UNIFORM DOUBLE WALL MICROSPHERES/MICROCAPSULES FOR PROTEIN DELIVERY

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THESIS

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Abstract

Biodegradable polymer devices have been utilized as a means to deliver drugs in a controlled and less invasive manner. Poly (lactide-co-glycolide) (PLG) and poly (lactic acid) (PLA) microparticles such as double-wall/single-wall microspheres and microcapsules were heavily investigated for controlled delivery of small molecule drugs as well as proteins and DNA. The size distribution of protein-loaded biodegradable polymer microparticle is a crucial factor for allowable routes of administration. Also, the geometric structures of microparticles can influence the resulted release profile. In this project, by using the Precision Particle Fabrication method, we produced uniform double-wall microspheres (DWMS) with a protein-loaded (Bovine Serum Albumin) PLG core and a drug-free PLA shell, which was expected to provide better encapsulation of the protein as well as to postpone the protein release. Different inherent viscosity (i.v.) of PLG and PLA and different organic solvent configurations were used to produce uniform DWMS. Also, by studying the in vitro release profiles and microscopy images, we found that using ethyl acetate as shell-phase solvent, dichloromethane as core-phase solvent and using lower PLG and PLA i.v., better encapsulation of the protein-loaded core as well as clearly core-shell structure can be achieved. We have also successfully produced uniform protein-loaded DWMS with different shell thickness.
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Chapter 1.  INTRODUCTION

1.1 Controlled Drug Release

With fast development in genomics and biotechnology, new protein and peptide drugs are being created. Because of many problems such as low solubility and poor stability, the delivery systems or methods for these drugs can dramatically impact the efficacy and clinical implementation as much as the nature of the drugs themselves.

Figure 1.1: Therapeutic window for drug delivery

For many years researchers have sought to develop drug delivery systems that can target drug to specific body sites or precisely control drug release rate for prolonged time [1]. To produce beneficial results, the in vivo concentration of drug should be maintained within the therapeutic window, which consists of a lower bound, the minimum effective concentration (MEC) and an upper bound, the minimum toxic concentration (MTC), as illustrated in Figure 1.1. Conventional drug delivery systems, such as oral dosing and injections typically generate concentration profiles with peaks and valleys, due to the release and exhaustion of the therapeutics. One of the reasons to consider controlled drug release is to maintain the drug concentration within the therapeutic window. Besides,
controlled release can reduce the dosage frequency and increase patient compliance.

In recent years, many controlled release system have been developed, such as polymer-based drug-delivery system, liposome-based delivery system and intelligent delivery systems [1]. These delivery systems can not only maintain drug concentration within the therapeutic window for an extended time after the initial dose but also protect the fragile therapeutics encapsulated [2]. From sophisticated microchip and implantable pumps to simple devices such as drug-encapsulated polymer microparticles, there are many ways for drug controlled release nowadays and many of them are commercialized.

1.2 Biodegradable Microparticles for Protein Delivery

Biodegradable polymer devices have been utilized for around 30 years as a means to deliver drugs in a controlled and less invasive manner [3-5]. Specifically, spherical microspheres and microparticles have been shown to provide controlled release for small molecules drugs as well as macromolecules such as proteins and DNA [6-9].

Microspheres and microparticles size ranging from a few to several hundred microns have received much attention in recent years. For example, monodisperse microspheres approximately 1-5 μm in diameter would be ideal for passive targeting of professional antigen-presenting cells [10, 11]. Microspheres 10-20 μm in diameter could be used to target the tortuous capillary bed of tumor tissues by chemo-embolization [12]. Microparticles 1-5 μm in diameter and highly porous particles 5-20 μm in diameter are effective pulmonary drug delivery vehicles [1]. Microspheres 20-100 μm are less myotoxic than smaller microspheres (less than 5 μm) [13]. Because of the simplicity and versatility of these devices, many commercialized products have been produced. For example, the Trelstar® injectable PLG microspheres and Lupron® depot for prostate cancer, the Sandostatin LAR® PLG depot for acromegaly and Gliadel® polyanhydrides wafer for brain cancer are all commercialized implantable or injectable polymer devices.
Also, biodegradable microparticles offer several advantages such as high local drug concentrations at the site of administration, good protection of fragile therapeutics and minimized side effect. For protein specifically, the simple formation process of microparticles and programmable degradation rate of biodegradable polymers make this a promising delivery system.

1.2.1 Choice of Polymers

Researchers have been using biodegradable polymers as depot vehicles for drug delivery for many years. Among them, three classes of polymers have been heavily investigated: polyesters, polyanhydrides and polyphosphoesters [15, 16].

The most frequently studied of these polymers are poly(lactide-co-glycolide) (PLG), and polylactide or poly(lactic acid) (PLA). These two polymers are both polyesters and their structures are shown in Figure 1.2. In this project, we will focus on PLG, because its degradation kinetics, drug encapsulation capability and biocompatibility are well understood and a number of PLG delivery systems have been FDA approved. PLG degrades by hydrolysis of its ester linkages in the presence of water. PLG chain scission by hydrolysis generates products with hydroxyl and acid end groups. These pieces may be further hydrolyzed to lactic and glycolic acid monomers. The schematic of PLG degradation is shown in Figure 1.3.

![Figure 1.2 Structures of PLG (left) and PLA (right)](image-url)
The degradation rate of PLG depends on its monomer ratio: the lower the content of glycolide units, the slower the degradation because increased lactide content leads to increased hydrophobicity of lactide over glycolide [17]. In addition, ester-end polymer degrades slower than carboxylic acid-end polymer because the acidic microenvironment interior the hydrolyzing PLG matrices could potentially accelerate the degradation rate [17, 18].

1.2.2 Fabrication Methods

Fabrication of protein-loaded PLG microspheres/microparticles includes solvent extraction/evaporation [19], polymer extrusion [20], spray drying [21] and coacervation or precipitation [22]. Although there are differences of these methods for size range and drug encapsulated, several things are in common. Initially, PLG should be dissolved in a suitable solvent such as dichloromethane. Then, the drug is co-dissolved with PLG,
suspended as solid particulate or dissolved in another solvent and emulsified with the PLG solution. The drug polymer solution or emulsion is broken into droplets which are allowed to harden according to different fabrication methods.

Solvent extraction/evaporation is one of the most common methods used for producing drug-loaded microspheres. In this method, the PLG solution is emulsified in a non-solvent phase and broken into small droplets. After emulsification, the polymer-drug droplets are stirred in the non-solvent bath so that the solvent may be extracted and allowed to evaporate. This method is simple, but the size distribution of resulting particles is relatively broad. In Chapter 2, we will discuss the method of Precision Particle Fabrication which is an alteration of solvent extraction/evaporation that provides highly monodisperse microspheres.

Extrusion methods form microspheres by forcing the microsphere constituents through a nozzle or an orifice. This method can achieve relatively narrow size distribution of microspheres [23, 24]. However, the high velocity of the stream inside the orifice and the shear force imparted may damage the encapsulated proteins or other therapeutics.

For spray drying, an atomizer produces fine droplets of the PLG mixture, and a carrier stream of hot air is used for extracting the solvent. The size distribution is relatively narrow because no other chemicals are involved during fabrication. However, the relatively high temperature and shear force of the air stream can damage the protein inside the particles [25].

Coacervation methods rely on careful selection of PLG solvents as well as non-solvent. The solvent and non-solvent will together promote phase separation of polymer into droplets around the protein encapsulated [26, 27]. The success of this process necessitates an understanding of the complex thermodynamics of phase separation and the resulting size distribution is typically broad.
1.2.3 Protein Release Mechanism

For protein release from PLG microparticles, there are generally three mechanisms: diffusion, chemical reaction and solvent activation [2].

Diffusion is the most common release mechanism for small molecule drugs encapsulated in polymer depots. The encapsulated small molecule drug migrates from its initial position in the polymeric system to the polymer’s surface and finally to the body [2]. Release by drug diffusion is generally based on the size of PLG depots. For protein, however, the dimension of the protein makes diffusion not so easy because proteins are too large and hydrophilic to diffuse through most polymeric materials. During degradation and erosion of polymeric materials, water-filled pores would form through which proteins could diffuse. So the diffusion rate or the effective diffusivity of protein is controlled by the rate of formation of water-filled pores.

Chemical reaction is accomplished by polymer degradation and erosion. Degradation is the act of individual polymer chain cleavage and erosion is mass loss from the overall polymer matrix. There are two types of erosion for polymers used in the area of controlled release: surface erosion and bulk erosion. Surface erosion polymers degrade and lose material primarily from the exposed surface area while bulk erosion polymers degrade throughout the entire polymer matrix simultaneously. PLG is a bulk erosion polymer, which means protein may move through a complex porous path during bulk erosion. Also the erosion of PLG will affect the porous structure and accelerate the release.

Solvent activation involves either swelling of polymer or osmotic effects. Devices that control the flow of protein solutions utilize osmotic potential gradients across PLG barriers to generate pressurized chambers containing aqueous solutions of protein. This pressure is relieved by the flow of the protein solution out of the delivery device [28].
For protein-loaded PLG microspheres, the release mechanism can be combinations of the three mechanisms. Upon immersing the microsphere within an aqueous buffer, water penetrates toward the center of the microsphere. Since PLG is a bulk erosion polymer, the rate of water penetration is faster than the rate of polymer hydrolysis and degradation of PLG copolymers is occurring throughout the microsphere volume. During this degradation and swelling phase, water-filled pores form and grow in size, and the effective diffusivity of protein increases. The protein has to diffuse through water-filled pores and the release is controlled by effective diffusivity and rates of pore formation. The release profiles basically comprise three phases: the initial burst, the lag phase and the steady release phase [29-31]. The initial burst, a relatively fast release of protein in the first few days, may be due to the protein attached to the surface of microspheres, protein near the periphery, and protein encapsulated inside small microspheres. The initial burst is followed by a lag phase of slow protein release which may be caused by the low initial porosity of PLG and a final phase of steady, relatively rapid drug release governed by higher effective diffusivity through water-filled pores. A typical protein release profile is shown in Figure 1.4.

PLG microparticles diameter is intricately related to the degradation rate and protein release properties. For larger particles, water penetration takes longer time and the formation of water-filled pores is slow. The relatively long diffusion distance and slow pores formation rate may lead to the conclusion of slower protein release. However, larger PLG microparticles will accumulate an increased amount of acidic degradation byproduct, leading to an acidic microenvironment inside [32]. This reduced pH will further catalyze the degradation and erosion of PLG. This process is called autocatalysis and will lead to the counter-intuitive result of fast protein release in large microspheres[17].
Figure 1.4 Typical protein release profile from PLG microspheres

1.3 Double-Wall Microspheres and Microcapsules

Double-wall microspheres (DWMS), comprising two distinct polymer core and shell phases and microcapsules (MC), comprising aqueous/oil core and polymer shell phase, are useful controlled release systems. The core-shell structure of DWMS and MC may provide unique opportunities to control drug release rates [33-39]. For example, particle diameter and shell thickness have been shown to affect drug release rates [40]. Also, the degradation and erosion mechanisms of the shell and core materials add a tunable parameter.

1.3.1 Conventional Fabrication of Double-Wall Microspheres and Microcapsules

There are various approaches to produce DWMS and MC. For producing DWMS, the
traditional solvent evaporation method can be used. Two polymers are dissolved in a volatile organic solvent such as dichloromethane. The solution is then added into an aqueous solution containing surfactant and stirred. As the polymers become more concentrated, they begin to phase separate and form the core-shell structure DWMS [34]. This oil-in-water (O/W) method can produce DWMS with core and shell polymers at their thermodynamically stable configurations according to the spreading coefficient theory [41, 42]. The oil-in-oil-in-water (O/O/W) method was used for producing DWMS by different researchers. Lee et al. fabricated etanizadole-loaded DWMS using dichloromethane as organic solvent. First, separate solutions of PLA and PLG in dichloromethane were prepared. The etanizadole was co-dissolved to dichloromethane with PLG. The two polymeric solutions were then added together and sonicated or homogenized to create an oil-in-oil (O/O) emulsion. Addition of the emulsion dropwise into non-solvent solution created an O/O/W emulsion. The emulsion was stirred to allow for the extraction and evaporation of dichloromethane as well as the hardening of the DWMS [38]. Kokai et al. used a similar O/O/W emulsion method to produce solid lysozyme-loaded DWMS [43]. Sanchez et al. used the O/O/W emulsion method to produce DWMS with solid protein powder-loaded oil core MC. Fine particles of protein powder were dispersed in mineral oil using high-speed homogenizer. The suspension was dispersed in PLG acetonitrile/ethyl acetate mixture solution with agitation to produce the O/O emulsion. The resulting organic phase was poured through a narrow orifice into the aqueous non-solvent solution with stirring to produce the O/O/W oil core MC [35].

Besides emulsion methods, layer-by-layer deposition on sacrificial template particles was also used for producing nano- or micro-scale MC [44]. This method involves the deposition of layer-by-layer film components onto the outer surface of colloidal particles that are subsequently removed via chemical or thermal means [45, 46].

For these methods, the control of DWMS and MC dimensions such as outer
diameter and shell thickness are typically poor. The emulsion method can produce DWMS and MC with relatively broad size distribution and the polymer orientation for core and shell may change during fabrication. For layer-by-layer coating, the diameter and shell thickness of MC can be very uniform but these dimensions are controlled by the templates. Besides, this method possesses limitations when generating thick layers or encapsulating a liquid core [47].

1.3.2 Protein Encapsulation and Release in Double-Wall Microspheres and Microcapsules

DWMS and MC have been used for protein delivery. Sanchez et al. reported using oil-core PLG MC for tetanus toxoid delivery. Tetanus toxoid powder was suspended in the mineral oil core phase and surrounded by PLG shell phase. After an initial burst, the systems released tetanus toxoid in a pulsatile manner. Kim et al. produced insulin-loaded PLG MC using a monoaxial ultrasonic atomizer. When the protein solution and the PLG solution were mixed, a water-in-oil (W/O) emulsion was formed within a few seconds. The atomization process resulted in the formation of microdroplets of aqueous solution surrounded by PLG solution. The *in vitro* release profile of insulin consists of two parts: a fast initial burst on the first day, followed by a slow, smooth release for up to 30 days [48]. Kokai et al. using the oil-in-oil-in-water (O/O/W) emulsion method produced DWMS with two polymers PLA and PLG. Initial studies with DWMS encapsulating a fluorescently tagged protein, FITC-BSA, indicated that protein localization was restricted to the PLG core. Protein *in vitro* release was performed using DWMS with the model protein lysozyme encapsulated alone or with the surfactant docusate sodium salt (AOT). Degradation studies showed that DWMS encapsulating lysozyme alone resulted in a core composition of PLG and a shell composition of PLA. In contrast, the polymer orientation of core and shell were reversed due to AOT addition in the PLG solution [43].
1.4 Project Objectives

In this project, we have used the Precision Particle Fabrication method, which will be discussed in detail in Chapter 2, to produce uniform DWMS or MC.

We studied the release profile of a model protein, Bovine Serum Albumin (BSA), encapsulated in the core of DWMS or MC. By applying an additional drug-free polymer layer, we can further control the protein release profile not only by changing degradation rate of the core but also by changing the shell thickness of the drug-free layer and initial drug distribution within the matrices. Figure 1.5 shows the schematic of the DWMS and MC.

For the DWMS structure, we have used PLG as the core material and BSA encapsulated within PLG using double emulsion methods. The shell material is PLA which degrades slower than PLG [49, 50]. We studied the relation between PLA shell/PLG core (denoted as PLA (PLG)) double-wall structure and the BSA release profile. For example, we tried to change the PLA shell thickness and the outer diameter of the DWMS to study the influence on BSA release. Besides, the drug-free PLA shell might change the initial BSA distribution within the DWMS.

For PLG shell/water or oil core microcapsules, we want to study the relation between aqueous core structure and the release rate of protein. By using oil core with suspended lyophilized protein inside, we expect to achieve higher protein loading and better encapsulation efficiency because of less organic solvent contact with BSA in this method. A previous study demonstrated that the distinct phase separation of an aqueous/oil core loaded with BSA and PLG shell resulted in pulsed release upon sufficient degradation of the PLGA shell [47]. By changing PLG shell thickness, we expect to achieve different BSA release profiles.
Figure 1.5 Structures of Double-Wall PLA (PLG) microspheres and PLG (water/oil) microcapsules
Chapter 2. NOZZLE CONFIGURATION FOR PRECISION PARTICLE FABRICATION

2.1 Precision Particle Fabrication

2.1.1 Method Description

The Precision Particle Fabrication (PPF) is a technology developed to produce monodisperse particles of a variety of materials [51-54] and adapted by our group for fabrication of controlled-release devices comprising biodegradable polymers [29, 40, 47, 55-60]. Figure 2.1 shows the set up for PPF.

![Diagram of Precision Particle Fabrication system](image)

Figure 2.1 Schematic diagram of Precision Particle Fabrication system

The PPF system consists of pump system, frequency generator, nozzle system,
visualization system and collecting system. The pump system has one gear pump (IP65, ISMATEC) which is used to carry the non-solvent carrier stream and two syringe pumps (Pump 11, Harvard Apparatus) which are used to carry the polymer/drug solution. For double-wall microsphere (DWMS) and microcapsule (MC) fabrication, two syringe pumps are engaged: one for the core phase and the other for the shell phase. The non-solvent carrier stream (usually aqueous poly (vinyl alcohol) solution) is carried by the gear pump. For single-wall microsphere fabrication, only one syringe pump is engaged. The frequency generator (Agilent 33220A) and piezoelectric transducer (cv33, Sonic & Materials Inc.) generate an acoustic wave on the nozzle to break the laminar polymer-based stream into droplets. We can change the type, amplitude and frequency of the acoustic wave together with the polymer and carrier stream flow rates to produce droplets of desired diameters [29, 47, 55].

The nozzle system is the most important part of PPF. We manually produce double glass nozzle and single glass nozzle systems for producing DWMS or MC and single-wall microspheres. For the double glass nozzle system, we use a hypodermic needle (PrecisionGlide, Becton Dickinson Co.) as the inner metal nozzle, which is surrounded coaxially by the inner glass nozzle made from a glass capillary. The outer glass nozzle surrounds the inner glass nozzle and is made of Pyrex glass. For DWMS and MC fabrication, the core phase polymer/aqueous/oil stream comes through the inner metal nozzle, and the shell phase polymer stream comes through the inner glass nozzle. The outer glass nozzle is for non-solvent carrier stream, which is used to facilitate the forming of round shape particles and providing “drag force” in order to produce particles smaller than the nozzle opening [57, 58]. For the single glass nozzle system, one hypodermic needle is used as inner metal nozzle which is surrounded by the inner glass nozzle. There is no outer glass nozzle in this setting. The drug-polymer stream comes through the inner metal nozzle, and carrier stream comes through inner glass nozzle.
The visualization system consists of a strobe light (Nova Strobe BA, Monarch Instrument) and a video camera (EO Edmund, industrial optics) which is connected to a monitor. By adjusting the frequency of the strobe light the same as the frequency of the acoustic wave, we can get a steady picture of the droplets stream and so monitor the formation of microparticles. The collecting system which is used to collect the nascent particles consists of a glass vial with a stirring bar. Enough non-solvent in the collecting vial should be provided to extract the organic solvent inside the particles [50, 61].

2.1.2 Theory of PPF

The main apparatus of PPF, which provides fabrication of monodisperse microparticles, is based on passing a stream containing the sphere materials and any drug to be encapsulated through a small (10-100 μm) orifice in the nozzle system to form a smooth, cylindrical stream. To break the stream into droplets, the nozzle is vibrated by a piezoelectric transducer driven by an acoustic wave generator at certain frequency. The acoustic energy along the stream generates periodic instabilities that break the stream into a train of uniform droplets. With only the nozzle system, the minimum particle size achievable is slightly larger than the nozzle opening [40, 55, 56]. By employing an annular flow of a non-solvent phase, known as the carrier stream, we can further control the shape and size of the microspheres. The carrier stream is pumped by the gear pump at a linear velocity greater than that of the inner polymer stream. So, the frictional contact between the two streams generates an additional downward force which “pulls” the polymer stream away from the nozzle. Accelerated by this force, the polymer stream is thinned to a degree depending on the difference in linear velocities of the two streams.

To achieve the desired droplet diameter, we need to understand the theory of droplet formation in this system. Lord Rayleigh first derived the jet instability equations for a cylindrical jet subject to disturbance [62]. Lord Rayleigh found that the most unstable
wavelength ($\lambda_{\text{max}}$) of a disturbance imposed on a jet surface is:

$$\lambda_{\text{max}} = 9.016r_j$$  \hspace{1cm} (1)

where $r_j$ is the radius of the undisturbed jet. The theoretical range of wavelengths that still results in the production of uniform droplets was derived by Lord Raleigh to be:

$$7r_j < \lambda < \infty$$  \hspace{1cm} (2)

Above a certain wavelength, the instability growth is so small that noise near the wavelength of the applied acoustic wave causes random breakup of the jet. So the actual range of acoustic wavelengths which can break up a liquid jet into uniform droplets was experimentally determined to be [63]:

$$7r_j < \lambda < 36r_j$$  \hspace{1cm} (3)

The frequency generator used here allows for control of the acoustic wave frequency and amplitude. The wavelength produced by a set frequency is given by:

$$f = \frac{v_j}{\lambda}$$  \hspace{1cm} (4)

where $v_j$ is the linear velocity of the liquid jet. Knowing that the volume of the sphere made should be equal to the volume of the cylindrical element of the jet (5), the length of which is defined by the acoustic wavelength, we can find that the droplet radius, $r_d$, is given by (6).

$$\frac{4}{3} \cdot \pi \cdot r_d^3 = \pi \cdot r_j^2 \cdot \lambda = \pi \cdot r_j^2 \cdot \frac{v_j}{f}$$  \hspace{1cm} (5)

$$r_d = \left(3r_j^2v_j/4f\right)^{1/3}$$  \hspace{1cm} (6)

At the optimum wavelength (put equation (1) into equation (6)), $r_{d,\text{max}}=1.891r_j$. Thus, by imposing acoustic wave on the nozzle, we can control the breakup of the stream into droplets and predict the nozzle opening size ($\sim r_j$), solution flow rate ($v_j$) and acoustic frequency ($f$) needed to generate the desired sphere size [58].

For this project, we use double glass nozzle or single glass nozzle configuration at fixed nozzle opening to produce different samples. By changing the flow rate of the
polymer stream, we can get the droplet size close to what we desired. Using equation (6), by changing acoustic wave frequency, we can make the minor adjustment of the droplet size to what we want to within 1 micron.

2.2 Nozzle Screening for PPF

As mentioned before, the nozzle system is the most important part of the whole PPF. A “well-behaved”, reliable and sturdy nozzle is the key part of successful PPF particle fabrication. We tried several combinations of the outer glass nozzle, inner glass nozzle and inner metal nozzle and found that the dimension and the inner curvature of the glass nozzles are important for successful particle fabrication.

2.2.1 Curvature of Nozzles

The outer Pyrex glass nozzle and inner glass nozzle are both made by hand. The manufacturing processes are similar. For outer Pyrex glass nozzle, we cut Pyrex glass tube (Kimax) into approximately 1 inch long piece. The outer diameter of the tube is 2.5 mm and the wall thickness is 0.5 mm. We slowly rotated and melted one end of the Pyrex tube by propane flame until the end was sealed, and then sanded the melted end on a sandpaper until a small opening (1/5 to 1/4 of the inner diameter) was exposed. The dimension of the opening might not be either too small (blocked frequently) or too big (cannot generate smooth jet). For inner glass nozzle, we use commercialized Borosilicate glass capillary (World Precision Instrument, Inc.). There are six types of capillary with different outer diameter and wall thickness: 1.0 mm R (outer diameter 1.0 mm and regular wall thickness), 1.0 mm TW (outer diameter 1.0 mm and thin wall thickness), 1.2 mm R (outer diameter 1.2 mm and regular wall thickness), 1.2 mm TW (outer diameter 1.2 mm and thin wall thickness), 1.5 mm R (outer diameter 1.5 mm and regular wall thickness), and 1.5 mm TW (outer diameter 1.5 mm and thin wall thickness). Figure 2.2
shows the outer glass nozzle and six types of inner glass nozzles.

Figure 2.2 Outer and inner glass nozzles

Figure 2.3 “Bad” (left) and “Good” (right) nozzles

We used PLG solution (10% w/v in dichloromethane) and 0.5% (w/v) poly (vinyl alcohol) (PVA) as the carrier stream to test the behavior of nozzles to form steady, uniform droplets using PPF. We found that the inner curvatures of outer and inner glass nozzles played an important role in forming steady trains of droplets. The “well-behaved” nozzles all had “blunt” inner curvatures while the “poorly-behaved” nozzles had “sharp”
inner curvatures (Figure 2.3).

The reason for this phenomenon might be that the “blunt” inner curvature would lead the laminar flow of polymer solution in a smooth way through the nozzle opening while the “sharp” inner curvature would cause turbulence when the flow was squeezed by the nozzle. Also for inner glass nozzle, “blunt” inner curvature was crucial and if the outside of the nozzle head was tempered to give more room for carrier stream within outer and inner glass nozzles (Figure 2.2 1.5 mm R), the jets coming through the nozzles would be more steady and smooth.

2.2.2 Dimension of Nozzles

The dimension of inner glass nozzle and inner metal nozzles were also important for forming steady monodisperse droplet steams. We had only one type of outer glass nozzle which was Pyrex glass tube (outer diameter=2.5 mm, wall thickness=0.5 mm), six types of inner glass capillary (1.0 mm R, 1.0 mm TW, 1.2 mm R, 1.2 mm TW, 1.5 mm R, 1.5 mm TW) and three types of hypodermic needle as inner metal nozzles (Gauge 23, Gauge 25, Gauge 27). By screening different combinations using PLG solution (10% w/v in dichloromethane), we found the proper configuration. Table 2.1 shows the results of the nozzle screening.

Table 2.1 Nozzle configuration screening for PPF

<table>
<thead>
<tr>
<th>Outer Glass Nozzle</th>
<th>Inner Glass Nozzle</th>
<th>Inner Metal Nozzle</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD=2.5 mm WT=0.5 mm</td>
<td>1.0 mm TW</td>
<td>Gauge 27</td>
<td>Inner glass nozzle broke frequently</td>
</tr>
<tr>
<td>OD=2.5 mm WT=0.5 mm</td>
<td>1.0 mm R</td>
<td>Gauge 27</td>
<td>Could not form droplets</td>
</tr>
<tr>
<td>OD=2.5 mm WT=0.5 mm</td>
<td>1.2 mm TW</td>
<td>Gauge 25, 27</td>
<td>Inner glass nozzle broke frequently</td>
</tr>
<tr>
<td>OD=2.5 mm WT=0.5 mm</td>
<td>1.2 mm R</td>
<td>Gauge 25, 27</td>
<td>Hard to form droplets</td>
</tr>
<tr>
<td>OD=2.5 mm WT=0.5 mm</td>
<td>1.5 mm TW</td>
<td>Gauge 23, 25, 27</td>
<td>Inner glass nozzle broke frequently</td>
</tr>
<tr>
<td>OD=2.5 mm WT=0.5 mm</td>
<td>1.5 mm R</td>
<td>Gauge 23, 25</td>
<td>No major problems</td>
</tr>
<tr>
<td>N/A</td>
<td>1.5 mm R</td>
<td>Gauge 23, 25</td>
<td>No major problems</td>
</tr>
</tbody>
</table>
From screening results, we found that for double glass nozzle configuration, the optimum nozzle setting was: outer glass nozzle (OD=2.5 mm WT=0.5 mm), inner glass nozzle (1.5 mm R), inner metal nozzle (gauge 23, 25). For single glass nozzle configuration, the optimum nozzle setting was simply the optimum double glass nozzle configuration without outer glass nozzle.

2.3 Conclusions

Precision Particle Fabrication can produce monodisperse microparticles at desired diameter by changing nozzle opening, polymer flow rate and frequency of acoustic wave.

The nozzle system is the most important part of the whole PPF and is responsible for successful uniform particle fabrication. Through several trials, we found that the “blunt” inner curvature nozzles’ performance in producing uniform particles was much better than that of nozzles with “sharp” inner curvatures. Also, different combinations of the inner glass nozzles and inner metal nozzles have tremendous effect on nozzle behavior.

After screening, we found the optimum configurations of double glass nozzle and single glass nozzle shown in Table 2.2. Micrographs of optimized double glass nozzle and single glass nozzle are shown in Figure 2.4. Optimized double and single glass nozzle settings are used for future DWMS and single-wall microspheres fabrication.

<table>
<thead>
<tr>
<th>Single Glass Nozzle Configuration</th>
<th>Double Glass Nozzle Configuration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outer Glass Nozzle</td>
<td>Outer Glass Nozzle OD=2.5 mm WT=0.5 mm</td>
</tr>
<tr>
<td>Inner Glass Nozzle</td>
<td>Inner Glass Nozzle 1.5 mm R</td>
</tr>
<tr>
<td>Inner Metal Nozzle</td>
<td>Inner Metal Nozzle Gauge 23</td>
</tr>
</tbody>
</table>

Table 2.2 Single/Double glass nozzles configuration for PPF
Figure 2.4 Optimized single and double glass nozzle configurations
Chapter 3. UNIFORM BOVINE SERUM ALBUMIN LOADED DOUBLE-WALL/SINGLE WALL MICROSPHERES FABRICATION USING PPF

3.1 Precision Particle Fabrication Parameters

Using PPF, we can produce monodisperse double-wall microspheres (DWMS) and single-wall microspheres at desired outer diameter and shell thickness [49, 55, 58, 61]. By changing the polymer-drug solution flow rates, we can change the laminar jet diameter coming through the nozzle systems; thus, we can change the droplets diameter in a broad range. Then, by adjusting the PPF parameters such as frequency, amplitude and the flow rate of the carrier stream, we can achieve the round shape droplets at desired diameter within 1 micron [47, 58, 60].

3.1.1 Double-Wall Microspheres PPF Parameters

For producing DWMS, the double glass nozzle configuration was used (Table 2.2). The drug-loaded or drug-free poly (lactide-co-glycolide) (PLG) solution stream passed through the inner metal nozzle and the drug free poly (lactic acid) (PLA) solution stream passed through the inner glass nozzle. The outer glass nozzle was used for carrier stream (0.5% (w/v) PVA solution). The frequency generator of PPF can form acoustic waves of different type (sine, square, ramp, pulse, noise and arbitrary), amplitude and frequency. By changing these parameters together with the carrier stream flow rates, we can generate uniform droplets of different size. The experimental parameters are shown in table 3.1.

3.1.2 Single-Wall Microspheres PPF Parameters

For single-wall microspheres, the single glass nozzle configuration was used (Table 2.2). There was no outer glass nozzle and drug-loaded PLG stream passed through the inner metal nozzle while the inner glass nozzle was used for the carrier stream. The
experimental parameters are also shown in Table 3.1.

Table 3.1 PPF parameters for single-wall,double-wall microspheres

<table>
<thead>
<tr>
<th></th>
<th>Single-Wall Microspheres</th>
<th>Double-Wall Microspheres</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nozzle Configuration</td>
<td>Single Glass Nozzle</td>
<td>Double Glass Nozzle</td>
</tr>
<tr>
<td>Shell Flow Rate (mL/hr.)</td>
<td>0</td>
<td>36</td>
</tr>
<tr>
<td>Core Flow Rate (mL/hr.)</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Ultrasonic Wave Amplitude (V)</td>
<td>+5.00 (Maximum)</td>
<td>+5.00 (Maximum)</td>
</tr>
<tr>
<td>Ultrasonic Wave Frequency (KHz)</td>
<td>7.5</td>
<td>5</td>
</tr>
<tr>
<td>Ultrasonic Wave Type</td>
<td>Sine</td>
<td>Sine</td>
</tr>
<tr>
<td>PVA Flow Rate (mL/hr.)</td>
<td>150-500</td>
<td>500-1100</td>
</tr>
</tbody>
</table>

3.2 Materials and Methods

3.2.1 Materials

The polymers used for making double-wall/single wall microspheres are PLG for core phase and PLA for shell phase. The biodegradable polymers were purchased from LACTEL Absorbable Polymers. The inherent viscosity (i.v.) of PLG, PLA and the corresponding molecular weight is shown in Table 3.2. For PLA, when inherent viscosity is low, 0.34-0.70 dL/g, the chiral structure is poly (D, L-lactide). For inherent viscosity of 1.05 dL/g, the chiral structure was poly (L-lactide). Chromatography grade of ethyl acetate and dichloromethane were obtained from Sigma-Aldrich. Bovine Serum Albumin (BSA or “Fraction V”, Molecular Weight 66,700 Da) purchased from Fisher Scientific was used as model protein.

3.2.2 PPF Fabrication Method

Using the PPF system with frequency generator, pump system, visualization system and collecting system, we employed a double glass nozzle to create core-shell DWMS. The core phase contains Bovine Serum Albumin (BSA) water solution emulsified with
PLG/dichloromethane (DCM) solution. This “double-emulsion” method began with dissolving BSA into deionized water at a concentration of 100 mg/mL. The BSA/water solution was emulsified with PLG/DCM solution (10% (w/v) PLG) at a volume ratio of 1:10 using Branson Ultrasonic tip at 60% amplitude for 1 minute. The drug-polymer emulsion should be used for PPF within 3 hours. The shell phase is 3% (w/v) PLA dissolved in either ethyl acetate (EtAc) or DCM.

Table 3.2 PLG and PLA inherent viscosity to molecular weight

<table>
<thead>
<tr>
<th></th>
<th>Inherent Viscosity (i.v., dL/g)</th>
<th>Molecular Weight (MW, Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLG (poly (lactide-co-glycolide))</td>
<td>0.20</td>
<td>4,200</td>
</tr>
<tr>
<td>PLG (poly (lactide-co-glycolide))</td>
<td>0.38</td>
<td>15,000</td>
</tr>
<tr>
<td>PLG (poly (lactide-co-glycolide))</td>
<td>0.61</td>
<td>38,000</td>
</tr>
<tr>
<td>PLA (poly (D, L-lactide))</td>
<td>0.34</td>
<td>38,000</td>
</tr>
<tr>
<td>PLA (poly (D, L-lactide))</td>
<td>0.37</td>
<td>43,000</td>
</tr>
<tr>
<td>PLA (poly (D, L-lactide))</td>
<td>0.70</td>
<td>106,000</td>
</tr>
<tr>
<td>PLA (poly (L-lactide))</td>
<td>1.05</td>
<td>192,000</td>
</tr>
</tbody>
</table>

The core BSA/water-PLG/DCM emulsion was put into one of the syringe pump in PPF system and the PLA EtAc or DCM solution was put into the other syringe pump. The 0.5% (w/v) poly (vinyl alcohol) (PVA) water solution as carrier stream was put into the gear pump. Each solution was pumped to the nozzle at specific flow rates shown in Table 3.1. The frequency generator was turned on after the stream coming out of the nozzle system was steady. The frequency, amplitude and PVA flow rate were adjusted to produce a steady, uniform droplets stream. To visualize and monitor the fabrication process, turned on the strobe light and set the frequency of the strobe light the same as the acoustic wave frequency. The droplets stream was visualized by the video camera connected to the computer/TV monitor. Nascent DWMS were collected in a 500 mL beaker with 200-500 mL of 0.5% (w/v) PVA solution and were stirred for another 3 hours for organic solvent extraction/evaporation. Then the particles were filtered (Filter Paper
#4, Whatman) and washed three times by deionized water and lyophilized for 48 hours. Samples were stored until use in a -20 °C freezer with desiccant.

For single-wall microspheres, single glass nozzle was employed and BSA-loaded PLG stream was put into one syringe pump. The concentration of BSA/water solution and PLG/DCM solution was the same as DWMS fabrication. And so was the process for emulsification. No shell phase was needed for single-wall microspheres and using parameters in Table 3.1, uniform particles could be formed at desired diameter. Nascent single-wall microspheres in 200-500 mL of 0.5% (w/v) PVA solution were stirred for another 3 hours. The particles were filtered, washed by deionized water, as above, and lyophilized for 48 hours. Samples were stored in a -20 °C freezer with desiccant.

3.2.3 Size Distribution

The size distributions of nascent microspheres (wet particles before lyophilizing) were determined using a Beckman Coulter Multisizer III. The particle size of nascent droplets would decrease as the organic solvent was extracted by the PVA solution. After 30-40 minutes, the size of microparticles remained constant, and then we tested the size distributions using a 200 micron aperture [58]. More than 10,000 spheres were measured for every sample.

3.2.4 Solvent Selection for Double-Wall Microspheres

For dissolving biodegradable polymers such as PLG and PLA, organic solvents such as ethyl acetate (EtAc) and dichloromethane (DCM) are normally used [2, 19, 31]. Figure 3.1 shows the size distribution of DWMS with EtAc and DCM as shell or core solvents.

Using DCM as both core and shell solvent (denoted as DCM (DCM), Figure 3.1 A), the primary particle size distribution was narrow and the uniformity of DWMS was good. Using EtAc as shell solvent and using DCM as core solvent (EtAc (DCM), Figure 3.1 B),
the primary particle size distribution was also narrow and uniformity was good although not as good as DCM (DCM). There were “bumps” just before the main peak, showing some particles smaller than desired diameter formed in this solvent configuration. This was probably due to the fast extraction rate of EtAc in the dilute shell phase (3% (w/v) PLA in EtAc) by PVA solution. Not fully encapsulated core-shell structured DWMS were produced as a result [64]. In both of these cases, the volume percent of the main peaks were high (around 10%). However, when EtAc was used as core solvent and whether the shell solvent was DCM or EtAc (DCM (EtAc), EtAc (EtAc)), the uniformity was poor. This was probably because the fast extraction of EtAc from the condensed core to the PVA solution jeopardized the formation of the core-shell structure. When DCM was used as core solvent, due to the slow evaporation rate of DCM to PVA solution, the condensed core is better confined during solvent evaporation, and monodisperse DWMS could be formed. So the DCM (DCM) and EtAc (DCM) solvent configurations are good choices for producing monodisperse DWMS using PPF.
Figure 3.1 Size distributions of different solvent configurations: (A) DCM as both shell and core solvent; (B) EtAc as shell solvent and DCM as core solvent; (C) DCM as shell solvent and EtAc as core solvent; (D) EtAc as both shell and core solvent.
3.2.5 Double-Wall Microspheres Fabrication

3.2.5.1 EtAc (DCM) Double-Wall Microspheres

The solvent configuration of EtAc (DCM) was used to produce monodisperse DWMS. The core phase was 100 mg/mL BSA/water solution emulsified with 10% (w/v) PLG/DCM solution at a volume ratio of 1:10. The shell phase was 3% (w/v) PLA solution. PVA (0.5%) was used as carrier stream. The flow rates of core, shell and carrier stream were 4 mL/hour, 36 mL/hour and 500-1100 mL/hour. Other PPF parameters are shown in Table 3.1.

Firstly, we kept the core polymer PLG inherent viscosity at a constant 0.20 dL/g and increased the shell PLA inherent viscosity from 0.37 dL/g to 1.05 dL/g. Figure 3.2 shows the size distribution for these samples. Figure 3.2 A is the size distribution for PLG i.v. 0.20 and PLA i.v. 0.37 (denoted as Sample A1), the uniformity is good and the measured outer diameter by Coulter Multisizer III is $55.1 \pm 2.0 \mu m$. Using the material balance of PLG and PLA, and assuming 100% yield of particles and total phase separation of PLG and PLA, the calculated PLG core diameter is $35.8 \mu m$ and the thickness of PLA drug free shell of A1 is $9.7 \mu m$ (denoted as $9.7 \ (35.8) \mu m$). Figure 3.2 B is the size distribution for PLG i.v. 0.20 dL/g and PLA i.v. 0.70 dL/g (Sample A2). Good monodispersity and high volume percent of the main peak are shown in the distribution curve. Measured diameter is $56.8 \pm 2.8 \mu m$, and calculated core diameter and shell thickness are 36.9 and 10.0 μm (10.0 (36.9) μm). PLA i.v. 1.05 dL/g could not dissolved in EtAc.

Secondly, we kept the shell polymer PLA inherent viscosity constant at 0.34 dL/g and increased PLG inherent viscosity from 0.20 dL/g to 0.38 dL/g and 0.61 dL/g (samples A3, A4 and A5). Figure 3.3 shows the distributions of these samples (A-A3, B-A4 and C-A5).
All three samples exhibit good uniformity and high main peak volume percent. The measured outer diameters are 56.4±2.4 µm, 55.5±2.0 µm and 55.0±1.6 µm for A3, A4 and A5. Still using the 100% yield and total phase separation assumption, the calculated core diameter and shell thicknesses are 9.9 (36.7) µm, 9.7 (36.1) µm and 9.6 (35.7) µm, respectively.
Figure 3.2 Size distributions of EtAc (DCM) DWMS: (A) Sample A1, PLG i.v. 0.20 and PLA i.v. 0.37; (B) Sample A2, PLG i.v. 0.20 and PLA i.v. 0.70
Figure 3.3 Size distributions of EtAc (DCM) DWMS: (A) Sample A3, PLG i.v. 0.20 and PLA i.v. 0.34; (B) Sample A4, PLG i.v. 0.38 and PLA i.v. 0.34; (C) Sample A5, PLG i.v. 0.61 and PLA i.v. 0.34.
3.2.5.2 DCM (DCM) Double-Wall Microspheres

For DCM (DCM) configuration, we employed shell polymer PLA inherent viscosities of 0.37 dL/g, 0.70 dL/g and 1.05 dL/g and kept the core polymer PLG inherent viscosity constant at 0.20 dL/g (sample C1, C2 and C3). Figure 3.4 shows the size distributions of these samples (A-C1, B-C2 and C-C3). All three samples had very good uniformity except some small droplets were formed in the 40-50 µm range. The measured outer diameter of C1, C2 and C3 are 54.8±1.4 µm, 55.4±1.7 µm and 56.6±2.1 µm while the calculated core diameter and shell thickness are 9.6 (35.6) µm, 9.7 (36.0) µm and 9.9 (36.8) respectively.

3.2.6 Single-Wall Microspheres Fabrication

PLG single-wall microspheres were produced using PPF to mimic the PLG core in DWMS. From the above experiments, the calculated diameters of PLG cores were 35-37 µm which was set as the desired diameter. Using only one pump and PPF parameters show in Table 3.1, we produced uniform single-wall microspheres with diameter 35.2±1.0 µm (Sample E1). Figure 3.5 shows the distribution curve.

3.3 Conclusions

Using PPF, we produced monodisperse DWMS with PLG-BSA core and PLA shell. For organic solvents DCM and EtAc, we found that using DCM as the core PLG phase solvent and either DCM or EtAc as the shell PLA phase solvent could produce monodisperse DWMS as desired. However, using EtAc as core PLG phase solvent would lead to DWMS with poor monodispersity.

Using EtAc (DCM) and DCM (DCM) we produced monodisperse DWMS with different PLG and PLA inherent viscosity. The outer diameters of these samples are
similar (within 2 microns). We also produced uniform single-wall microspheres of the same diameter as the calculated core diameter of DWMS for comparison. Table 3.3 shows the outer diameters, calculated core diameters and calculated shell thickness of these samples.
Figure 3.4 Size distributions of DCM (DCM) DWMS: (A) Sample C1, PLG i.v. 0.20 and PLA i.v. 0.37; (B) Sample C2, PLG i.v. 0.20 and PLA i.v. 0.70; (C) Sample C3, PLG i.v. 0.20 and PLA i.v. 1.05.
Figure 3.5 Size distribution of single-wall microspheres E1, PLG i.v. 0.20
<table>
<thead>
<tr>
<th>Code</th>
<th>Solvent Selection Shell (Core)</th>
<th>PDLL or PLL Shell i.v.</th>
<th>PLG core i.v.</th>
<th>Outer Diameter Measured (μm)</th>
<th>Core Diameter Calculated (μm)</th>
<th>Shell Thickness Calculated (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>EtAc (DCM)</td>
<td>0.37</td>
<td>0.20</td>
<td>55.1±2.0</td>
<td>35.8</td>
<td>9.7</td>
</tr>
<tr>
<td>A2</td>
<td>EtAc (DCM)</td>
<td>0.70</td>
<td>0.20</td>
<td>56.8±2.8</td>
<td>36.9</td>
<td>10.0</td>
</tr>
<tr>
<td>N/A</td>
<td>EtAc (DCM)</td>
<td>1.05</td>
<td>0.20</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>A3</td>
<td>EtAc (DCM)</td>
<td>0.34</td>
<td>0.20</td>
<td>56.4±2.4</td>
<td>36.7</td>
<td>9.9</td>
</tr>
<tr>
<td>A4</td>
<td>EtAc (DCM)</td>
<td>0.34</td>
<td>0.38</td>
<td>55.5±2.0</td>
<td>36.1</td>
<td>9.7</td>
</tr>
<tr>
<td>A5</td>
<td>EtAc (DCM)</td>
<td>0.34</td>
<td>0.61</td>
<td>55.0±1.6</td>
<td>35.7</td>
<td>9.6</td>
</tr>
<tr>
<td>C1</td>
<td>DCM (DCM)</td>
<td>0.37</td>
<td>0.20</td>
<td>54.8±1.4</td>
<td>35.6</td>
<td>9.6</td>
</tr>
<tr>
<td>C2</td>
<td>DCM (DCM)</td>
<td>0.70</td>
<td>0.20</td>
<td>55.4±1.7</td>
<td>36.0</td>
<td>9.7</td>
</tr>
<tr>
<td>C3</td>
<td>DCM (DCM)</td>
<td>1.05</td>
<td>0.20</td>
<td>56.6±2.1</td>
<td>36.8</td>
<td>9.9</td>
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<tr>
<td>E1</td>
<td>(DCM)</td>
<td>N/A</td>
<td>0.20</td>
<td>35.2 ±1.0</td>
<td>35.2</td>
<td>0</td>
</tr>
</tbody>
</table>
Chapter 4. CHARACTERIZATION AND IN VITRO RELEASE OF UNIFORM BSA LOADED DOUBLE-WALL/SINGLE-WALL MICROSPHERES

4.1 Loading Test

To test the loading of each batch of microparticles produced in Chapter 3, a sample of approximately 5 mg was dissolved in 100 µL dimethyl sulfoxide (DMSO). After complete dissolution, the solution was pipetted into 1 mL of phosphate-buffered saline (PBS, pH 7.4±0.05) then incubated for 1 hour in 37 °C incubator shaking at 240 rpm. Next, the mixture was centrifuged for 10 minutes at 10,000 rpm to settle the precipitate. BSA concentration in the supernatant was determined using BCA assay (Pierce) according to the protocol provided by the manufacturer. All absorbance measurements were taken on a SpectraMax 340 PC equipped with SoFTMax Pro software. The loading of each batch equaled the mass of BSA measured by absorbance per mass of particles. The encapsulation efficiency of each batch of microspheres equaled the actual loading divided by theoretical BSA loading multiplied by 100%. Table 4.1, Figure 4.1 and 4.2 show the loading and encapsulation efficiency of different batches.

Table 4.1 Loading and encapsulation efficiency of double-wall/single-wall microspheres

<table>
<thead>
<tr>
<th>Code</th>
<th>Solvent Selection</th>
<th>PLA Shell (Core)</th>
<th>PLG Shell i.v.</th>
<th>BSA Loading (µg BSA/mg Particle)</th>
<th>Encapsulation Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>EtAc (DCM)</td>
<td>0.37</td>
<td>0.20</td>
<td>10.81</td>
<td>42.73</td>
</tr>
<tr>
<td>A2</td>
<td>EtAc (DCM)</td>
<td>0.70</td>
<td>0.20</td>
<td>13.82</td>
<td>54.66</td>
</tr>
<tr>
<td>N/A</td>
<td>EtAc (DCM)</td>
<td>1.05</td>
<td>0.20</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>A3</td>
<td>EtAc (DCM)</td>
<td>0.34</td>
<td>0.20</td>
<td>8.43</td>
<td>33.35</td>
</tr>
<tr>
<td>A4</td>
<td>EtAc (DCM)</td>
<td>0.34</td>
<td>0.38</td>
<td>7.37</td>
<td>29.13</td>
</tr>
<tr>
<td>A5</td>
<td>EtAc (DCM)</td>
<td>0.34</td>
<td>0.61</td>
<td>6.42</td>
<td>25.37</td>
</tr>
<tr>
<td>C1</td>
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<td>0.20</td>
<td>5.00</td>
<td>19.78</td>
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<tr>
<td>C2</td>
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<td>5.18</td>
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<tr>
<td>C3</td>
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<td>8.31</td>
<td>32.86</td>
</tr>
<tr>
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<td>(DCM)</td>
<td>N/A</td>
<td>0.20</td>
<td>17.66</td>
<td>19.42</td>
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</table>
Figure 4.1 BSA loading of double-wall/single-wall microspheres: (A1) EtAc (DCM), PLG i.v. 0.20 and PLA i.v. 0.37; (A2) EtAc (DCM), PLG i.v. 0.20 and PLA i.v. 0.70; (A3) EtAc (DCM), PLG i.v. 0.20 and PLA i.v. 0.34; (A4) EtAc (DCM), PLG i.v. 0.38 and PLA i.v. 0.34; (A5) EtAc (DCM), PLG i.v. 0.61 and PLA i.v. 0.34; (C1) DCM (DCM), PLG i.v. 0.20 and PLA i.v. 0.37; (C2) DCM (DCM), PLG i.v. 0.20 and PLA i.v. 0.70; (C3) DCM (DCM), PLG i.v. 0.20 and PLA i.v. 1.05; (E1) (DCM), PLG i.v. 0.20.
Figure 4.2 BSA encapsulation efficiencies of double-wall/single-wall microspheres: (A1) EtAc (DCM), PLG i.v. 0.20 and PLA i.v. 0.37; (A2) EtAc (DCM), PLG i.v. 0.20 and PLA i.v. 0.70; (A3) EtAc (DCM), PLG i.v. 0.20 and PLA i.v. 0.34; (A4) EtAc(DCM), PLG i.v. 0.38 and PLA i.v. 0.34; (A5) EtAc (DCM), PLG i.v. 0.61 and PLA i.v. 0.34; (C1) DCM (DCM), PLG i.v. 0.20 and PLA i.v. 0.37; (C2) DCM (DCM), PLG i.v. 0.20 and PLA i.v. 1.05; (E1) (DCM), PLG i.v. 0.20.

DCM has low boiling point and subsequently fast evaporation rate. Because of its low water solubility (1.6% w/w), the primary particle hardening mechanism is by solvent evaporation. The assumption has been made here that the rate of extraction of solvent from the microparticles is not limiting compared to the rate of solvent evaporation [64]. For EtAc, the increased water solubility (8.7% w/w) leads to a much faster removal of solvent from nascent particles. However, EtAc has a higher boiling point thus the evaporation process will be significantly slowed. Despite this offsetting extraction/evaporation interplay, a previous study has shown that EtAc-based
microspheres harden faster than those formed with DCM [65]. From Figure 4.1 and 4.2, we found the BSA loading and encapsulation efficiency of EtAc (DCM) DWMS A1, A2, A3, A4 and A5 were in general higher than DCM (DCM) DWMS C1 and C2 but not C3. This is probably due to fast extraction of the shell solvent EtAc, which can provide better encapsulation of the BSA-PLG core and thus keep as much BSA as possible within the microspheres during solvent extraction/evaporation. When DCM was used as shell solvent, the slow evaporation of DCM in both shell and core would allow BSA diffusion toward the particle surface and poor encapsulation of the BSA-PLG core by PLA.

For EtAc (DCM) DWMS, increasing shell PLA inherent viscosity from 0.37 dL/g (Mw 43,000 Da) to 0.70 dL/g (Mw 106,000 Da) with the core PLG inherent viscosity constant at 0.20 dL/g (Mw 4,200), increased the loading and encapsulation efficiency. This is also the case for DCM (DCM) DWMS when shell PLA inherent viscosity increased from 0.37 dL/g (Mw 43,000 Da) to 0.70 dL/g (Mw 106,000 Da) and 1.05 dL/g (Mw 192,000 Da). For sample C3, which contained 1.05 dL/g PLA shell, the chirality changed from poly (D, L-lactide) to poly (L-lactide), the BSA loading and encapsulation efficiency increased dramatically compared to C1 and C2. For both EtAc (DCM) and DCM (DCM), increasing shell PLA inherent viscosity (molecular weight) would lead to higher loading and encapsulation. This is probably because the higher molecular weight PLA shell was more hydrophobic and could better confine the BSA/water particulate into the PLG core region [66]. For samples A3, A4 and A5, using EtAc (DCM) and constant shell PLA inherent viscosity of 0.34 dL/g (Mw 38,000 Da) while increasing core PLG inherent viscosity from 0.20 dL/g (Mw 4,200 Da), 0.38 dL/g (Mw 15,000 Da) to 0.61 dL/g (Mw 38,000 Da), the loading and encapsulation efficiency decreased. We cannot yet explain this phenomenon. However, the microscopy study of the DWMS reported below will provide additional insight.

For PLG single-wall microspheres at inherent viscosity 0.20 dL/g, the loading was
higher than all DWMS because there was no drug-free PLA layer. On the other hand, the encapsulation efficiency of single-wall microspheres was lower than all DWMS. The reason for this might be that the lack of drug-free PLA layer could lead to easier diffusion or escape of BSA out of the microspheres and thus poorer encapsulation of the BSA.

4.2 Microscopy

4.2.1 Scanning Electron Microscope

Hardened double-wall and single-wall microspheres were prepared for imaging by placing a droplet of an aqueous microsphere suspension on a silicon stub. The samples were dried overnight and were sputter coated with gold and platinum prior to imaging [58].

In order to image the cross-section of the microspheres, we first froze microspheres in a 1.7 mL micro-centrifuge tube immersed in liquid nitrogen. Then we chopped the frozen microspheres using a blade on a glass slide. The JOEL 6060 LV Scanning Electron Microscope was used at an acceleration voltage of 5-20 kV.
Figure 4.3 SEM general view, close view and cross-section view of DWMS: (A, B, C) Sample A1, EtAc (DCM), PLG i.v. 0.20 and PLA i.v. 0.37; (D, E, F) Sample A2, EtAc (DCM), PLG i.v. 0.20 and PLA i.v. 0.70.
Figure 4.4 SEM general view, close view and cross-section view of DWMS: (A, B, C) Sample A3, EtAc (DCM), PLG i.v. 0.20 and PLA i.v. 0.34; (D, E, F) Sample A4, EtAc (DCM), PLG i.v. 0.38 and PLA i.v. 0.34; (G, H, I) Sample A5, EtAc (DCM), PLG i.v. 0.61 and PLA i.v. 0.34.
Figure 4.5 SEM general view, close view and cross-section view of DWMS: (A, B, C) Sample C1, DCM (DCM), PLG i.v. 0.20 and PLA i.v. 0.37; (D, E, F) Sample C2, DCM (DCM), PLG i.v. 0.20 and PLA i.v. 0.70; (G, H, I) Sample C3, DCM (DCM), PLG i.v. 0.20 and PLA i.v. 1.05.

Figure 4.6 SEM general view, close view and cross-section view of single-wall microspheres: (A, B, C) Sample E1, (DCM), PLG i.v. 0.20.

Figure 4.3 shows the general view, close view and cross-section view of DWMS samples A1 and A2. From the general view and close view, we found for EtAc (DCM) solvent configuration, the DWMS exhibited relatively smooth surfaces. There were small pores on the surface of A1 and A2, which were probably caused by EtAc extraction. For A2, large concave indentions were observed on the surface, which might be caused by the
dense PLA shell collapsing toward the porous PLG inner core during solvent extraction/evaporation. For A1 and A2, there were clearly porous core areas which might be caused by aggregation of BSA/water droplets in the relatively hydrophilic low molecular weight PLG. The dense PLA shell areas were approximately 10 µm thick as calculated before.

Figure 4.4 shows the general view, close view and cross-section view of DWMS samples A3-A5 with solvent configuration of EtAc (DCM). For A3, the DWMS still exhibited smooth surfaces. There were porous core area and dense PLA shell area in cross-section of A3 like A1 and A2. However, for A4 and A5, small “bumps” could be seen on the surface. This might be because for A3 as well as A1 and A2, the core PLG inherent viscosity was low (0.20 dL/g, Mw 4,200 Da) and good core encapsulation was achieved by the PLA shell, while for A4 and A5, the core PLG inherent viscosity was high (0.38 dL/g, Mw 15,000 Da, 0.61 dL/g, Mw 38,000 Da) and the relatively hydrophobic nature of high molecular weight PLG would cause poor encapsulation by PLA. The “bumps” visible on the surface might be PLG. Also, there were several big “craters” or pores on the surfaces of A4 and A5, which likely result from aggregated BSA/water emulsion droplets reaching the surfaces. The cross-section images show several large pores, presumably resulting from BSA/water droplets, very near the particle surface, which supports the above hypothesis. For A4 and A5, the cross-section images showed no evidence of a porous core and a relatively dense shell area. This might be because the hydrophobic high molecular weight PLG could not be effectively confined by PLA and BSA/water particulate aggregations spread throughout the particles.

Figure 4.5 shows the general view, close view and cross-section view of DWMS samples C1-C3 with solvent configuration of DCM (DCM). The particle surfaces were much more porous than A1-A5 with many large holes. No clearly defined core and shell regions could be identified in the cross-sections. This probably was because the slow
evaporation rate of DCM in both shell and core areas would allow the BSA/water domains to diffuse throughout the microspheres. The encapsulation and confinement of the BSA-PLG core were poor compared to EtAc (DCM) solvent configuration.

Figure 4.6 shows the SEM images of single-wall microspheres with low PLG molecular weight (i.v. 0.20 dL/g, Mw 4,200 Da) mimicking the core area of DWMS. The cross-section view also showed the porous inner structures.

4.2.2 Confocal Fluorescent Microscope

The BSA used for double-wall/single-wall microspheres fabrication was labeled with fluorescent dye 5-(and-6)-Carboxytetramethylrhodamine succinimidyl ester (TAMRA) obtained from Molecular Probes. Twenty milligrams of BSA were dissolved in 2 mL of sodium bicarbonate (Fisher) at pH 8.3±0.05. A dye solution of 1 mg TAMRA in 100 µL DMSO (Fisher) was then pipetted into a foil-wrapped vial containing the BSA solution. The solution was stirred for 60 minutes at room temperature, and then separated using PD-10 desalting column (GE Healthcare). The labeled protein was collected from the column, frozen, and lyophilized. The degree of labeling (DoL), which is the number of TAMRA molecules attached to each protein molecule, was determined by the Molecular Probes established protocol using NanoDrop spectrophotometer (Thermo Scientific). The DoL for BSA-TAMRA used for DWMS fabrication was 3.40. And during fabrication, 5% of TAMRA labeled BSA and 95% unlabeled BSA were used.

Optical and fluorescent images of the protein-loaded double-wall/single-wall microspheres were taken with a Leica SP2 visible laser confocal microscope. Images were obtained with a 63x oil immersion lens. The fluorescent settings on the microscope include a PMT 600-750 V, and the excitation and emission wavelength of TAMRA are 555 nm and 580 nm, respectively.
Figure 4.7 Confocal images of DWMS: (A, B) Sample A1, EtAc (DCM), PLG i.v. 0.20 and PLA i.v. 0.37; (C, D) Sample A2, EtAc (DCM), PLG i.v. 0.20 and PLA i.v. 0.70.
Figure 4.8 Confocal images of DWMS: (A, B) Sample A3, EtAc (DCM), PLG i.v. 0.20 and PLA i.v. 0.34; (C, D) Sample A4, EtAc (DCM), PLG i.v. 0.38 and PLA i.v. 0.34; (E, F) Sample A5, EtAc (DCM), PLG i.v. 0.61 and PLA i.v. 0.34.
Figures 4.7, 4.8 and 4.9 show the confocal fluorescence images of DWMS A1-A2, A3-A5 and C1-C3, respectively. Figure 4.10 shows the confocal fluorescence images of single-wall microspheres E1. In Figures 4.7 and 4.8, the TAMRA-BSA fluorescence was concentrated in the core area. Obvious drug-free PLA layers were observed for samples A1, A2 and A3, which were EtAc (DCM) DWMS with low PLG inherent viscosity or molecular weight. For A4 and A5 (EtAc (DCM) DWMS with higher PLG molecular weight), although A5 showed the relatively concentrated BSA region, the fluorescence intensity was relatively low, while in A4 the TAMRA-BSA spread throughout the whole microspheres. This result was in accordance with previous SEM study.

Also in Figure 4.9, showing the DCM (DCM) DWMS, TAMRA-BSA was not confined in a concentrated core; instead, the protein appeared to spread throughout the microspheres and tended to be concentrated near the surface. This was also in accordance with previous SEM study in which BSA/water domains were observed throughout the DWMS.

Figure 4.10 shows the TAMRA-BSA distribution within single-wall microspheres mimicking the low molecular (i.v. 0.20 dL/g) PLG core. The TAMRA-BSA distribution was relatively even across the microspheres, as expected. Some fluorescence dark regions were observed within microspheres which might be due to the overlapping of microspheres.
Figure 4.9 Confocal images of DWMS: (A, B) Sample C1, DCM (DCM), PLG i.v. 0.20 and PLA i.v. 0.37; (C, D) Sample C2, DCM (DCM), PLG i.v. 0.20 and PLA i.v. 0.70; (E, F) Sample C3, DCM (DCM), PLG i.v. 0.20 and PLA i.v. 1.05.
4.3 *In Vitro* Release Study

*In vitro* BSA release was measured for the samples described in Table 4.1. For each batch of double-wall or single-wall microspheres, a sample of approximately 30 mg was suspended in 1.25 mL release buffer consisting of 0.05% (v/v) Tween 80 (Fisher) and PBS. These samples were incubated for a three-month period at 37 °C with shaking (240 rpm). At various time points, 1.0 mL supernatant was removed from each sample and replaced with fresh media in order to maintain constant pH sink condition. The release study was performed in triplicate, and BSA concentrations in the collected supernatants were measured using BCA assay (Pierce) [67].

Figure 4.11 shows the release profiles of DWMS A1, A2 and single-wall microspheres E1 mimicking the PLG core. We found that all three profiles showed the tri-phase release: an initial release, a lag phase and a final steady release. The release profiles of A1 and A2 which had EtAc (DCM) configuration and low molecular weight PLG core were almost identical. But compared to E1, the single-wall microsphere the same size as calculated PLG core in A1 and A2, the lag phase and the final steady release phase were delayed for A1 and A2. For E1, 100% release occurred at around 55 days,
while for A1 and A2, complete release was delayed to 70 days. Also, the BSA release rates in the steady release phase for A1 and A2 were slower than that of E1. These release profiles showed that the presence of PLA drug-free shell did postpone the BSA release rate from the PLG core, but the molecular weight of the PLA shell (i.v. 0.37 dL/g, Mw 43,000 Da for A1, i.v. 0.70 dL/g, Mw 106,000 Da for A2) had no influence. This probably was due to the slow degradation rate of PLA compared to PLG. Even at inherent viscosity 0.37 dL/g, the degradation was too slow to be discernible from inherent viscosity 0.70 dL/g. Another thing to be noticed is that the initial release rates of A1, A2 and E1 were also the same. For A1 and A2, this might mean that the BSA-PLG cores were both encapsulated by the PLA shell at the same extent.

Figure 4.12 shows the release profiles of A3, A4 and A5, DWMS with same PLA inherent viscosity (0.34 dL/g) and increasing PLG inherent viscosity. For A3, the release profile was similar to E1, although the initial release rate was slightly faster than E1 and the final steady release rate was slower overall. However, when core PLG inherent viscosity was increased from 0.20 dL/g to 0.38 dL/g and 0.61 dL/g, the release rate increased dramatically. From previous SEM and confocal study, we could conclude that because of the poorer encapsulation of the BSA-PLG core when core PLG inherent viscosity increased, the BSA initial distribution would not be confined within the PLG core. Thus the BSA release rates were faster and the loadings were lower.

Figure 4.13 shows the release profiles of C1, C2 and C3 which had DCM (DCM) solvent configuration. From SEM and confocal images, we know that the BSA initial distribution for DCM (DCM) were all near the surface of the microspheres. Thus, the fast release rates compared to E1 were understandable. For C3, the slow degradation of PLA at inherent viscosity 1.05 dL/g slowed the BSA release, although most BSA was concentrated near the surface.
4.4 Conclusions

Testing the loading and encapsulation efficiency of EtAc (DCM) DWMS and DCM (DCM) DWMS, we found that using EtAc as shell solvent, the loading and encapsulation efficiency were relatively high compared to microspheres using DCM as shell solvent. This might be because the faster extraction rate of EtAc could better encapsulate and confine the BSA-PLG core within the PLA shell during solvent extraction/evaporation. Increasing shell PLA inherent viscosity while keeping the core PLG inherent viscosity constant in EtAc (DCM) solvent configuration (Sample A1 and A2), the loading and encapsulation increased slightly due to better protection of the BSA-PLG core. But for A2, the round shape of DWMS was compromised when the surface collapsed and large indentions were formed. When increasing PLG core inherent viscosity and keeping shell PLA inherent viscosity constant (Sample A3, A4 and A5), the loading and encapsulation efficiency decreased. From SEM and confocal images, high PLG inherent viscosity would change the BSA initial distribution from being concentrated in the core area to spread throughout the microspheres. The reason might be the high molecular weight PLG tended to be more hydrophobic, thus “pushing” the BSA/water droplets away from the core area, while the low molecular weight PLG tended to be more hydrophilic and kept the BSA/water droplets within the core area. This BSA distribution change was also revealed in release profiles. When inherent viscosity of PLG core increased, the BSA release rates were faster than the release profile of PLG single-wall microspheres mimicking the PLG cores (Sample E1). The low inherent viscosity PLG core DWMS encapsulated in PLA shell in EtAc (DCM) solvent configuration exhibited a postponed BSA release compared to E1.

For DCM (DCM) solvent configuration, loading and encapsulation efficiency were lower than EtAc (DCM) counterparts. This might be because the slower evaporation of
the DCM in the dilute shell and dense core would allow the BSA to migrate from the core area to the surface and could easily diffuse out of the microspheres. SEM and confocal images confirmed this hypothesis, and no clear core-shell structures were observed. The fast release of BSA was consistent with the lack of good encapsulation of the BSA-PLG core within the PLA shell. As PLA shell inherent viscosity (Sample C1, C2 and C3) increased, BSA release rates decreased due to the slower degradation rate of the higher molecular weight PLA.

As a result, using EtAc (DCM) solvent configuration, lower inherent viscosity PLG with relatively lower inherent viscosity PLA shell will form the optimal DWMS with core-shell structures, exhibiting higher loading and encapsulation efficiency.

Figure 4.11 In vitro release of double-wall/single-wall microspheres: Sample A1, EtAc (DCM), PLG i.v. 0.20 and PLA i.v. 0.37; Sample A2, EtAc (DCM), PLG i.v. 0.20 and PLA i.v. 0.70; Sample E1, (DCM), PLG i.v. 0.20.
Figure 4.12 \textit{In vitro} release of double-wall/single-wall microspheres: Sample A3, EtAc (DCM), PLG i.v. 0.20 and PLA i.v. 0.34; Sample A4, EtAc (DCM), PLG i.v. 0.38 and PLA i.v. 0.34; Sample A5, EtAc (DCM), PLG i.v. 0.61 and PLA i.v. 0.34; Sample E1, (DCM), PLG i.v. 0.20.
Figure 4.13 In vitro release of double-wall/single-wall microspheres: Sample C1, DCM (DCM), PLG i.v. 0.20 and PLA i.v. 0.37; Sample C2, DCM (DCM), PLG i.v. 0.20 and PLA i.v. 0.70; Sample C3, DCM (DCM), PLG i.v. 0.20 and PLA i.v. 1.05; Sample E1, (DCM), PLG i.v. 0.20.
Chapter 5.  FUTURE WORK

5.1 Double-Wall Microspheres with Different Shell Thickness

DWMS with core-shell structure can provide better control of the release profile of the therapeutics encapsulated. Besides changing the polymer chemistry, degradation rate of the core and shell phase, the addition of shell phase will also give researchers opportunities to tailor release profile by changing the geometric properties such as outer diameter, inner core diameter and shell thickness. Our hypothesis is that by keeping core diameter constant, increasing shell thickness may better postpone the release of the therapeutics encapsulated in the core.

From previous study, we found that by using EtAc (DCM) solvent configuration, low PLG inherent viscosity, and low PLA inherent viscosity, we could produce DWMS with a clear core-shell structure and high loading and encapsulation efficiency of protein. The shell thickness was set at approximately 10 µm (calculated).

Our next plan is to try to change the shell thickness and study the influence of shell thickness on protein loading, encapsulation efficiency and release profiles. We tried to increase shell thickness at a core concentration of 10% (w/v) PLG (i.v. 0.20) in DCM in a previous study. Several attempts were performed, and it was hard to get steady, uniform DWMS stream using PPF. So we first increased the PLG core concentration from 10% (w/v) in DCM to 30% (w/v) in DCM. By increasing shell PLA (i.v. 0.37) concentration from 3%, 6% to 9% (w/v), we formed uniform DWMS in both DCM (DCM) and EtAc (DCM) solvent configurations.
Table 5.1 DCM (DCM) DWMS with different shell thickness

<table>
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<th>Code</th>
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<th>Outer Diameter Measured (μm)</th>
<th>Core Diameter Calculated (μm)</th>
<th>Shell Thickness Calculated (μm)</th>
<th>BSA Loading (μg BSA/mg)</th>
<th>Encapsulation Efficiency (%)</th>
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<td>6.4</td>
<td>4.54</td>
<td>28.99</td>
</tr>
<tr>
<td>G2</td>
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<td>71.1±1.3</td>
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<td>10.9</td>
<td>2.70</td>
<td>25.65</td>
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<tr>
<td>G3</td>
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<td>76.8±1.3</td>
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<td>14.0</td>
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<td>25.56</td>
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Table 5.2 EtAc (DCM) DWMS with different shell thickness

<table>
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<th>Outer Diameter Measured (μm)</th>
<th>Core Diameter Calculated (μm)</th>
<th>Shell Thickness Calculated (μm)</th>
<th>BSA Loading (μg BSA/mg)</th>
<th>Encapsulation Efficiency (%)</th>
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<tr>
<td>I1</td>
<td>1.09</td>
<td>60.5±2.2</td>
<td>47.9</td>
<td>6.28</td>
<td>7.58</td>
<td>48.36</td>
</tr>
<tr>
<td>I2</td>
<td>2.14</td>
<td>69.8±1.6</td>
<td>48.5</td>
<td>10.64</td>
<td>5.02</td>
<td>47.74</td>
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<tr>
<td>I3</td>
<td>3.04</td>
<td>76.3±1.9</td>
<td>48.5</td>
<td>13.93</td>
<td>3.76</td>
<td>47.15</td>
</tr>
</tbody>
</table>
Table 5.1 and 5.2 show the mass ratio, measured outer diameter, calculated core diameter, calculated shell thickness, loading and encapsulation efficiency of these microspheres. Figure 5.1, 5.2 shows the size distributions.

![Size distributions of DWMS (DCM (DCM)) with different shell thickness](image)

Figure 5.1 Size distributions of DWMS (DCM (DCM)) with different shell thickness
The next step is to determine the loading and encapsulation efficiency of these DWMS and using SEM and confocal fluorescence microscopy to characterize them. Also, in vitro release will be performed to test whether the change of shell thickness will have effects on BSA release rate.

5.2 Aqueous/Oil Core PLG Microcapsules for Protein Delivery

Our next goal is to produce microcapsules with PLG shell and oil/aqueous core. By using oil core and suspend lyophilized protein inside or aqueous core of protein solution, we expect to achieve higher protein loading and better encapsulation efficiency because of less organic solvent contact with BSA.

First step of this goal is to produce monodisperse aqueous/oil core PLG microcapsules for protein delivery. The PPF setting will be similar to the DWMS except
that the core phase is protein aqueous solution or solid protein dispersed in oil [58].

Former research from our lab shows that the monodisperse PLG microcapsules for protein delivery can form pulsatile release profiles [47]. Our future work will be changing the PLG shell thickness and studying the effect of shell thickness on protein release rate.
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