

The second problem is due to the fact that microcapsules based on the above mentioned templates need post-loading of drugs. Post-loading involves loading of drugs into the complete microcapsule, which can have some adverse effects. (1) Resulting in disassembly of the microcapsules. The already-formed microcapsules need to be subjected to harsh conditions like low pH and high ionic strength which will induce opening of the capsule shell for drug molecules to diffuse inside. After that, the conditions are restored to heal the capsules. This process may cause irreversible disassembly of the microcapsules. (2) Poor encapsulation efficiency. (3) Only limited categories of drugs can be loaded.

The third problem is that hydrophobic drugs cannot be encapsulated into LbL polyelectrolyte microcapsules, mainly due to their aqueous solution based fabrication and encapsulation process. Hydrophobic drugs roughly describe a heterogeneous group of molecules that exhibit poor water solubility. It has been reported that about 40% of new pharmacologically active substances identified through screening of combinatorial libraries have poor water solubility [55]. To date, the main strategy of encapsulating hydrophobic drugs

into LbL polyelectrolytes microcapsules is to embed hydrophobic drug loaded micelles or liposome subunits into the microcapsules. However, there are some limitations associated with these methods such as the difficulty of handling soft liquefied droplets, necessity of an oil phase, cytotoxicity of micelles and breakage of liposomes.

1.3 Objectives and scopes

The primary goal of this project is to develop LbL polyelectrolyte microcapsules with a simplified formation procedure, versatile encapsulation capability and tunable release ability. To achieve this goal, the project is divided into two broad sections.

The first section involves development of LbL polyelectrolyte microcapsules using pre-loaded RBCs as template. This section will investigate 4 aspects. (1) hydrophilic compounds loading into RBCs using hypotonic dialysis method (2) coating of polyelectrolyte layers onto the pre-loaded RBCs via LbL deposition procedure (3) release behaviors of the loaded compounds against different polyelectrolyte layer numbers (4) fitting the release behaviors using established mathematical models to elucidate the release mechanism.

The second section involves development of LbL polyelectrolyte microcapsules using hydrophobic drugs loaded CaCO_3 microparticles as template. This section will investigate 5 aspects. (1) coating hydrophobic drugs loaded PLGA layer onto CaCO_3 using PLGA *in situ* precipitation method (2)

further coating different numbers of polyelectrolyte layers onto the loaded CaCO_3 microparticles (3) release of the hydrophobic drugs against different polyelectrolyte layer numbers (4) modeling the release behaviors with established mathematical models (5) the interior of the microcapsules is also loaded with hydrophilic drugs and magnetic nanoparticles to build a magnetically responsive dual drug release microcapsules.

1.4 Hypothesis

Versatile encapsulation capability and tunable release ability are two desirable features for drug carriers. We hypothesize that improving the compounds loading and changing the coated polyelectrolyte layer numbers can produce LbL polyelectrolyte microcapsules with these important features.

Regarding the release kinetics control, we hypothesize that coating the polyelectrolyte layers onto the drug loaded template cores can control their release profiles. (1) Coating the polyelectrolyte layers onto the bare loaded cores can reduce their burst release and thus produce microcapsules with sustainable release manners. (2) Increasing the coated polyelectrolyte layer numbers can further reduce the burst release and tune the release kinetics of these microcapsules to meet different requirements.

Regarding the drug encapsulation capability, we hypothesize that pre-loaded template cores can simplify the fabrication process, enable loading of hydrophobic drugs and produce multifunctional dual-drug releasing

microcapsules. (1) Drug loaded vesicles (RBCs ghosts) can be better templates than solid polymeric templates, since they can eliminate the core dissolution and substances post-loading steps. (2) PLGA, which is a good candidate for hydrophobic drugs encapsulation, can be introduced into LbL polyelectrolyte microcapsules via combining porous CaCO_3 cores and PLGA *in situ* precipitation method.

1.5 Organization of the thesis

Chapter 2 Literature Review

2.1 Rationales of drug encapsulation

Drug encapsulation can maintain blood level of a drug to be therapeutically active while at the same time minimize adverse side effects (**Figure 2**). When a single dose of drug is administered, the blood level of the drug will increase rapidly and then decay exponentially as the drug is metabolized (**Figure 2**). Such process can lead to a drug overdose and it might require repeated drug administration [27-28]. On the other hand, appropriate encapsulation of drugs can maximize their effectiveness, maintain stable drug concentrations and reduce the administration frequency (**Figure 2**) [29].

Figure 2 Change of blood level of drugs with (*yellow dash line*) and without (*purple dash line*) encapsulation after administration

Besides controlling the blood level of drugs, encapsulation may also be beneficial to enhance their bioavailability. For example, encapsulating hydrophobic drugs with liposomes can enhance their solubilization in the gastrointestinal tract and then facilitates their delivery to the targeted site, which addresses the problems of low solubility and permeability of hydrophobic drugs. Encapsulation also makes drug targeting possible. For instance, LbL polyelectrolyte microcapsules modified with monoclonal antibodies show much higher uptake efficiency by targeted cancer cells than the unmodified ones [30-31]. Consequently, increased bioavailability and accumulation of drugs at the target site can be achieved after encapsulation.

Reduction of toxicity and protection of drugs from *in vivo* cellular degradation is another benefit of drug encapsulation [32-35]. One typical example is the prevention of orally-administered drugs from acid degradation in the stomach before they reach the intestines. This can be achieved via encapsulating drugs into gelatin capsules, which are hardly soluble in stomach acidic environment. Furthermore, there are increasing numbers of therapeutic agents based on active biomacromolecules such as peptides, proteins, DNA and siRNA. These bioactives are susceptible to extracellular enzyme and acid breakdown, making the protection of them via appropriate encapsulation techniques a necessity [32-35].

2.2 LbL polymeric microcapsules for drug encapsulation

2.2.1 Formation of LbL polymeric microcapsules

LbL polyelectrolyte microcapsules as possible drug carriers were initially invented in 1998-1999 at the Max Plank Institute in Golm, Germany by Sukhorukov, Donath, Caruso and M $\ddot{u}$ hlwald. The process of preparing LbL polyelectrolyte microcapsules is explained in **Figure 3** [36-38].

Figure 3 Schematic illustration showing the preparation process of LbL polyelectrolyte microcapsules (i) & (ii) the charged colloid template is alternately coated with oppositely charged polyelectrolytes (iii) & (iv) the template is decomposed to produce hollow LbL polyelectrolyte microcapsules

Figure 4 Schematic illustration showing the drug encapsulation and functionalization of LbL polyelectrolyte microcapsules

For preparation of empty LbL polyelectrolyte microcapsules, a spherical charged colloidal particle is used as supporting template core and sequentially coated with polyelectrolyte layers from the aqueous solution (**Figure 3**). Electrostatic attraction, hydrogen bonding and covalent linking can be used as driving force to bond the deposited layers [39-40]. After desired layer numbers are reached, the template core can be dissolved to yield hollow microcapsules (**Figure 3**). Functionalization of microcapsules with magnetic, silver, gold, quantum dots, carbon nanotubes et al can be realized within the microcapsules cavity or between the microcapsule walls (**Figure 4**). Attachment

of targeting molecules or polyethylene glycol (PEG) moieties can be performed at the external surface of the capsules (**Figure 4**). [41-43]

2.2.2 Template cores for LbL polyelectrolyte microcapsules

Template cores used for forming LbL polyelectrolyte microcapsules can be broadly divided into solid polymeric microparticles, inorganic porous microparticles and biological cells [44-47]. The most frequently used solid polymeric cores are weakly cross-linked melamine formaldehyde lattices (MF) and polystyrene latex (PS) microparticles. Benefit of these templates is their excellent monodispersity. However, microcapsules generated from them have some problems such as the requirement of substance post-loading, residue of toxic oligomers, necessity of toxic solvents for dissolution and rupture of microcapsules [44-45]. Consequently, biomedical application of LbL polyelectrolyte microcapsules from such template cores is limited.

Compared to MF and PS, porous inorganic microparticles such as CaCO_3 and silica microparticles have some unique merits. Regarding mesoporous silica microparticles, drugs solution or nanoparticles can be infiltrated and immobilized inside the microparticles. After subsequent LbL shell coating, the cores are decomposed in dilute hydrofluoric acid (HF) solution and the loaded compounds can remain in the interior of the LbL microcapsules. Mesoporous silica microparticles can not only be coated with polyelectrolytes at normal pH but can also be exposed to coating materials which require low pH conditions. One example is poly

(N-vinylpyrrolidone)/poly (methacrylic acid) (PVPON/PMA) LbL microcapsules where coating of PVPON and PMA requires low pH values. These PVPON/PMA microcapsules show interesting pH responsive properties, which can be utilized for applications as intracellular delivery vesicles [46].

Regarding CaCO_3 microparticles, substances can be pre-loaded via co-crystallization or infiltration. The unique benefits of CaCO_3 microparticles as template cores are their excellent biocompatibility, complete biodegradability, simplicity of fabrication, mild conditions of decomposition without the toxic residues. Furthermore, CaCO_3 microparticles are useful for forming LbL polyelectrolyte microcapsules having more sophisticated structures. For example, multi-compartment or “capsule-in-capsule” LbL polyelectrolyte microcapsules, which might be useful as micro-reactors have been produced using CaCO_3 as the supporting templates [47].

Regarding biological cells as templates, red blood cells (RBCs) are the most frequently used ones. They are available in large quantities, with good dispersity and possibility of substance pre-loading. However, their cell membrane stability is poor requiring fixation with glutaraldehyde prior to LbL polyelectrolyte coating. Furthermore, decomposition of fixed RBCs requires harsh conditions such as a pH 12 sodium hypochlorite solution, which will also destroy the polyelectrolyte coating [11, 48].

2.2.3 LbL coating onto template cores

Beside template cores, the coating materials also play a pivotal role for LbL microcapsules. Electrostatic attraction, hydrogen bonding and covalent bonding are the three major driving forces for the LbL assembly (**Figure 5**). [49-52]

Figure 5 The three major driving forces for LbL assembly of coating materials during preparation process of LbL microcapsules.

Electrostatic attraction

Majorities of LbL microcapsules are formed from polyelectrolytes where electrostatic attraction brings the poly-cation and poly-anion to form insoluble complexes (**Figure 5**). In the beginning, most studies were devoted to use synthetic and non-degradable polyelectrolytes to produce LbL polyelectrolyte microcapsules. Representative examples are poly (styrene

sulfonate) (PSS), poly (allylamine hydrochloride) (PAH), poly (acrylic acid) (PAA) and poly (ethyleneimine) (PEI). Later, researchers explored natural and biodegradable polyelectrolytes to manufacture LbL polyelectrolyte microcapsules. Examples include alginate, hyalurate, chondroitin sulfate, heparin, dextran sulfate, carboxymethyl cellulose, polyaminoacids (PLL, PGA et al), chitosan, collagens, protamine sulfate and gelatin [49-50].

Hydrogen bonding

In order to avoid using potentially toxic poly-cations, LbL microcapsules based on hydrogen bonding were also developed. The frequently-used one is PVPON/PMA microcapsule (**Figure 5**). At low pH values, both PVPON and PMA are in quasi-uncharged state, where PVPON is the hydrogen bond acceptor and PMA is the hydrogen bond donor (**Figure 5**). These unique microcapsules show interesting pH-dependent assembly/disassembly behaviors. PVPON/PMA microcapsule may be a good carrier for intracellular delivery, since the pH difference between extracellular and intracellular environment. The PVPON/PMA microcapsules will only disassemble in acidic environment like the acid lysosome compartments inside the cell and can be used as intracellular drug release depot [51].

Covalent bonding

Although electrostatic interaction is the major force for forming the LbL polyelectrolyte microcapsules, they are sensitive to the changes of

environmental conditions. In contrast, covalent reaction is a more effective way to stabilize LbL polyelectrolyte microcapsules (**Figure 5**). Previous studies prepared covalently linked polyelectrolyte multilayer microcapsules using glutaraldehyde to covalently link the alternatively coated polyelectrolyte layers having primary amines groups through forming imines linkage. The reaction can be performed in short time at mild conditions. However, residues of toxic cross-linkers are the major problem of this method. Different from glutaraldehyde, Caruso and co-workers developed a bio-reducible disulfide cross-linker which is less toxic (**Figure 5**). In their studies, thiol-modified PMA is first assembled with PVPON via hydrogen bonding. After desired layer numbers are reached, thiol groups are converted into disulfide linkages using chloramine T. In a reductive environment, the disulfide bonds will be broken into single thiol again, causing disassembly of the microcapsules, which may be utilized for bio-responsive delivery systems [51-52].

2.2.4 Loading drugs into LbL polyelectrolyte microcapsules

Post-loading, pre-loading and direct coating of polyelectrolytes onto drug crystals are the three major methods to load drugs into LbL polyelectrolyte microcapsules [53-57]. Post-loading involves loading drugs into the already-formed LbL polyelectrolyte microcapsules through alternating the permeability of microcapsules shell. For polyelectrolyte microcapsules, factors affecting permeability including pH and ionic strength of mediums are used to

induce the loading of drugs (**Figure 6**) [53-54]. There are some problems for this loading method. (1) Encapsulation efficiency of post-loading method is quite low. (2) Only limited categories of drugs can be loaded using this method. (3) Causing rupture and irreversible disassembly of the microcapsules due to the harsh conditions during this treatment.

Figure 6 Schematic illustration showing the post-loading of drugs into the already- formed LbL polyelectrolyte microcapsules

On the other hand, pre-loading is able to achieve higher loading efficiency. Pre-loading can be realized via two different ways [55-56]. (1) Drug solution can be infiltrated and immobilized in porous solid cores prior to LbL coating. Subsequently, the cores are dissolved and the loaded drugs get trapped inside the cavity of the microcapsules (**Figure 7**). (2) Drugs are loaded into smaller subunits such as liposomes and then incorporated into the cores or between the shell walls. This way is effective for loading hydrophobic drugs into LbL microcapsules. Usually, LbL microcapsules are used to encapsulate hydrophilic drugs due to their aqueous solution based assembly process. This method greatly broadens the application field of LbL polyelectrolyte microcapsules in drug encapsulation and delivery [55-56].

Figure 7 Schematic illustration showing the pre-loading method

The final method involves direct coating of multilayer polyelectrolytes onto drug crystals. Initially, the coating was carried out on unmodified drug crystals. Unstable surface charge and irregular shape are two problems of this method [57]. Later, these two problems are solved using the solvent emulsification method. In the improved method, micro-size drug crystals are firstly dissolved in organic solvents and emulsified with positively or negatively charged polyelectrolytes solution. By adding poor solvents, drug crystals with regular shape grow again and are further coated with multilayer polyelectrolytes [58].

2.2.5 Control of drug release from LbL polyelectrolyte microcapsules

Permeability of LbL polyelectrolyte microcapsules is essential in determining their drug release properties. Layer thickness, layer composition and layer interaction are three important factors influencing the microcapsule shell permeability, which are frequently used to tune the microcapsules release properties [59-67].

Layer thickness

Alternating the polyelectrolyte layer numbers is a convenient way to tune the layer thickness. It is generally observed that increase of layer numbers induces thicker microcapsule shell which decreases the penetration of drug molecules. Depending on the polyelectrolyte layer numbers, the resulting hollow microcapsules usually have a wall thickness of between a few tens and hundreds of nanometers. For a single polyelectrolyte layer, the typical thickness is usually ranging from 2nm to 8nm as indicated by small angle neutron scattering (SANS) and scanning force microscope (SFM) in most of previous researches. The thickness of single polyelectrolyte layer is determined by the nature of polyelectrolyte, pH of the solution, molecular weight of the polyelectrolyte, coating time, washing solution and ionic strength of the solution. Recent experiments on the PAH/PSS multilayers have shown that the thickness of the polyelectrolyte multilayers in aqueous solution determined by SANS is very similar to that of the corresponding polyelectrolyte layer thickness in a dried state measured by SFM [59]. Poorly water-soluble drugs such as ibuprofen (IBU) are good choices for studying impacts of layer thickness [59-60].

In Qiu's study, IBU was coated with different numbers of PAH/PSS layers [59-60]. They found that permeability coefficient of IBU to PAH/PSS decrease from $7.6 \times 10^{-9} \text{ cm}^2/\text{s}$ to $3.4 \times 10^{-9} \text{ cm}^2/\text{s}$ when the layer numbers increase from 10 to 16. Besides IBU, other hydrophobic drugs crystals such as

fluorescein, paclitaxel and curcumin were also coated with different numbers of polyelectrolytes. The thickness dependence of permeability was also observed.

The thickness dependence of permeability was explained by polyelectrolytes layers homogenization. When the deposited layers are few, defects may exist in the shell, which will give rise to the permeability. On the other hand, after more layers are coated, the defects are sealed and a more homogeneous shell is achieved, which can reduce drug release [61].

Layer composition

Changing the polyelectrolyte layer composition is another effective way to adjust the layer permeability. There are two major ways to control the layer composition. (1) Change of coating polyelectrolyte (2) Addition of lipids [62-63].

Generally, microcapsules made from strong polyelectrolytes are less permeable than those formed from weak polyelectrolytes. For example, IBU were coated with PAH/DES or PAH/PSS at the same layer numbers (10 layers) for studying the release. It was found that PAH/PSS has a permeability coefficient of $7.6 \times 10^{-9} \text{ cm/s}$ while that for PAH/DES is up to $29 \times 10^{-9} \text{ cm/s}$. Polyelectrolytes multilayer assembly is linked via electrostatic attraction. The permeability is regulated via the entanglement density (i.e. magnitude of electrostatic attraction) [62-63]. Consequently, assembly formed with polyelectrolytes having higher charge density as PAH/PSS combination can form tighter and more homogeneous film which is less permeable. Otherwise,

assembly made from PAH/DES has a defect-rich film, which has higher drug release rate.

Combination of polyelectrolytes and lipids provides another promising way to control permeability. Assembling lipids into polyelectrolyte microcapsules shell has brought a prominent reduction of microcapsules shell permeability. It was found that the time of 6-carboxy-fluorescein (6-CF) equilibrating through the PAH/PSS microcapsules wall is 10s while that for lipids added PAH/PSS microcapsules is 10~100min. After intercalating lipid components in-between the polyelectrolyte layers, the increased drug-lipid hydrophobic interaction and refuse of water access are two main factors for reducing the permeability [64].

Layer interaction

Electrostatic interaction is the major binding force to form multilayer polyelectrolytes assembly. However, it is sensitive to the changes of external pH and ionic strength conditions, which will affect the permeability. Introduction of covalent bonding and thermally defined annealing are two major ways to maintain microcapsules permeability in different conditions [65].

A convenient way of introducing covalent bonding is to use low molecular weight cross-linking agents such as glutaraldehyde (GA). It was demonstrated that PAH/PSS microcapsule can be remarkably stabilized via cross-linking the PAH component with GA. Schiff base between the aldehyde groups of GA and the amine groups of PAH is formed. These microcapsules can

withstand 0.1M NaOH treatment for 24h. Moreover, the permeability coefficient for FITC-dextran was decreased by 3 magnitudes as compared to the control microcapsules stabilized with only electrostatic interaction [66].

LbL polyelectrolyte microcapsules can be subjected to thermal treatment, which greatly influences their permeability. As demonstrated by Ibarz et al, the permeability coefficient of PAH/PSS microcapsules can be lowered down by two order of magnitude after annealing at 80°C for 30min. It was proposed that the density of polyelectrolyte assembly may become more homogeneous due to the rearrangement of polyelectrolyte chains during annealing, which can eliminate defects [67].

2.3 Cellular carriers as natural microcapsules for drug delivery

Although synthetic drugs carriers are widely studied, it remains important to explore natural cells for drugs encapsulation and delivery. Natural cellular carriers include bacteria, red blood cells (RBCs), islets, hepatocytes, macrophages and fibroblasts. Compared to synthetic carriers, cellular carriers have some unique merits [68-76]. For example, bacteria have developed some special surface markers to avoid immune system recognition and can enter target cells without being cleared. Because of the special shape and self-markers on the membrane, RBCs have remarkable ability for long-time circulation and delivery drugs [68-70].

Amongst various cellular carriers, using RBCs to delivery drugs is

gaining more interest due to their beneficial features. (1) Rich resources of RBCs, they are the largest population of cells in human body. (2) RBCs have flexible membranes; they can pass through capillaries without fracturing [71]. (3) RBCs have good biocompatibility, long circulation time and degradability without leaving toxin. Additionally, RBCs have a biconcave shape imparting RBCs sufficient space for encapsulating drugs solution or even nanoparticles (**Figure 8**). [72]

Figure 8 Schematic illustration showing the size and morphology of RBCs

Currently, there are numerous methods to load drugs into RBCs. All the methods are aiming to achieve a higher loading efficiency while causing fewest alterations to RBCs [73-74]. This is to ensure that the loaded RBCs can survive and circulate as the native RBCs. Osmosis-based, electroporation and drug-induced endocytosis have been developed to load drugs into RBCs. Osmosis-based loading is the widest used method. It has several variations like hypotonic dilution, hypotonic pre-swelling, osmotic pulse, hypotonic hemolysis and hypotonic dialysis with the latter one being used most frequently (**Figure 9**).

Figure 9 The loading of drugs into RBCs using hypotonic dialysis method

Considering their natural function and prolonged circulation time, RBCs carriers are regarded as suitable delivery systems for intravenous drugs which need long circulation time. Anti-retroviral drug, anti-parasitic drug, anti-neoplastic drugs, steroids and cardiovascular drugs have been loaded into RBCs. Besides, RBCs carriers can also be loaded with magnetic particles for organs targeting [75-76].

2.4 PLGA-based carriers for hydrophobic drugs delivery

LbL polyelectrolyte microcapsules and natural cellular carriers are suitable for hydrophilic drugs encapsulation and delivery. On the other hand, carriers made from biodegradable polyesters such as PLGA are better choice for hydrophobic drugs encapsulation and delivery [77-85].

Hydrophobic drugs or poorly water-soluble drugs roughly describe a

heterogeneous group of molecules that exhibit poor water solubility but typically are soluble in various organic solvents. It was reported that about 40% of newly-identified drugs through screening of combinatorial libraries have poor water solubility [77-79]. Limited water solubility and restricted bioavailability are the main problems of hydrophobic drugs, despite their pharmacological activity. Consequently, encapsulation of hydrophobic drugs continues to be an important formulation strategy among scientists and pharmaceuticals companies [77-79].

PLGA have been using widely to delivery hydrophobic drugs. This is because of their numbers of appealing features like biodegradability, biocompatibility, FDA (U.S. Food and Drug Administration) approval, commercial availability and their hydrophobic nature which can encapsulate various hydrophobic compounds with high encapsulation efficiency [80-84].

PLGA based devices such as sutures, microcapsules, microsphere, nanoparticles, pellets, implants and films have been manufactured to delivery various hydrophobic drugs. One of important features of PLGA is that they are convenient to make into drugs carrying devices. Drug-eluting stent is one of the best examples. In drug eluting stent, restenosis inhibiting drugs are dissolved in PLGA and coated onto the metallic stent. Such drug containing stents have been found to outperform bare metal stents [80-83].

Unlike large PLGA implants requiring surgical insertion, injectable PLGA microcapsules represent another important and convenient way for drug

delivery. Emulsion solvent-extraction/evaporation method is the most frequently used method to prepare hollow PLGA microcapsules (**Figure 10**).

Figure 10 PLGA microcapsules made by emulsion solvent evaporation method

In this method, PLGA can be initially dissolved in a mixture combining good solvents (like chloroform) and poor solvents (called oil, like higher alkane). The mixture is then emulsified with water phase containing a surfactant. After evaporation of the good solvent and extraction of oil droplet core, hollow PLGA microcapsules are formed. The drugs can either be encapsulated inside the hollow cavity or in the polymer shell. Due to the ease of preparation and high drug payload, PLGA microcapsules have been of great interests in medicine, catalysis, cosmetics and foods. Besides being as drug carriers, blank hollow PLGA microcapsules are also used as ultrasound contrast agents, which exhibit better properties than the traditional lipid-shell agents [83-85].

2.5 Mathematical models of drugs release from microcapsules

Mathematical models of drugs delivery are important in understanding the drugs release mechanism, optimizing the design of carriers and predicting the drugs release behaviors. The first effort of mathematically modeling drug

delivery is carried out by Takeru Higuchi in 1961. Higuchi equation was initially used for describing the drug release behaviors from ointment with film geometry. One character of the ointment is having an initial excess amount of dispersed drugs within the matrix. Nowadays, there are many mathematical equations in the literatures. Some of the representative examples are listed in **Table 1** [86-96]

Table 1 Some representative models for studying the drug delivery

Models	Equation
Zero-order	$Q = Q_o + k_o t$
First-order	$Q = Q_{\infty}(1 + e^{-k_1 t})$
Higuchi	$Q = k_H t^{1/2}$
Korsmeyer-Peppas	$Q/Q_{\infty} = kt^n$
Baker-Londale	$(3/2)[1 - (1 - Q)^{2/3}] - Q = kt$

Notes:

Q (mg) is the amounts of drugs release at time t (h); Q_o (mg) is the amounts of drugs in the solution at time $t = 0$; Q_{∞} (mg) is the total amount of drug; k_o is the zero-order kinetic constant; k_1 is the first order kinetic constant; k_H is the Higuchi rate constant; n is the release exponent; k is the release constant for Korsmeyer-Peppas or Baker-Londale.

The mathematical models for describing drug release can be broadly divided into empirical/semi-empirical models and mechanistic models.

Empirical/semi-empirical models are not based on real biological, physical and chemical phenomenon. Generally, the predictive power of such models is low. On the other hand, mechanistic models are based on real phenomenon such as diffusion, dissolution, swelling, erosion, precipitation and degradation. Mechanistic mathematical models are good at determining the system parameters which can help to understand drug release mechanisms. The mechanistic mathematical models are useful for products development, since they can provide insight about effects of fabrication parameters on the drug release manners [86-90].

For empirical/semi-empirical mathematical models, equation developed by Peppas et al (named Korsmeyer-Peppas Model) may be the most famous one.

Korsmeyer-Peppas Model is expressed as $M_t/M_\infty = kt^n$, where M_t (mg) is the amounts of drug release at time t (h), M_∞ (mg) is the total amounts of drugs, k is a constant incorporating characteristics of the system and n is the release exponent indicative of the release mechanism. Release of curcumin and paclitaxel from LbL polyelectrolyte microcapsules with different layer numbers was fitted using this model. It was found that the validity of the model increase with the increase of polyelectrolytes layer numbers for both curcumin and paclitaxel [58, 91].

The Higuchi equation and the Hopfenberg model are other examples of empirical models. Higuchi's equation is expressed as $Q = 2C_0(Dt/\pi)^{1/2}$, where Q

(mg) is the amounts of drug released per unit area (cm^2), $Q=C/A$, C (mg) is the amount of drugs release at time t (h), A (cm^2) is the total area of the capsules, C_o (mg/ml) is the initial drug concentration and D (cm^2/sec) is the apparent diffusion coefficient. Release of doxorubicin and daunorubicin from PAH/PSS multilayer microcapsules were analyzed by this equation. At the initial stage ($\sim 200\text{min}$), the release was fitted quite well by this equation, indicating that the drug release behaviors obeys a diffusion controlled rules [92-93].

For mechanistic mathematical models, their analysis of drugs release is often based on theories of Fick's law of diffusion. Generally, LbL polyelectrolyte microcapsules can be regarded as membrane-controlled release system. After assuming no swelling or dissolution of the membranes, availability of perfect sink condition and constant drugs permeability through the barriers membrane, Antipov et al developed a mechanistic model based on Fick's law to study the fluorescein release from PAH/PSS microcapsules. They built an equation as $(dC/dt) \times V_o = J = P(c_i - c_e)S$ where P is the permeability coefficient, V_o (cm^3) is the volume of solution, J is the fluorescein flux through the capsules wall with the surface area of S (cm^2) and $(c_i - c_e)$ is the difference of the fluorescein concentration inside (c_i) (mg/ml) and outside (c_e) (mg/ml) the capsules. At the initial stage of release, $c_i \gg c_e$ and thus $c_i - c_e$ can be equal to c_i and the flux is steady. Consequently, the concentration increment in time (dC/dt) is the slope of linear part of the release curves and the permeability coefficient (P) can be obtained from the slop of the linear part. By using this model, a

series of studies concerning IBU release against different polyelectrolytes layer numbers, different polyelectrolytes components, different release conditions (pH and ionic strength) were conducted. Informative parameters like effective diffusion coefficient of multilayer polyelectrolytes film were obtained [94-96].

Chapter 3 Materials and Methods

Materials

Fluorescein isothiocyanate labeled dextran (FITC-dextran) (4000Da), FITC-dextran (70,000Da), poly-l-arginine hydrochloride (PAG) (70,000~130,000Da), poly-l-lysine hydrobromide (PLL) (30,000–70,000 Da), 25% glutaraldehyde solution were purchased from Sigma–Aldrich. Dextran sulfate (DXS) (36,000~50,000Da) was purchased from MP Biomedicals. Poly (*dl*-lactide-*co*-glycolide) 53:47 (PLGA) (IV=1.05) was obtained from Purac Biochem (Gorinchem, Netherlands) and used as received. Poly (allylamine hydrochloride) (PAH) (average M_w ~56,000), poly (styrene sulfonate) (PSS) (average M_w ~70,000), Ibuprofen, Nile red and Coumarin6 were purchased from Sigma-Aldrich.

Metoclopramide monohydrochloride (Meto-salt) is from Sigma, and metoclopramide base (Meto-base) is converted from Meto-salt by adding equal mole of NaOH in the lab. Briefly, Meto-salt was dissolved in DI H₂O and kept stirring until fully dissolved. NaOH solution was added into the Meto-salt solution dropwise. White precipitate (Meto-base) will appear immediately. Equal amounts of NaOH were added and keep stirring until no white precipitate can be observed. The white precipitate were then separated from the solution using filtration and washed with DI H₂O until the filtrate has a neutral pH value. The Meto-base was dried under vacuum and kept at room temperature for

further use.

FITC tagged PAH was carried out according to the following protocol. 100mg PAH was dissolved in an aqueous solution of 1M sodium hydrogencarbonate and mixed with concentrated solution of FITC in methanol. The reaction was performed at room temperature under stirring for 6h. The labeled polymer was purified by dialysis (exclusion MW 15 000g/mol) until no FITC monomers were detectable in the washed water by fluorescence spectroscopy. The dialyzed polymer solution was freeze dried and kept at -20°C for further use.

Methods

(1) Preparation of PAG/DES multilayer microcapsules based on FITC-dextran loaded RBCs ghosts

RBCs ghosts represent empty RBCs where most of hemoglobin is removed via first adding hypotonic buffer and then healing with isotonic buffer. The healing process is like the following: the swelling RBC where hemoglobin are removed out are collected and washed with 1X PBS (isotonic buffer). The RBC ghosts are then separated from the washing buffer through centrifuging at 4,000×g for 5 min. The collected ghosts are then dispersed in 1ml isotonic buffer and incubated at 37°C for 1h to allow the RBC membrane to reseal. The whole process (loading and coating) can be explained with the following figure **(Figure 11)**.

Figure 11 Schematic illustration showing the preparation of RBCs ghosts, loading of FITC-dextran into the RBCs ghosts and LbL coating of polyelectrolyte (PAG/DXS) onto the pre-loaded RBCs ghosts

The above process involves the following steps:

(a) Preparation of RBCs ghosts

Blood samples were withdrawn by venipuncture from healthy volunteers, with informed consent approved according to the procedures of the institutional reviewer board, and transferred to ethylene diamine tetraacetic acid (EDTA) coated tubes. After centrifugation at $1800\times g$ for 10 min at 4°C , the plasma and buffy coat was removed and the remaining packed RBCs were washed three times with isotonic PBS (1XPBS). 1 ml of packed RBCs were washed twice in 10ml of 150 mOsm/kg hypotonic PBS via centrifugation at $20,000\times g$ for 10 min at 4°C followed by another three washings of the ghost membrane pellet in 10 ml of 100mOsm/kg hypotonic PBS. The resulting ghost

membranes were then dispersed in isotonic PBS and incubated at 37°C for 1 h to allow the membranes to reseal.

The morphology and integrity of RBCs ghosts were investigated using scanning electron microscopy (JSM-5310, Jeol Ltd. Japan). Standard procedure for preparing the samples for SEM observation was employed. In brief, one drop of the native RBCs or RBCs ghosts suspension were put onto a clean silicon wafer and dried at 37°C and coated with a fine gold film and observed under 5kV accelerating voltage.

(b) Loading of FITC-dextran into RBCs ghosts

The RBCs ghosts were re-dispersed in 50 mOsm/kg PBS containing FITC-dextran at the appropriate concentration and transferred into dialysis bags with 500~1000 Da molecular mass cutoff for FITC-dextran. The RBCs ghosts were then dialyzed against 30mOsm/kg hypotonic PBS for 2h at room temperature in order to facilitate loading of the FITC-dextran. Subsequently, the 30mOsm/kg PBS was changed to isotonic PBS and dialyzed for another 2h at 37°C to reseal ghosts. Thereafter, the loaded vesicles were washed 4 times with isotonic PBS.

In order to quantitatively study the amounts of FITC-dextran loaded into RBCs ghosts, aliquots of the loaded vesicles were dispensed into 1.5ml Eppendorf tubes and 1% Triton X-100 was added to completely lyse the loaded RBCs ghosts. The supernatant was placed into black 96 well plates and the intensity of each well was read at an excitation and emission wavelength of 490

and 520 nm, respectively, using a UV spectrofluorophotometer microplate reader (Perkin Elmer Victor 1420).

In order to investigate the FITC-dextran distribution within the RBCs ghost, confocal laser scanning microscope (CLSM) was used. The 200ug fed RBCs ghosts were suspended in 1ml 1X PBS and mixed thoroughly. 200ul of the loaded RBCs ghosts were then transferred to the 8 well chamber slides (ibidi, Singapore) and let the loaded RBCs ghosts settle down for 10min. The chamber slides were then loaded onto CLSM (Leica, Singapore) and observed with 60X oil objective. The excitation wavelength is 488nm and the emission wavelength is set to green light (520nm). In order to know the distribution of FITC-dextran within the RBCs ghosts, CLSM pictures of the loaded RBCs ghosts were taken in 15 planes separated by 0.5 μm along with the z-axis from the bottom part to the top layers of the carriers.

(c) Coating of polyelectrolytes onto drug loaded RBCs ghosts

Prior to the LbL polyelectrolyte coating, the loaded RBCs ghosts were fixed with 2.5% glutaraldehyde solution for 1h at 4°C. For the LbL coating, the fixed RBCs ghosts were added to a solution containing 0.5mg/ml of the respective polyelectrolyte in 0.1M NaCl. After incubating the vesicles with the polyelectrolyte solution for 10 min at room temperature, they were washed twice in isotonic PBS to remove non-adsorbed polyelectrolyte before coating the vesicles with the next polyelectrolyte layer. LbL consecutive adsorption of positively and negatively charged polyelectrolytes was carried out by repeating

these steps in sequence until the desired layer number was reached.

In order to monitor the layering process, the electrophoretic mobility of the bare and coated vesicles was measured using a Malvern Zetasizer (Malvern Zetasizer Nano, UK). The electrophoretic mobility was converted into the respective ζ -potential using the Smoluchowski relationship.

The bare loaded RBCs ghosts were incubated with 1ml of PLL (dissolved in 0.5M NaCl) for 10min at room temperature, the supernatant was then collected by separating the RBCs ghosts with centrifugation. The supernatant was then stored in 4°C before testing with fluorescence spectrometer. The PLL coated RBCs ghosts were then washed with 0.5M NaCl and the supernatant was also stored in 4°C before testing. The same protocol was repeated for DXS coating until desired layer numbers are achieved. The collected supernatants were then tested with fluorescence spectrometer and the FITC-dextran amounts are calculated to know the lost amounts of FITC-dextran during coating and washing steps

(d) Release of FITC-dextran from the LbL microcapsules

The release of FITC-dextran was studied and compared between bare and LbL polyelectrolyte coated RBCs ghosts. For this purpose, equal amounts of coated and uncoated RBCs ghosts were added to 500 μ l isotonic PBS and kept at 37°C. At defined time intervals, the samples were centrifuged and 500 μ l of supernatant was collected to analyze for the fluorescence intensity and the ghosts were re-suspended in 500 μ l of fresh isotonic PBS. The collected

supernatants were stored in 4°C and tested with fluorescence spectrometer with excitation at 490nm and emission at 520nm. 200ul of the supernatant was transferred into 96 well black plates and each sample was tested for three times. The total release time is 300h.

2. Preparation of PLL/DES multilayer microcapsules based on RITC-lysozyme loaded intact RBCs

(a) Isolation of erythrocytes

Blood samples were withdrawn by venipuncture from healthy volunteers, with informed consent approved according to the procedures of the institutional review board of Nanyang Technological University (NTU) and transferred to EDTA coated tubes. After centrifugation at 1800g for 10 min at 4°C, the plasma and buffy coat were removed and the remaining packed erythrocytes were washed three times with isotonic PBS.

(b) Loading of erythrocyte

The isolated erythrocyte were re-dispersed in 50 mOsm/kg PBS containing RITC-lysozyme at the appropriate concentrations and transferred into dialysis bags with 5000~10,000 Da molecular mass cut off. The whole set were then dialyzed against 30 mOsm/kg PBS for 6h at 37°C. Subsequently, the 30 mOsm/kg PBS was changed to isotonic PBS and dialyzed for another 2h at 37°C to reseal the erythrocyte. Thereafter the loaded erythrocytes (i.e. erythrocyte carriers) were washed 4 times with isotonic PBS to separate them

from free, un-encapsulated macromolecules.

(c) Polymer coating of erythrocyte carriers

Prior to the layer-by-layer adsorption, the erythrocyte carriers were fixed with 0.125% glutaraldehyde solution for 5 min at 4°C. For the layer-by-layer adsorption, the fixed erythrocyte carriers were added to a solution containing 0.5mg/ml of the respective polyelectrolyte in 0.5M NaCl. After incubating the erythrocyte carriers with the polymer solution for 10 min at room temperature, they were washed twice with 0.5M NaCl to remove non-adsorbed polyelectrolyte before coating the erythrocyte carriers with the next polyelectrolyte layer. Layer-by-layer consecutive adsorption of positively and negatively charged polyelectrolyte was carried out by repeating these steps in sequence until the desired layer number was achieved.

(d) Characterization of coated erythrocyte carriers

In order to quantitatively study the amounts of RITC-lysozyme loaded into the erythrocyte carriers, aliquots of the erythrocyte carriers were lysed with water and the supernatant was placed into 96-microwell plates and the intensity was read using a UV spectrofluorophotometer microplate reader (Infinite 200, Tecan, Switzerland). For quantifying lost amounts of RITC-lysozyme from erythrocyte carriers during each polyelectrolyte coating and washing steps, the polyelectrolyte and NaCl supernatant was collected and tested for the RITC-lysozyme presented.

(e) Microscopic techniques

Confocal laser scanning microscope (CLSM) (Leica) was used to study the distribution of RITC-lysozyme within the erythrocyte carriers and polyelectrolyte multilayer coating. One drop of the RITC-lysozyme loaded and polyelectrolytes coated RBCs carriers were transferred into 8-well chamber slide. For observation of RITC-lysozyme, the excitation was set at 560nm and emission was set at 620nm. For observation of FITC-PAH coating onto the surface of RBCs carriers, the excitation is set at 488nm and emission wavelength is set at 520nm. Scanning electron microscopy (SEM) (JSM-5310, Jeol Ltd) was employed to study the morphology of polyelectrolyte coated erythrocyte carriers. Standard procedure for preparing the sample for SEM was employed. In brief, one drop of the sample suspension was put onto a clean silicon wafer and dried at 37°C and coated with a fine gold film and observed under a 5kV accelerating voltage.

(f) Electrophoretic mobility measurement

In order to monitor the layering process, the electrophoretic mobilities of the bare and coated erythrocyte carriers were measured using a Malvern Zetasizer (Malvern Zetasizer Nano, UK). The electrophoretic mobility was converted into the respective ζ -potentials using the Smoluchowski relationship.

(3) Preparation of LbL PLGA/polyelectrolyte hybrid microcapsules for encapsulation of hydrophobic drugs

In order to enable the LbL polyelectrolyte microcapsules for encapsulation of hydrophobic compounds, PLGA which is a good candidate for hydrophobic drug encapsulation and delivery is introduced here to form PLGA/polyelectrolyte hybrid microcapsules. The process is illustrated in the following figure (**Figure 12**).

Figure 12 Schematic illustration showing the preparation procedure of LbL PLGA/polyelectrolyte hybrid microcapsules for encapsulation of hydrophobic drugs, where porous CaCO_3 microparticles are first coated with PLGA layer containing hydrophobic drugs using PLGA *in situ* precipitation technique, the PLGA coated CaCO_3 microparticles are further coated with multilayer polyelectrolytes to control the drug release kinetics.

The above procedure can be explained in details by the following steps:

(a) Preparation of porous CaCO_3 microparticles

CaCO_3 microparticles were prepared by colloidal crystallization from supersaturated solution [7]. Briefly, 10ml 0.33M Na_2CO_3 solution was quickly mixed with an equal volume of 0.33M CaCl_2 solution at room temperature under intense agitation on a magnetic stirrer. After stirring for 5min, the precipitate (CaCO_3 microparticles) was filtered off and washed with H_2O for 3 times. Following which, the precipitate was washed using absolute ethanol for two times and dried under vacuum and stored at room temperature for further use [7]. SEM was used to investigate the surface morphology of CaCO_3 microparticles. One drop of CaCO_3 ethanol suspension was put onto a clean silicon wafer and dried under 37°C vacuum for 30min. The CaCO_3 adhered silicon wafer was coated with Pt and observed under SEM with a 5Kv accelerating voltage. In order to see the internal structure of the CaCO_3 microparticles, the as-prepared CaCO_3 microparticles were suspended in acetone and treated with probe ultrasonic for 5min under ice bath.

(b) Coating CaCO_3 microparticles with PLGA layer containing hydrophobic drugs

Hydrophobic compounds like Nile red, Coumarin 6, ibuprofen and Meto-base are dissolved in NMP or acetone where PLGA is also dissolved. 2ml of PLGA/hydrophobic compounds NMP or acetone solution is incubated with 10mg CaCO_3 microparticles under stirring for ~1h. The particles immobilized

with PLGA/hydrophobic compounds NMP or acetone solution were separated by centrifugation and exposed to 0.02% PVA solution. Due to the diffusion of NMP or acetone to H₂O, PLGA precipitates to form a solidified matrix containing the hydrophobic compounds. The PLGA coated CaCO₃ microparticles were then mixed with 0.1M HCl for 1-2 min, bubbles will produce due to the reaction between CaCO₃ and HCl. The PLGA microcapsules were then separated from the supernatant by centrifugation at 5000 ×g for 5 min. The PLGA microcapsules were then washed with 0.01% PVA solution for 2 times. PLGA microcapsules loaded with Coumarin6 were observed using CLSM. 200μl PLGA microcapsules suspensions were transferred into 8 well chamber slide and observed at excitation 488nm and emission at 520nm.

The PLGA microcapsules were also observed with SEM and atomic force microscope (AFM) for their surface morphology. For SEM observation, one drop of the PLGA microcapsules suspension in H₂O was put onto clean silicon wafer and dried under vacuum for 30min. The PLGA microcapsules adhered silicon wafer was coated with Pt and observed under SEM with 5Kv accelerating voltage. Preparation of samples for AFM observation is the same like that for SEM observation except the Pt coating step. Tapping mode of AFM was used to obtain the images. For surface roughness analysis, the arithmetic average roughness Ra was calculated according to the equipment attached software.

(c) Coating polyelectrolytes (PAH/PSS) onto PLGA coated CaCO₃

microparticles

The above obtained CaCO_3 microparticles covered with solidified PLGA layer containing hydrophobic compounds are further coated with multilayer polyelectrolytes. For the coating, the microparticles are added to PSS solution (1mg/ml in 0.5 M NaCl) for 10 min at room temperature. Subsequently, the microparticles are separated by centrifugation and washed with 0.5M NaCl twice. Following that, the microparticles are coated with PAH with the same conditions used for PSS coating. LbL consecutive adsorption of PAH and PSS is carried out by repeating these steps in sequence until the desired layer number is achieved.

(d) Drug release from the hybrid microcapsules

The release behaviors of ibuprofen (IBU) or meto-base from the hybrid microcapsules is carried out in 2ml PBS (pH 7.2) at 37°C under constant shaking. After defined time intervals, the samples were centrifuged and the supernatant was collected and replaced by fresh PBS. The drug release amounts are analyzed using UV spectroscopy.

Besides loading of hydrophobic compounds, the interior of the hybrid microcapsules can also be loaded with hydrophilic substance (FITC-dextran) and magnetic nanoparticles, which make the microcapsule to be multifunctional. Formation of such multifunctional microcapsules can be referred to the following figure (**Figure 13**).

Figure 13 Schematic illustration showing the preparation of multifunctional hybrid microcapsules via combining PLGA *in situ* precipitation technique and LbL polyelectrolyte deposition (I) NMP or acetone solution containing PLGA and hydrophobic compounds infiltrated onto CaCO_3 template (II) The loaded microparticles are exposed to H_2O , which results in precipitation of PLGA containing hydrophobic compounds. (III) Further LbL polyelectrolyte coating to control the compounds release profiles. (IV) CaCO_3 template cores were removed to produce hollow capsules. (V) Interior of the capsules can also be pre-filled with hydrophilic compounds (FITC-Dextran, denoted in green) and inorganic nanoparticles (Fe_3O_4 denoted in black dot) via encapsulating these substances into CaCO_3 template core beforehand, thus produce multifunctional microcapsules.

In order to pre-load FITC-dextran into CaCO_3 microparticles, co-crystallization method is used. Briefly, 10ml 0.33M Na_2CO_3 solution was rapidly poured into an equal volume of 0.33M CaCl_2 solution containing 5mg FITC-dextran at room temperature under intense agitation on a magnetic stirrer [7]. After stirring for 5min, the precipitate (CaCO_3 microparticles loaded with FITC-dextran) was filtered off and washed with H_2O for 3 times. Following which, the precipitate was washed using absolute ethanol for two times and dried under vacuum and stored at room temperature for further use. In order to investigate the FITC-dextran distribution within the CaCO_3 microparticles, CLSM was used. 5mg of FITC-dextran loaded CaCO_3 microparticles were suspended in absolute ethanol and then one drop of the suspension was put onto the a cover slip and dried for 10min in 37°C oven. The cover slip was then loaded onto CLSM (Lecia, Singapore) and observed using 60 x oil objectives. The excitation wave length is 488nm and emission wavelength is 520nm. Along the z-axis, 8 planes from the bottom to the middle of the CaCO_3 microparticles were made to see the FITC-dextran distribution within the microparticles. Fe_3O_4 can be loaded into the CaCO_3 microparticle via incubating the Fe_3O_4 nanoparticles suspension with the dried porous CaCO_3 microparticles for 30 min [43, 46]. Briefly, 10mg of 5-10nm Fe_3O_4 nanoparticles (from Sigma-Aldrich) were suspended in 1ml 0.05mg/ml PSS solution and then incubated with 20mg dried porous CaCO_3 microparticles for 30min at room temperature. The mixture was then

centrifuged at $500\times g$ to settle down the Fe_3O_4 attached CaCO_3 microparticles. The CaCO_3 microparticles were then washed with DI H_2O and centrifuge to discard the supernatant. The wash and centrifuge were repeated two times. The CaCO_3 microparticles were then coated with 1mg/ml PSS solution for 10-15min, washed with 0.5M NaCl solution. Following that, the PSS coated $\text{Fe}_3\text{O}_4/\text{CaCO}_3$ microparticles were coated with 1mg/ml PAH solution, washed with 0.5M NaCl solution. These steps were repeated until desired numbers of polyelectrolyte layers are reached.

Chapter 4 Results and Discussion

4. Results and Discussion

4.1 LbL polyelectrolyte microcapsules with pre-loaded RBCs ghosts as template

The process for the preparation of LbL polyelectrolyte microcapsules with FITC-dextran loaded RBCs ghosts as template core is shown in **Figure 11**.

4.1.1 Preparation of RBCs ghosts

RBCs ghosts refer to re-sealed RBCs membrane after partial or complete removal of hemoglobin from the native RBCs. RBCs ghosts can be likely regarded as natural empty microcapsules consisting of lipids, proteins and polysaccharides [97].

Figure 14 shows scanning electron microscopy (SEM) images of native RBCs (**Figure 14a**) and RBCs ghosts treated with hypotonic buffers having different osmotic pressure (**Figure 14b, c and d**). The native RBCs show the typical discoid shape and have a flat surface (**Figure 14a**). In contrast, RBCs ghosts appear flat with several folds and crease on the surface (**Figure 14b and c**).

In order to investigate the effects of the osmolality on the vesicle shape and integrity, RBCs were also suspended in hypotonic PBS having a much lower osmolality of 20mOsm/kg followed by resealing in isotonic PBS. As shown in **Figure 14d**, most of these RBCs ghost vesicles cannot retain their shape and integrity, it can thus be concluded that when the osmolality is

reduced below a certain limit (20mOsm/kg), irreversible ruptures and openings of the membrane will occur.

Hypotonic solution based technique for preparation of resealed RBCs ghosts may be first invented by Dodge, Mitchell and Hanahan in 1963. The encapsulation of substances in RBC by hypotonic dialysis may be first proposed by Deloach and Ihler in 1977. However, the exact mechanism for the open and resealing of the RBCs ghosts membrane remained unknown and even has some controversial observations. The most widely cited mechanism may be the reversible pores opening and closing.

Cell membrane of RBCs mainly consisted of cytoskeleton proteins and lipid bilayer. When the RBCs are treated with hypotonic buffers having certain osmolarity, osmotic shock of the buffers induces cell swelling and formation of pores in the lipid bilayer, without causing lysis where cytoskeleton proteins are disrupted. The presence of pores allows the release of hemoglobin and encapsulating agents to be incorporated inside the RBCs. When normal condition of osmotic pressure are reinstated by putting the RBCs to isotonic buffer, the open lipid bilayers gain the ability to form closed vesicles spontaneously and reseal the open pores. When RBCs are treated with hypotonic buffer having lower osmolarity, the excessive swellings causes disruption of the cytoskeleton protein and result in irreversible rupture of the cell membrane as seen in Figure 14d, since proteins lack the ability to form closed vesicles in aqueous solution like lipid bilayer.

Figure 14 SEM investigation of morphology for RBCs and RBCs ghosts (a) native RBCs showing typical biconcave discoid shape with a size of around 7 μm; (b) re-sealed RBCs ghosts showing a flat surface and crease; (c) enlarged view of b; (d) poorly re-sealed RBCs ghosts showing ruptured and discrete membrane.

4.1.2 Loading of FITC-dextran into RBCs ghosts

FITC-dextran was loaded into RBCs ghosts as model compounds via hypotonic dialysis method as schematically illustrated in **Figure 11**. SEM image of the loaded vesicles shows that these loaded RBCs ghosts still resemble to the original shape (**Figure 14b** and **Figure 15a**), indicating that the loading procedure does not induce morphological changes, which is important for the

subsequent LbL polyelectrolyte coating step. The fluorescence microscopy picture also confirms the successful loading of FITC-dextran into the RBCs ghosts, where RBCs ghosts are fully filled with the green compounds (FITC-dextran) (**Figure 15b**).

Figure 15 SEM and CLSM investigation for the loading of FITC-dextran into re-sealed RBCs ghosts (a) SEM observation showing the similarity between the FITC-dextran loaded and unloaded RBCs ghosts; (b) CLSM observation confirming the successful loading of green FITC-dextran into the RBCs ghosts.

In order to know the distribution of FITC-dextran within the RBCs ghosts, confocal laser scanning microscopy (CLSM) pictures of the loaded carriers were taken in 15 planes separated by $0.5\ \mu\text{m}$ (**Figure 16**). At each plane, especially the planes from $2.5\ \mu\text{m}$ to $4.5\ \mu\text{m}$ which corresponding to the middle parts of the loaded RBCs ghosts, most of the vesicles are completely and uniformly filled with green FITC-dextran, confirming the successful encapsulation of these macromolecules.

Figure 16 Different planes of CLSM pictures (separated by 0.5 μm) showing the FITC-dextran distribution within the RBCs ghosts. Scale bar = 5 μm

Table 2 summarizes the encapsulation efficiency (EE) and the loading amounts (LA) with the EE denoting the percentage ratio of the loaded FITC-dextran to the initially added amounts, whereas the LA corresponds to the total amounts of FITC-dextran encapsulated. The EE shows only small variations independent on the initial feed amounts of 28~31%. Consequently, the loaded amounts increase steadily with the increasing feed amounts (**Table 2**). It should be noted that the EE is in the similar range as reported in other studies where RBCs ghosts were used to encapsulate other agents like enzymes, peptides, proteins, polysaccharides and DNA [70].

Table 2 Encapsulation efficiency and loading amounts of FITC-dextran encapsulation into RBCs ghosts using hypotonic dialysis method

Initial Feeding (μg)	EE (%)	LA (pg/cell)
200	27.9 $\pm$ 1.6	12.08
400	30.9 $\pm$ 2.3	22.41
600	31.1 $\pm$ 0.4	39.11

4.1.3 Coating of polyelectrolytes onto RBCs ghosts

Considering the potential biomedical application of these LbL polyelectrolyte microcapsules, only biopolymers including polypeptides (PAG) and polysaccharides (DXS) were used as the coating materials, since these polymers are widely regarded as biocompatible and biodegradable [98]. It should be noted that RBCs ghosts are rather soft and fragile, thus to avoid breakage of these vesicles due to coagulation and aggregation during the LbL deposition of polyelectrolytes, they were stabilized via glutaraldehyde fixation prior to the polyelectrolytes coating and gently re-suspended and centrifuged after each coating step. To monitor the polyelectrolytes coating process, the ζ -potential was recorded after each coating step. As shown in **Figure 17**, RBCs ghosts have a negative ζ -potential of -50mV, which changes to a positive value of +38mV after the deposition of the first cationic polyelectrolyte layer (PAG). This indicates that the surface charge of the RBCs ghosts is overcompensated by the PAG molecules. The charge overcompensation resulting from PAG facilitates the subsequent deposition of anionic polyelectrolytes layer (DXS). From **Figure 17**, a net reversal of surface charge between positive and negative

values is evident for each polyelectrolytes layer, suggesting the successful coating of polyelectrolytes onto the RBCs ghosts.

Figure 17 Change of ζ -potential during the polyelectrolyte coating process (data points represent mean $\pm$ standard deviation of data from three independent samples)

To quantify the amounts of the remaining FITC-dextran in the RBCs ghosts after the LbL polyelectrolyte coating procedure, the amounts of FITC-dextran in the supernatant after the each coating steps were quantified. The **Figure 18** shows the relative amounts of FITC-dextran left inside the RBCs ghosts after layering, with 100% denoting the amounts presenting in the RBCs ghosts before coating. It is noted that the remaining FITC-dextran is in the range of 35-39%, which is as the actual loading amounts for the later release studies.

Figure 18 Amounts of FITC-dextran remaining in the RBCs ghosts after coating of polyelectrolyte with different numbers of layers (data points represent mean $\pm$ standard deviation of data from three independent samples)

4.1.4 Release of FITC-dextran from LbL polyelectrolyte microcapsules

The above results indicate the successful formation of LbL polyelectrolyte microcapsules with pre-loaded RBCs ghosts as template for the encapsulation of FITC-dextran, where cores dissolution and compounds post-loading for the traditional LbL polyelectrolyte microcapsules can be eliminated. The next step is to study the release profiles of the encapsulated FITC-dextran. **Figure 19** shows the FITC-dextran release from bare and LbL polyelectrolyte coated RBCs ghosts.

Figure 19 FITC-dextran release kinetics of RBCs ghosts coated with different numbers of polyelectrolyte layers (data points represent mean $\pm$ standard deviation of data from three independent samples)

Within the initial 2h, approximately 40% of the FITC-dextran is released from uncoated ghosts followed by a significant reduction of the release rate, with about another 43% of the FITC-dextran being released within the next 300h (**Figure 19**). This release behavior is very similar to that of other nano- or microparticles delivery systems based on polymeric matrices, where a burst release is usually followed by a slower release. In many cases, the burst release is undesirable, as it may lead to toxicity or wastage of the encapsulated agents.

On the other hand, the LbL polyelectrolyte coating of the RBCs ghosts suppresses the initial burst release of FITC-dextran with only three

polyelectrolytes layers as shown in **Figure 19**. Since the FITC-dextran release was determined in the same buffer as used for the polyelectrolyte deposition, it can be concluded that a tight polyelectrolyte complex between PAG and DXS is formed that blocks the FITC-dextran release. Due to this retardation, which is primarily a partitioning effect coupled to diffusion, only 39% of the FITC-dextran has been released after ~290h from the vesicles coated with 3 polyelectrolytes layers. For 5, 7, and 9 layer-coated vesicles, the FITC-dextran release is further reduced to 30%, 25% and 21% during the same time interval. The *in vitro* release behavior demonstrates that controlled release can be achieved simply by adjusting the layer numbers (**Figure 19**).

The un-coated RBCs ghosts can be considered as monolithic solution/dispersion system, while the 3, 5, 7, and 9 layers of polyelectrolytes coated RBCs ghosts can be considered as membrane-controlled reservoir system. For the membrane-controlled reservoir systems, the release patterns can be fitted with the following equation (Eq. 1) [103-104], when the drug loading amounts are below its solubility limit in the reservoir (also named as reservoir devices with non-constant activity source) [103-104].

$$M_t = M_o \left[1 - \exp \left(-\frac{ADKt}{Vl} \right) \right] \quad \text{Eq. 1}$$

Where A (cm^2) is the surface area, D is the diffusion coefficient (cm^2/sec), K is partition coefficient, V (cm^3) is the volume and l is the membrane thickness (nm). DK can be replaced with P (P is termed as

permeability of the membrane, $P = DK$).

For the coated RBCs ghosts, the FITC-dextran loading amounts (12~30pg/cell) here is far below the solubility limit of FITC-dextran in the RBCs ghosts reservoir (50 mg/ml). Consequently, the release profiles of 3, 5 7 and 9 layers of polyelectrolytes coated RBCs ghosts were fitted with the above equation (Eq. 1), the R^2 values and DK values were summarized in **Table 3**. It was seen that very good agreement between the theoretical and experimental curves can be obtained (**Figure 20b**) using Eq. 1.

For monolithic devices (un-coated RBCs ghosts), the release pattern was fitted with Peppas equation (Eq. 2) [91].

$$M_t/M_\infty = kt^n \quad \text{Eq. 2}$$

Here, M_t (mg) is the accumulated released drug amounts at time t (h), M_∞ (mg) is the released drug amounts at infinite time, k is a constant relating to the properties of drugs, structural and geometric characteristics of the devices and n is the release exponent which characterizes the different release mechanisms.

Peppas equation (Eq. 2) has been used to describe the drug release from discs, cylinder, slabs and spheres (monolithic devices), regardless of the release mechanisms [91]. For discs-shaped carriers, the Fickian diffusion mechanism is described by $0.43 < n < 0.5$.

The un-coated RBCs ghosts here are typically discs-shaped ($2a = 7\mu\text{m}$ and l is about $0.5\mu\text{m}$), characterized by $2a/l \gg 1$ (Figure 14). The FITC-dextran

release from the un-coated RBCs ghosts is fitted by the Peppas equation (**Figure 20a**). For comparison, the 3, 5, 7 and 9 layers of polyelectrolytes coated carriers were also fitted by this equation (Eq. 2) and R^2 are summarized in **Table 3**.

Figure 20 Fitting curves of FITC-dextran release from different layers of polyelectrolytes coated microcapsules using (a) Peppas equation (b) equation 1.

Table 3 Summarized R^2 values for Eq. 1 and Eq. 2 fitting of FITC-dextran release

Layer No.	R^2		DK
	Eq. 1	Eq. 2	Eq.1
0	0.85	0.99	/
3	0.96	0.95	$0.007rl_3$
5	0.97	0.93	$0.007rl_5$
7	0.97	0.96	$0.007rl_7$
9	0.98	0.95	$0.007rl_9$

Notes: Eq. 1 is $M_t = M_o \left[1 - \exp\left(-\frac{ADKt}{Vl}\right) \right]$; r is radius of RBCs and l is the layer thickness in Eq.1.

Eq. 2 is $M_t/M_\infty = kt^n$ (Peppas equation)

It was found that the FITC-dextran release from the un-coated RBCs ghosts can be ideally fitted with Peppas equation (Eq. 2) with a R^2 value at 0.992, while Peppas equation cannot describe the release of coated RBCs ghosts with reasonable R^2 values (**Table 3**). In contrast, the equation 1, which is used for describing the solute release from membrane-controlled reservoir system, has poor fitting for the un-coated RBCs ghosts ($R^2 = 0.85$). On the other hand, for the 3, 5 7 and 9 layers of polyelectrolyte coated RBCs ghosts, the equation 1 (Eq. 1) has better fitting than Peppas equation does (**Table 3**), indicating the membrane-controlled release of FITC-dextran from the coated

RBCs ghosts. The “DK” values extracted from Eq. 1 is constantly $0.007rl_{\#}$, where the single variable is the layer thickness, further indicating the membrane-controlled release behaviors. The good agreement between the fitting and experimental results for the polyelectrolytes coated RBCs ghosts can indicate that Eq. 1 is valid for the analysis of the release profiles and the FITC-dextran release is primarily controlled via its diffusion through the intact polyelectrolyte membrane.

In addition, the shell resistance to surfactant treatment was investigated by exposing the un-coated RBCs ghosts and LbL microcapsules to Triton X-100. 100% FITC-dextran release is observed after the treatment of un-coated ghosts, indicating complete membrane lysis. However, for the coated ones, the treatment cannot induce a complete release of FITC-dextran. Furthermore, with the increase of layer numbers, less FITC-dextran releases out. These results suggest that the deposited polyelectrolytes layer can effectively resist penetration of Triton X-100 and thus protect the cores from decomposition. This is an important feature in that it demonstrates that this method helps to preserve the bioactivity of the loaded compounds.

4.2 LbL polyelectrolyte microcapsules with pre-loaded intact RBCs as templates

Figure 21 Schematic illustration showing the loading of RITC-lysozyme into the intact RBCs via directly subjecting intact RBCs into RITC-lysozyme containing hypotonic dialysis buffer and the LbL coating of polyelectrolytes (PLL/DXS) onto the pre-loaded intact RBCs

In the first study, most of the hemoglobin of RBCs are removed out (such vesicles are called RBCs ghosts) before loading compounds (**Figure 11**). In order to further simplify the loading procedure, intact RBCs are directly subjected to hypotonic loading process here (**Figure 21**). Most of the previous studies had confirmed the successful loading of various water soluble substances into RBCs using this method [66-68]. It was also regarded as an improvement compared to loading compounds into RBCs ghosts, because this method is simple, effective, and can maintain the original shape of RBCs which is convenient for the following LbL polyelectrolyte coating steps. Here, RITC-lysozyme is loaded into intact RBCs which are subsequently coated with different layer numbers of polyelectrolytes (**Figure 21**). The loading of

RITC-lysozyme, coating of polyelectrolyte and the release of RITC-lysozyme against different layer numbers were studied.

4.2.1 Loading of RITC-lysozyme into intact RBCs

RITC-lysozyme was loaded into intact RBCs via the hypotonic dialysis method as illustrated in **Figure 21**. As seen from the SEM images, the hypotonic dialysis loading method for RITC-lysozyme encapsulation results in some morphological/topological changes of RBCs (**Figure 22**). However, the changes are much fewer than the previous study where RBCs ghost have a completely different morphology from the native RBCs (**Figure 14**). In this improved loading method, the unloaded normal RBCs have the typical biconcave discoid shape (**Figure 22**) while the loaded ones have a flatter surface.

Figure 22 SEM investigation of morphology for native RBCs and RBCs loaded with RITC-lysozyme via direct hypotonic dialysis loading (a) native RBCs showing typical biconcave discoid shape with a size of around 7 μm ; (b) RITC-lysozyme loaded RBCs showing a flatter surface

In order to visualize the distribution of RITC-lysozyme within the RBCs, CLSM pictures of the loaded RBCs were taken in several planes. As shown in **Figure 23**, most of the RBCs are completely and uniformly filled with red RITC-lysozyme. Moreover, as indicated by the CLSM, considerable encapsulation efficiency (41.6%) and loading amounts (12.7 pg/cell) is achieved. Subjecting the intact RBCs instead of re-sealed RBCs ghosts to hypotonic dialysis loading process retains the hemoglobin and membrane components, which helps to preserve the morphological characteristics of native RBCs. Consequently, such RBCs drug carriers that closely resemble native RBCs could serve as improved template cores for the LbL polyelectrolytes deposition.

Figure 23 Different planes of CLSM pictures showing the RITC-lysozyme distribution within the loaded RBCs cores

4.2.2 Coating polyelectrolytes onto the RITC-lysozyme loaded RBCs

ζ -potential was recorded after each coating to monitor the polyelectrolyte layering process (**Figure 24**). RBCs have a negative ζ -potential of about -60mV, which changes to positive value of +42mV after deposition of PLL. After depositing anionic DES, the ζ -potential becomes negative value of -65mV. From **Figure 24**, a net reversal of surface charge between positive and negative values is evident for each polyelectrolyte layer added.

Figure 24 Change of ζ -potential during the process of coating positive and negative polyelectrolyte onto the RITC-lysozyme loaded RBCs (data points represent mean $\pm$ standard deviation of data from three independent samples)

Besides monitoring the ζ -potential alternation, CLSM was also used to confirm the successful coating of polyelectrolyte layers onto the RITC-lysozyme loaded RBCs (**Figure 25**). After coating the RITC-lysozyme loaded RBCs with 3 layers of PLL/DES with negative DES as the outmost coated layer, a positive fluorescein isothiocyanate labeled poly (allyamine hydrochloride) (FITC-PAH) was coated onto the outmost layer. The spherical shape of RITC-lysozyme loaded RBCs, highlighted by the green fluorescence from the FITC-PAH adsorbed onto the walls indicate the successful coating of PLL/DES and retain of their spherical shape after the LbL coating of PLL and DES (**Figure 25**).

Figure 25 CLSM observation to investigate the loading of RITC-lysozyme into RBCs and the coating of PLL/DES onto the loaded RBCs (a) red channel of CLSM showing the loading of RITC-lysozyme and the maintenance of spherical shape of RBCs (b) green channel of CLSM showing the successful coating of PLL/DES onto the loaded RBCs where the green shell is visualized from the coated FITC-PAH

The amounts of hemoglobin and RITC-lysozyme lost during the polyelectrolytes coating process were also quantified (**Figure 26, 27**). **Figure 26** shows the hemoglobin contents in the supernatant after polyelectrolyte coating and washing, with 100% hemolysis denoting the hemoglobin contents of loaded RBCs, which were completely lysed. When polyelectrolyte coating was carried out on loaded RBCs with GA stabilization, negligible hemolysis was observed (**Figure 26**). In contrast, around 40% hemolysis occurred when only 2 layers of polyelectrolyte coating were applied onto loaded RBCs without GA stabilization, with increasing layer numbers, a higher degree of hemolysis was observed (**Figure 26**).

Figure 26 Percent hemolysis for the RITC-lysozyme loaded RBCs during the polyelectrolyte coating/washing process where 100% hemolysis denote the hemoglobin contents in the bare loaded RBCs completely lysed by pure H₂O (data points represent mean $\pm$ standard deviation of data from three independent samples)

(* $P < 0.05$ relative to erythrocyte carriers w/GA fixation)

Figure 27 illustrates the relative amounts of RITC-lysozyme left inside loaded RBCs after polyelectrolytes coating and washing, where 100% denotes the amounts of RITC-lysozyme in loaded RBCs before fixation and coating. For the fixed loaded RBCs, coating and washing did not result in a significant loss of RITC-lysozyme; almost 80% of the initial amounts remain within the loaded RBCs (**Figure 27**). However, for the unfixed ones, more than 75% of the loaded RITC-lysozyme was lost, most likely due to hemolysis of loaded RBCs.

Figure 27 Amounts of RITC-lysozyme remaining in the RBCs after coating of polyelectrolyte with different number of layers (data points represent mean $\pm$ standard deviation of data from three independent samples) (* < 0.05 relative to erythrocyte carriers w/o GA fixation)

Induction of hemolysis by polyelectrolytes, especially cationic polyelectrolyte has been frequently observed in previous researches. When a positive polyelectrolyte approaches the negatively charged RBCs surface, it experiences an attractive force and is thus bound to the RBCs membrane. This in turn perturbs the membrane integrity and impairs the connection between the

cytoskeleton and lipid bilayer, thereby inducing pore formation and hemolysis. This also explains the major loss of RITC-lysozyme from unfixed loaded RBCs after polyelectrolytes coating (**Figure 27**). Consequently, RITC-lysozyme loaded RBCs were fixed with glutaraldehyde prior to polyelectrolytes coating.

4.2.3 Release of RITC-lysozyme from LbL microcapsules

Figure 28 shows the RITC-lysozyme release from uncoated and polyelectrolytes coated RBCs. Within the initial 8h, about 65% of the RITC-lysozyme is released from the uncoated RBCs followed by significant reduction of release rate, with about 20% of the RITC-lysozyme being released within the next 140h. In contrast, the coated RBCs suppress the initial burst release of RITC-lysozyme down to 20% even with only 2 polyelectrolytes layers. For carriers coated with 6 layers, the RITC-lysozyme burst release is further reduces to about 10%. Besides suppressing the burst release, the RITC-lysozyme release after ~148h from the coated carriers was also lower than the uncoated ones, with about 13%, 8% and 6% reduction for carriers coated with 2, 4 and 6 layers of polyelectrolytes, respectively (**Figure 28**).

Figure 28 Release of RITC-lysozyme from RBC coated with different numbers of polyelectrolyte layers (data points represent mean $\pm$ standard deviation of data from three independent samples)

4.3 LbL polyelectrolyte/polyester hybrid microcapsules for encapsulation of hydrophobic drugs

In order to encapsulate hydrophobic drugs into LbL microcapsules, PLGA, which is a good candidate for hydrophobic drugs encapsulation is introduced here. By combining the PLGA *in situ* gelling technique and LbL polyelectrolytes deposition, Hybrid LbL microcapsules which are capable of loading hydrophobic drug were formed (See **Figure 12**). The whole process mainly involves the following steps: (1) Formation of porous CaCO_3 microparticles (2) Coating hydrophobic drugs loaded PLGA layer onto CaCO_3 microparticles (3) Further LbL coating of polyelectrolytes onto the PLGA coated CaCO_3 microparticles (4) Studying the release behaviors of hydrophobic drugs from the LbL hybrid microcapsules.

4.3.1 Formation of porous CaCO_3 microparticles

Study of crystallization of CaCO_3 from supersaturated solution is important in geo-/bio- and materials science. In addition, CaCO_3 nano-/micro-particles have found important applications in industry, technology, medicine and many other fields [99]. Generally, the direct mixing of soluble salt of Ca^{2+} and CO_3^{2-} results in an amorphous nano-precipitate instantly, which will transform into aggregated CaCO_3 microcrystals. The CaCO_3 microcrystals have three morphologies—calcite, aragonite and vaterite. Only vaterite are suitable templates for forming LbL polyelectrolyte microcapsules [99].

In this project, a simple and reproducible procedure for preparing spherical CaCO_3 microparticles (vaterite) developed by Volodkin et al was used [7]. In this method, the amorphous nano-precipitate (CaCO_3 nanoparticles) instantly formed when mixing CaCl_2 and Na_2CO_3 solution, which transforms into spherical CaCO_3 microparticles. Scanning electron microscopy (SEM) observation indicates that CaCO_3 microparticles have a size of about 3-5 μm (**Figure 29a 29b**). The cross-section pictures show that CaCO_3 microparticles were formed by the assembly of CaCO_3 nanoparticles (**Figure 29c 29d**). A rough surface with connected channels and interspaces are thus resulted from the stacking of CaCO_3 nanoparticles, which is essential to immobilize substances (**Figure 29a 29b**).

Figure 29 SEM observation of CaCO_3 microparticles morphology (a) surface topography of CaCO_3 microparticles (b) enlarged view of single CaCO_3 microparticles (c) & (d) cross-section observation to show the internal morphology of CaCO_3 microparticles

The co-crystallization process between CaCl_2 and Na_2CO_3 for forming CaCO_3 microparticle is also quite useful for hydrophilic drugs encapsulation (**Figure 30**), which makes CaCO_3 to be a desirable pre-loaded template core for LbL polyelectrolyte microcapsules. Here, hydrophilic compound (FITC-dextran) was loaded into CaCO_3 microparticles via dissolving it in CaCl_2 and then mixed with Na_2CO_3 . CLSM was used to investigate the distribution of FITC-dextran within the interior of the CaCO_3 microparticles (**Figure 31**). From the bottom to

the middle of the spherical microparticles, each plane along the z -axis made by the CLSM was fully filled with green fluorescence, indicating the successful loading of FITC-dextran into CaCO_3 microparticles.

Figure 30 schematic diagram showing the formation process of CaCO_3 microparticles and its encapsulation of water-soluble FITC-dextran via co-crystallization process

Figure 31 z -stack of CLSM observation along with the z axis from the bottom to the middle place of CaCO_3 microparticles to show the FITC-dextran distribution within the CaCO_3 microparticles, the scale bar = $5\ \mu\text{m}$

4.3.2 Coating hydrophobic compounds loaded PLGA onto CaCO_3 microparticles

The porous CaCO_3 microparticles were incubated with a solution of

PLGA and hydrophobic compounds dissolved in NMP or acetone under stirring. The porous microparticles immobilized with PLGA/hydrophobic compounds were separated from the polymer solution by centrifugation and then exposed to H₂O. Due to the diffusion of NMP or acetone to H₂O, PLGA precipitates to form a solidified matrix containing the hydrophobic compounds. After removal of the CaCO₃ template using 0.1 M HCL solution, PLGA microcapsules can be obtained. ζ -potential, CLSM, SEM, AFM and TGA were used to study this process.

ζ -potential study

ζ -potential test is a good indicator to show the changes of surface potential when studying surface modification with coating. The bare CaCO₃ microparticles have a positive surface potential about +16mV (**Figure 32**). It changes to -3mV (PLGA solidified from acetone solution) and -2mV (PLGA solidified from NMP solution) after covered by solidified PLGA film (**Figure 32**). Since PLGA is neutral or slightly negatively charged polymer, the ζ -potential changes reflected here indicate the successful coating of PLGA layer onto the surface of CaCO₃ microparticles. Furthermore, the slightly negative ζ -potential values were also maintained even after the CaCO₃ template cores are dissolved away (**Figure 32**). These results may also suggest that the PLGA microcapsules remain integrated after the dissolution of CaCO₃ template cores.

Figure 32 ζ -potential values for bare CaCO_3 microparticles, PLGA coated CaCO_3 microparticles and hollow PLGA microcapsules where CaCO_3 microparticles template cores are dissolved (data points represent mean $\pm$ standard deviation of data from three independent samples)

CLSM observation

In order to further confirm the successful coating of PLGA onto the surface of CaCO_3 and the integrity of PLGA microcapsules after templates removal by mixing with 2ml 0.1M HCl solution at room temperature for 2min and HCl can decompose the CaCO_3 template core and yield the hollow PLGA microcapsules. The CLSM was used to investigate the PLGA microcapsules suspension.

For CLSM study, a hydrophobic green fluorescent dye—Coumarin6 was added into PLGA acetone or NMP solution before incubating them with CaCO_3 microparticles. It was found that spherical and integrated green microcapsules were observed after the CaCO_3 templates removal (**Figure 33a**

33b), demonstrating that CaCO_3 microparticles were successfully coated with PLGA which is either solidified from acetone or NMP solution. These well-dispersed hollow PLGA microcapsules produced via *in situ* gelling of PLGA on the surface of CaCO_3 microparticles provides an alternative way to manufacture hollow PLGA microcapsules. Traditionally, hollow PLGA microcapsules were produced by emulsification/solvent evaporation method where a liquid oil core is necessary [83-85].

Unlike the free Coumarin6 crystals which are hardly soluble in water, these PLGA microcapsules which have Coumarin6 loaded in the capsule shell can be well dispersed in aqueous solution, exhibiting as highly homogenous green capsules shells (**Figure 33a 33b**). Consequently, the solubility, applicability and bioavailability of hydrophobic Coumarin6 can be enhanced after the encapsulation.

Figure 33 CLSM observation of Coumarin6 loaded PLGA microcapsules (a) PLGA microcapsules solidified from acetone (b) PLGA microcapsules solidified from NMP

SEM and AFM investigation

SEM was also used to investigate the surface morphology of these PLGA microcapsules in more details (**Figure 34a 34b 34c 34d**). It was noted that these PLGA microcapsules have less folds and creases than the traditional LbL polyelectrolyte microcapsules. The possible reason is that PLGA has stronger mechanical strength than polyelectrolyte, which can resist the collapse caused by the template removal. The enlarged view showed that PLGA microcapsules formed from NMP have a less porous surface (**Figure 34a 34b**) while those solidified from acetone exhibit spongy surface where obvious pores are observed (**Figure 34c 34d**).

Figure 34 SEM observation of surface morphology for the PLGA microcapsules solidified from acetone or NMP solution (a) PLGA microcapsules solidified from NMP solution (b) enlarged view of single PLGA microcapsules solidified from NMP solution (c) PLGA microcapsules solidified from acetone solution (d) enlarged view of single PLGA microcapsules solidified from acetone solution

Atomic force microscopy (AFM) was introduced to further confirm such SEM observation in more details. AFM investigation is well corresponding to the SEM observation (**Figure 35a 35b**). The mean roughness analysis by AFM implied that PLGA microcapsules solidified from acetone solution have a more porous surface (8~12 nm) than those formed from NMP solution (3~5 nm), indicating porous nature of acetone PLGA microcapsules (**Figure 35b**).

Figure 35 AFM investigation of surface morphology and surface roughness for the PLGA microcapsules (a) PLGA microcapsules solidified from NMP solution (b) PLGA microcapsules solidified from acetone solution (c) mean roughness of PLGA microcapsule solidified from NMP and acetone solution respectively, the data points represent mean $\pm$ standard deviation of data from three positions of the microcapsules wall

The morphology difference can be explained by phase inversion dynamics which is largely affected by the solvent quality. NMP has viscosity of 1.7cP while that for acetone is only 0.32cP. Furthermore, it was reported that diffusion coefficient of water to NMP ($D_{\text{water-NMP}}$) is $18 \text{ cm}^2/\text{s}$, which is much lower than that of acetone having a $D_{\text{water-acetone}}$ up to $88.6 \text{ cm}^2/\text{s}$. Consequently, PLGA NMP solution will induce slower water exchange than PLGA acetone solution [100]. Upon contact with water, lower water influx rate and lower PLGA gelation rate can be expected for PLGA NMP system, giving NMP PLGA microcapsules decreasing structure porosity (**Figure35a**).

TGA measurement

TGA was used to investigate the weight of PLGA coated onto CaCO_3 microparticles after their solidification from NMP or acetone solution (**Figure 36a 36b 36c**). **Figure 36a** shows the thermal degradation profiles of pure PLGA and pure CaCO_3 microparticles (**Figure 36a**). It was found that pure PLGA starts to degrade at about 250°C and finish the degradation at 350°C with no

residues left (**Figure 36a**). In contrast, CaCO_3 microparticles have a higher degradation temperature, where they start to degrade at about 650°C . Consequently, the PLGA coated CaCO_3 microparticles should have a two-stage degradation profile under TGA analysis, where the first degradation temperature is for PLGA while the second one is for CaCO_3 microparticles.

The TGA analysis again confirms the successful coating of PLGA onto CaCO_3 either from acetone or NMP (**Figure 36b 36c**). A typical two-stage degradation profile was observed for both cases (**Figure 36b 36c**). Through calculating the weight difference on the first stage of the TGA curve, the weight of PLGA coated onto CaCO_3 after their solidification can be obtained. For CaCO_3 microparticles coated with PLGA solidified from acetone solution, a 4.5% coating amounts of PLGA can be obtained (**Figure 36b**). On the other hand, a 2.1% coating amounts of PLGA was obtained for PLGA solidified from NMP solution (**Figure 36c**). The weight difference proves that more PLGA can be coated onto CaCO_3 microparticles when the PLGA are solidified from acetone solution. This is because of different viscosity between PLGA acetone and NMP solution. PLGA NMP solution has a higher viscosity than PLGA acetone solution. Higher viscosity solution is more difficult to be infiltrated and immobilized into porous materials.

Figure 36 Weight analysis of PLGA coated onto CaCO₃ microparticles with TGA (a) TGA degradation profiles for pure PLGA and pure CaCO₃ (b) TGA degradation profile of CaCO₃ coated with PLGA solidified from acetone solution (c) TGA degradation profile of CaCO₃ coated with PLGA solidified from NMP solution

4.3.3 Further LbL coating of polyelectrolytes onto the PLGA coated CaCO₃ microparticles

The above results fully confirm the coating of PLGA layer onto CaCO₃ microparticles, where hydrophobic compounds can be loaded within the PLGA layer. As **Figure 12** shown, further LbL coating of polyelectrolytes (PAH/PSS) was applied onto the PLGA coated CaCO₃ templates to control the release behaviors and produce LbL microcapsules (**Figure 12**).

ζ -potential was firstly used to study the polyelectrolytes layering process (**Figure 37**). The PLGA coated CaCO_3 microparticles have a slightly negative charge (-4mV), possibly resulting from the ionization of $-\text{COOH}$ groups in PLGA. After depositing the first positive polyelectrolyte layer (PAH), the negative surface potential change to be +10mV. The positive potential was again changes to -20mV after PSS coating. A net reversal of surface charge between positive and negative values is evident for each polyelectrolyte layer added for either PLGA solidified from NMP solution or acetone solution (**Figure 37**). The ζ -potential studies suggest the successful coating of PAH/PSS onto the PLGA coated CaCO_3 template.

Figure 37 ζ -potential studies for the PAH/PSS coating process onto the PLGA coated CaCO_3 microparticles (data points represent mean $\pm$ standard deviation of data from three independent samples)

Besides, the CLSM and AFM were also used to confirm the successful coating of PAH/PSS onto PLGA coated CaCO_3 microparticle template.

For CLSM observation, another hydrophobic red fluorescent dye—Nile Red was loaded into the NMP or acetone PLGA layer coated onto the CaCO_3 template. The coating of PAH/PSS was indicated by the FITC-PAH (FITC-PAH/PSS). NMP or acetone PLGA layers were successfully formed as implying from the red capsule shell (**Figure 38a 38d**). On the other hand, FITC-PAH/PSS coating was observed being coated closely onto NMP or acetone PLGA microcapsules as the green capsule shell (**Figure 37f 38e**). The overlay pictures demonstrated that the polyelectrolyte-PLGA hybrid microcapsules were successfully formed (**Figure 38c 38f**). The PAH/PSS coating may not only control the substance release profiles but also render PLGA microcapsules with an active surface which can be further functionalized.

Figure 38 CLSM observation to confirm the coating of PAH/PSS onto the PLGA coated CaCO_3 microparticles; (a)—(c) PLGA solidified from acetone solution, (d)—(f) PLGA solidified from NMP solution; (a) & (d) red shell to indicate the PLGA coating where Nile Red is loaded; (b) & (e) green shell to indicated the FITC-PAH/PSS coating onto the PLGA coated CaCO_3 microparticles; (c) & (f) the overlay red and green channel to show the successful coating of PAH/PSS onto PLGA. Scale bar = $10\mu\text{m}$

AFM was used to explore these hybrid microcapsules in more depth (**Figure 39a, 39b**). The different surface features between bare NMP and acetone PLGA microcapsules (**Figure 35a 35b**) disappear after coating with PAH/PSS (**Figure 39a, 39b**). Surface topography of the hybrid microcapsules is quite similar (**Figure 39a, 39b**), which is a reflection of the PAH/PSS coating morphology. The surface of PLGA microcapsules after coating polyelectrolyte becomes tight, flat and continuous. Unlike the bare PLGA microcapsules (**Figure 35a, 35b**), no pores can be observed for the hybrid microcapsules, even for the acetone PLGA microcapsules which has highly porous wall.

Figure 39 AFM observation for the polyelectrolyte/PLGA hybrid microcapsules (a) PAH/PSS coating onto the CaCO_3 microparticles coated with PLGA solidified from NMP solution (b) PAH/PSS coating onto the CaCO_3 microparticles coated with PLGA solidified from acetone solution

4.3.4 Ibuprofen release from the hybrid microcapsules

Ibuprofen (IBU) was used as a model hydrophobic substance to investigate the release kinetics of these hybrid microcapsules having different layer numbers of polyelectrolytes coating (**Figure 40a 40b**). A high actual loading amount was achieved for either acetone PLGA microcapsules (2.9%) or NMP PLGA microcapsules (2.2%). For both uncoated NMP and acetone microcapsules, a burst release of about 90% of ibuprofen was observed within the first 5 hours (**Figure 40a 40b**). After coating 2 layers of PAH/PSS, the burst release was reduced to around 60%. With the further coating of PAH/PSS to 4 and 6 layers, the burst release was further reduced and a more sustainable release was observed, indicating that the LbL PAH/PSS coating have the barrier properties to reduce the transport of IBU to outside medium and tunable release profiles were achieved by changing the layer numbers.

Beside reduction of burst release, the subsequent linear release phase after about 20h was also different as indicated in the inset of the graph for both NMP and acetone hybrid microcapsules (**Figure 40a 40b**). For uncoated NMP PLGA microcapsules, IBU release from 20h to 170h remain no change. After coating 2 layers of PAH/PSS, a linear increased release from 60% to 65% was

observed. For 4 layers and 6 layers coated ones, the linear increase of release is from 45%, 35% to 60%, 55%, respectively (**Figure 40a**). Similar trends were observed for acetone PLGA hybrid microcapsules (**Figure 40b**). These results imply that further LbL PAH/PSS coating onto the NMP or acetone microcapsules is able to control the hydrophobic drug release in a more sustainable manner for longer time.

Figure 40 Ibuprofen release profiles from the hybrid microcapsules having different layer numbers (a) release profiles of ibuprofen loaded in PLGA solidified from NMP (b) release profiles of ibuprofen loaded in PLGA solidified from acetone solution. The schematic illustration shows the loading position of ibuprofen in the hybrid microcapsules; the insets in figure (a) & (b) indicate ibuprofen release after 20h. (Data points represent mean $\pm$ standard deviation of data from three independent samples.

4.3.5 Formation of magnetically responsive, hydrophilic/hydrophobic dual drugs delivery microcapsules

Beside hydrophobic drugs, other components like hydrophilic drugs and inorganic nanoparticles can also be conveniently integrated into such hybrid microcapsules (**Figure 41**).

Figure 41 Schematic illustration showing the formation of magnetically responsive, hydrophilic/hydrophobic co-delivery microcapsules

Here, the interior of the microcapsules was filled in with hydrophilic substance (FITC-dextran) and Fe_3O_4 nanoparticles according to previous procedures [7-10]. **Figure 42** suggests that a dual-drug containing, magnetically responsive multifunctional microcapsule is successfully formed (**Figure 42a 42b 42d 42e**). The dual drugs releasing behaviors were studied and summarized in **Figure 42f**. **Figure 42f** showed that hydrophilic-hydrophobic dual drug release in a controllable manner from the microcapsules is achieved. For either ibuprofen or FITC-dextran, the release rate decreased with the increase of layer numbers. At the respective 2, 4 or 6 layers of PAH/PSS coating, ibuprofen

showed faster release than FITC-dextran, which may result from the smaller size of ibuprofen molecules.

Figure 42 (a) Green channel of CLSM showing the aligned multifunctional microcapsules under magnetic force, the green color is from the hydrophilic model compound (FITC-dextran) pre-filled in the interior of the microcapsules. (b) Enlarged view of capsules in the selected area of figure 41a. (c) Schematic illustration to show how the capsules are aligned. (d) Red channel of CLSM showing the aligned multifunctional microcapsules under magnetic force, the red color is from the hydrophobic model compound (Nile Red) incorporated in the acetone PLGA microcapsules. (e) Enlarged view of capsules in the selected area of figure 41d. (f) Dual-drug (Ibuprofen & FITC-dextran) release behaviors from the acetone PLGA hybrid microcapsules (data points represent mean $\pm$ standard deviation of data from three independent samples)

4.3.6 Effects of hydrophobicity and molecular weight of the drugs on the release profiles

The above results showed the possibility of forming multifunctional LbL hybrid microcapsules where hydrophobic drugs (ibuprofen) and hydrophilic compounds (FITC-dextran) can be simultaneously loaded and released. For 2, 4 and 6 layers of polyelectrolytes coated microcapsules, ibuprofen exhibited faster release than FITC-dextran did. This may be resulted from the lower molecular weight of ibuprofen than FITC-dextran. Compounds having higher molecular weight will have bigger size (estimated from the effective molecules radius), which generally will have slower release because of their increased difficulties to travel through the entangled polyelectrolytes layers.

However, ibuprofen and FITC-dextran are not comparable in chemical terms, since they have different hydrophobicity and different charge, which will also impact on their release rate through the LbL polyelectrolyte membrane.

In order to have a more precise explanation about the difference of release profiles and factors affecting the release profiles, other model compounds are re-selected and studied.

For studying the effects of hydrophobicity on the release, metoclopramide hydrochloride (Meto-salt) and metoclopramide base (Meto-base) are used. Meto-salt and meto-base have same molecular weight and charge (positive charge). The only difference is the aqueous solubility where meto-salt is quite soluble in water (480mg/ml) while meto-base is poorly soluble in water

(0.2mg/ml). Consequently, they were loaded in the solidified PLGA layers and coated with 2 layers of PAH/PSS (**Figure 43**). This is to investigate the effects of hydrophobicity of the compounds on the release at the same coated layer numbers.

Figure 43 The release profiles of Meto-salt and Meto-base from the polyelectrolyte/PLGA hybrid microcapsule with 2 layers of polyelectrolyte coating (data points represent mean $\pm$ standard deviation of data from three independent samples)

Figure 43 shows the release of meto-salt and meto-base through 2 layers of PAH/PSS microcapsules. It was found that both of them have similar release profiles where an initial fast burst release is followed by a slower release phase. However, meto-salt diffuses faster than meto-base does. Meto-salt has a burst release of 75% while that for meto-base is 60%. For the subsequent slow release phase, meto-salt has an equilibrium plateau of 85% release while that for

meto-base is about 70%. The results suggest that hydrophobic drugs can have a more sustainable release than their hydrophilic counterpart. Similar results were obtained in previous studies where hydrophobic doxorubicin base had a slower release than hydrophilic doxorubicin hydrochloride from PLGA fibers. The fast release of small hydrophilic drugs from carriers was suggested to be resulted from their good solubility in the aqueous release medium, which makes them dissociate from the carriers faster [86].

For PLGA-polyelectrolyte hybrid microcapsules, it can be considered as a type of controlled release device combining monolithic and reservoir systems. The meto-salt and meto-base loaded PLGA layer can be considered as monolithic solution device, while the PAH/PSS coating is the membrane reservoir system. In order to describe the release kinetic of such combinational system, the following equation (Eq.3) was developed [103-105].

$$\frac{dM_t}{dt} = \frac{AD_1D_2C_{1s}}{xD_2 - K\gamma D_1} \quad \text{Eq. 3}$$

In this equation, A (cm^2) is the surface area, D_1 and D_2 are the diffusion coefficients (cm^2/sec) of solutes in PLGA matrix and PAH/PSS membrane, C_{1s} is the solute concentration (mg/ml) at the membrane-matrix interface, x is the distance (cm) between the matrix and membrane, K is the partition coefficient and γ is the membrane thickness (nm)

It is known that, when the term $xD_2 \ll K\gamma D_1$, the release rate is characteristic of simple reservoir system. On the other hand, the release kinetic of reservoir systems (non-constant activity source) can be described by equation 1

(Eq. 1), as mentioned above [103-104].

$$M_t = M_o \left[1 - \exp \left(-\frac{ADKt}{Vl} \right) \right] \quad \text{Eq. 1}$$

DK can be replaced with P (P is termed as permeability of the membrane, $P = DK$)

From figure 38 and 39, it is known that the PAH/PSS coating and PLGA layer is tightly contacted with each other, where the distance between PLGA layer and PAH/PSS coating can be neglected, i.e. $xD_2 \ll K\gamma D_1$. Consequently, these devices can be considered as membrane-controlled reservoir systems. Furthermore, these carriers can also be regarded as membrane-controlled reservoir systems with non-constant activity source, since the loading of meto-salt (1.5%) and meto-base (1.2%) here is far below their solubility limit in PLGA matrix (6 mg/ml for meto-salt and 10mg/ml for meto-base). Consequently, the release kinetics of meto-salt and meto-base from the microcapsules can be fitted by the equation 1 (**Figure 44**).

Figure 44 Fitting the release curves of Meto-salt and Meto-base with the theoretical equation

Clearly, the equation fitting can be a reasonable estimate for both the meto-slat and meto-base release kinetics, where a relatively high R^2 (above 0.96) can be obtained. The permeability of membrane to meto-salt can be estimated to be $P = DK = 0.25\text{VI/A}$ while that for meto-base is $P = DK = 0.17\text{VI/A}$. The good agreement between the fitting and experimental results indicates that the solutes release is primarily controlled via drug diffusion and partitioning through the intact polyelectrolyte membrane. In fact, the difference in the two permeability values is a difference in K between metosalt and metobase partitioning from PLGA into the polyelectrolyte layer. Metobase K is approximately 30% lower than that of metosalt, which is reasonable considering their hydrophilicities

For studying the effects of molecular size on the release profiles, FITC-dextran with 4k molecular weight (FD-4K) and 70k molecular weight (FD-70K) were used. FITC-dextran of different molecular weight has the same hydrophobicity and charge. The only difference is the size of the molecules. FD-4k has an effective radius of $16.5\text{\AA}$ in aqueous solution while that for FD-70K is around $50.4\text{\AA}$ [101-102]. Consequently, they were loaded into the interior of the LbL microcapsules having 2 layers of PAH/PSS coating (**Figure 45**). Their release behaviors were studied and modeled.

Figure 45 The release profiles of FD-4K and FD-70K from the microcapsules (data points represent mean $\pm$ standard deviation of data from three independent samples)

FD-4k and FD-70K were used to investigate the effects of molecular size on the release profiles. **Figure 45** shows the actual release profiles of FD-4k and FD-70K through 2 layers of PAH/PSS microcapsules. As expected, FD-70K releases out in a much slower manner than FD-4K dose, because of its bigger size. FD-4K has an initial burst release of 55% following by a steady slow release up to 65% at 110h. In contrast, FD-70K has no burst release and shows a near zero-order release with a value of 40% release at 110h (**Figure 45**).

LbL polyelectrolyte microcapsules encapsulated with FITC-dextran are typical membrane-controlled reservoir devices. These microcapsules can be considered as reservoir devices with non-constant active source, since the low loading of FITC-dextran (0.48% & 0.52% respectively) and good water solubility of FITC-dextran (50 mg/ml). Consequently, the actual experimental

release profiles in figure 45 can be fitted by the equation 1 and obtain the fitted curves in figure 46.

Figure 46 Fitting the release curves of FD-4K and FD-70K with Eq. 1

As clearly shown in figure 46, very good agreement between the theoretical and experimental curves can be obtained, with R^2 values above 0.98. The membrane's permeability to FD-4K is estimated to be $P = DK = 0.078 \text{ VI/A}$ while that for FD-70K is $P = DK = 0.023 \text{ VI/A}$. The difference here is entirely due to differences in diffusion coefficient, D . Thus the D value for FD4K is approximately 3.4 times that of FD-70K. The good agreement also confirms that the equation here is valid and considers the most important mass transport steps in the control of solute release from the LbL polyelectrolyte microcapsules.

Chapter 5 Conclusions and future recommendations

5.1 Conclusions

In summary, we have shown in this project that the use of LbL polyelectrolyte coating of microcarriers enables encapsulation of both hydrophilic and hydrophobic compounds, while retaining good control over the release of these compounds. At the same time, the loading procedure is easy and mild.

This project raises two ways to develop LbL polyelectrolyte microcapsules:

- (1) Instead of using solid polymeric templates, drug-loaded RBCs are used as templates for the LbL polyelectrolytes layering process. Such LbL microcapsules eliminate the core dissolution process and substance post-loading.
- (2) PLGA, which is a good candidate for hydrophobic drugs encapsulation, is incorporated onto another carrier during the LbL deposition process. PLGA and hydrophobic drugs are dissolved in water miscible solvent like NMP or acetone and then immobilized onto the surface of porous CaCO_3 microparticles. Due to the solvent exchange, PLGA containing drug can be coated onto the surface of CaCO_3 microparticles. Further LbL polyelectrolytes coating is used to control the release rate.

The first fabrication way simplifies the preparation of pre-loaded LbL polyelectrolytes microcapsules, whereby core dissolution and substance

post-loading can be eliminated. Different numbers of layers of PAG and DES were deposited successfully onto the negatively charged RBCs ghosts. These polyelectrolyte multilayers successfully suppressed the burst and subsequent release of FITC-dextran. Besides controlling the release rate, these LbL polyelectrolyte microcapsules also offer the possibility to protect the bioactive agents from the external environment.

Besides using drug loaded RBCs ghosts which require hemoglobin removal and cell membrane resealing as template, intact RBCs are also directly loaded with hydrophilic compounds (RITC-lysozyme) and then coated with multilayer polyelectrolyte. This improvement further simplifies the fabrication process of LbL polyelectrolytes microcapsules having cellular carrier as template cores.

Compared with drug loaded RBCs, porous inorganic CaCO_3 microparticles possess some unique features which make them as good template for loading hydrophobic drugs into LbL polyelectrolytes microcapsules.

The porous feature of CaCO_3 microparticles provides them a good platform for immobilizing substances. PLGA, which is a good candidate for encapsulating hydrophobic drugs, are dissolved in water miscible solvent and then immobilized onto the surface of porous CaCO_3 microparticles. Due to the exchange of solvents, PLGA microcapsules with hydrophobic drugs in the microcapsules wall are formed. Further LbL polyelectrolytes coating can tune the release rate of loaded hydrophobic drugs.

The use of porous CaCO_3 microparticles coated with hydrophobic drugs loaded PLGA layers as templates enable the production of hydrophobic drug loaded LbL polyelectrolyte microcapsules. Production of such PLGA/polyelectrolyte hybrid microcapsules expands both the application fields of PLGA delivery system and LbL polyelectrolyte microcapsules.

The use of the hybrid microcarrier also expands the range of usefulness. For example, hydrophilic drugs and other inorganic nanoparticles like silver, gold and magnetic nanoparticles can also be incorporated into the interior of the hybrid microcapsules. This multifunctional platform simultaneously has the ability to control drug release as well as be responsive to external stimuli. It may find wide application in gene and siRNA delivery systems, as well as in chemotherapeutic delivery..

5.2 Future recommendations

Based on the obtained results about the formation, characterization and application of the above novel LbL polyelectrolyte microcapsules, some more future recommendation can be made.

(1) Eliminating the use of cross-linker for the RBCs templates

In this project, the drug loaded RBCs template are cross-linked and fixed with glutaraldehyde prior to the subsequent LbL polyelectrolytes coating process due to the soft and fragile nature of RBCs. However, toxicity resulting from the residue of cross-linker may hinder their biomedical application. Eliminating the use of cross-linker for the RBCs template may be achieved by

using PEG-modified polyelectrolytes as some research suggested. In a past research carried out by S. Mansouri et. al [13], poly-L-lysine-graft-polyethylene glycol (PLL-PEG) and chitosan-graft-phosphorylcholine (CH-PC) were alternatively coated onto live human red blood cell to reduce their immune response during the blood transfusion. They found that the coating induced negligible hemolysis and agglutination and the coated RBCs preserved their viability and functionality. The coating materials may be used to produce fully biocompatible LbL microcapsules.

(2) Using titration method to coat polyelectrolytes onto the loaded RBCs

In this project, centrifugation method is used to coat multilayer polyelectrolytes onto the RBCs templates. For this method, excess amount of polyelectrolytes solution is incubated with RBCs templates. The redundant polyelectrolytes solution is then removed via centrifugation. Centrifugation force may result in breakdown of RBCs template. On the other hand, titration coating is a milder method. For titration method, exactly-needed amounts of polyelectrolytes are added onto the RBCs templates, which will not produce centrifugal force to the fragile RBCs template. Furthermore, titration coating method can also eliminate the washing steps which are needed for centrifugation method.

(3) Making RBCs to be able to load hydrophobic drugs

Although use of drug loaded RBCs as template can simplify the

preparation of LbL microcapsules by eliminating the core dissolution and drug post-loading, loading of hydrophobic drugs into RBCs remains impossible via the traditional hypotonic dialysis loading method. Consequently, it would be difficult to load hydrophobic drugs into LbL microcapsules which use RBCs as template cores. This problem would be solved by attaching hydrophobic drugs loaded PLGA nanoparticles onto the surface of the RBCs and then coated with multilayer of polyelectrolytes. Some previous research has suggested that polystyrene particles of different size can be attached to the surface of live RBCs [72-75].

- (4) Tagging the targeting moieties onto the microcapsules and studying their cellular interaction

Due to the rich active chemical groups like amine groups, the surface of these formed LbL microcapsules can be tagged with targeting molecules like monoclonal antibody to give them targeting ability. Besides the detailed studies about the release behaviors, the interaction between various kinds of cells and the LbL microcapsules loaded with different drugs can also be carried

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- (2) **Rongcong Luo**, Bjorn Neu, Subbu Venkatraman. Surface Functionalization of Nanoparticles to Control Cell Interaction and Drug Release. *Small*, 8 (16), 2585-2594, 2012.
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(Equal Contribution)

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