ABSTRACT

Title of Document: SELF-DESTRUCTING "MOTHERSHIP" CAPSULES FOR TIMED RELEASE OF ENCAPSULATED CONTENTS

Anand S. Bagal, Master of Science, 2012

Directed by: Prof. Srinivasa R. Raghavan
Department of Chemical & Biomolecular Engineering

This thesis explores a new class of hierarchical containers that are formed via single-step assembly, and at a later time, self-destruct due to their packaged contents. These containers are spherical capsules formed by electrostatic complexation of the anionic biopolymer, gellan gum with the cationic biopolymer, chitosan. The capsules are termed "motherships", and are engineered to carry a payload of much smaller containers, i.e., nanoscale vesicles ("babyships"), within its lumen. By also packaging an enzyme, chitosanase, inside the capsule, we create "motherships" that self-destruct, releasing their payload of "babyships" in a pulsatile manner. The timescale for self-destruction can be engineered based on the internal concentration of enzyme. The "motherships" are stable when stored in a freeze-dried form and can be readily dispersed into buffer solutions at a later time, whereupon their "internal clock" for self-destruction is initiated. The above concept could be useful for the controlled release of a variety of payloads including drugs, biological therapeutics, cosmetics, or flavor ingredients.

Self-Destructing "Mothership" Capsules for Timed Release of Encapsulated Contents

Anand S. Bagal

Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Master of Science
2012

Advisory Committee:

Prof. Srinivasa R. Raghavan, Dept. of Chemical & Biomolecular Engineering, Chair

Prof. Sheryl Ehrman, Dept. of Chemical & Biomolecular Engineering

Prof. Chunsheng Wang, Dept. of Chemical & Biomolecular Engineering

Prof. Gregory Payne, Fischell Department of Bioengineering

Dedication

This thesis is dedicated to my wonderful family for the immense love and unconditional support they have shown me in every endeavor that I have undertaken

Acknowledgements

First and foremost, I would like to thank my advisor Dr. Srinivasa Raghavan for the opportunity to conduct research in his group. His guidance and support throughout my graduate studies has been invaluable and I am grateful to him for that. I appreciate the concern he has shown for my work in the lab as well as my career. His approach towards research and problem solving has had a deep impact on me. He has been of immense support to me and I would like to thank him for all his help and concern.

I would like to thank my committee members, Dr. Sheryl Ehrman, Dr. Chunsheng Wang and Dr. Gregory Payne for their support and guidance during the course of this thesis. In particular, Dr. Ehrman has been of great support and I would like to thank her for her concerns.

I would also like to register my thanks to all my fellow members of the Complex Fluids Group. Annie, Apoorv, Ariel, Bani, Chanda, Charles, Hee-Young, Hyuntaek, Jasmin, Kevin, Kunal, Kunqiang, Neville, Stephen, Veena, Veidhas and Vishal have been great fun to work with and I have enjoyed their company. It was a fantastic experience working with such talented and intelligent individuals and I would like to wish them luck in their endeavors. I would also like to register my thanks to Dr. Matthew Dowling in particular, for his insights and recommendations in this thesis.

Finally, and most importantly, I would like to acknowledge my parents, my sister and my friends who have been immensely supportive through the course of this thesis. I cannot thank them enough for their support and encouragement through all the tough times that I have faced.

TABLE OF CONTENTS

Chapter 1: INTRODUCTION & OVERVIEW

A major research topic in academia and the pharmaceutical industry has been the delivery of drugs. With regard to drug carriers, two popular types of such vehicles are vesicles and capsules. Vesicles (also termed liposomes) are typically nanoscale structures formed by the self-assembly of amphiphilic molecules such as lipids or surfactants.[1, 2] The inner core of vesicles is an aqueous solution and a wide range of molecules can be encapsulated within this core. However, vesicles have the disadvantage that they are vulnerable and can be easily broken down in vivo due to the fact that they are held together by relatively weak bonds. On the other hand, capsules are structures having a liquid core surrounded by a solid shell (typically made of polymers or inorganic molecules). Capsules are typically much larger (microscale or milliscale), but they are much more robust compared to vesicles. Once again, the core of capsules can hold a variety of drugs, and the slow permeability of the packaged drugs across the shell can be used to deliver the drugs in a sustained manner.

This thesis seeks to create a hybrid of the above two structures, i.e. capsules containing vesicles. Such a hybrid would be attractive as a drug delivery vehicle, as it offers to combine the advantages of both whilst minimizing the drawbacks of each.[3] We term these hybrids as "mothership" capsules which are hierarchical structures containing encapsulated vesicles or "babyships". An overview of this type of structure is shown in figure 1.1. By including nano-sized babyships within motherships, we ensure that capsules protect susceptible vesicles from external factors. Motherships can be made

more versatile by including additional drugs within its lumen. We will demonstrate a single-step method to form mothership capsules by electrostatic complexation of oppositely charged polymers in the presence of vesicles. Our method employs mild settings, avoiding the use of extreme conditions or harsh solvents.

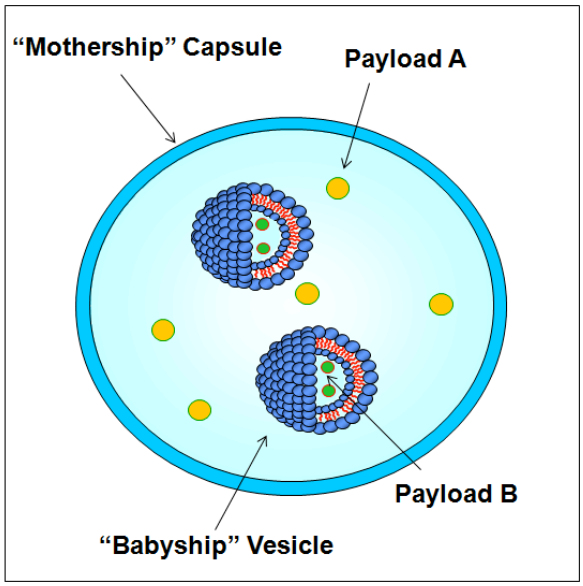

Figure 1.1. Concept of "mothership" capsule with internalized "babyships" and payload.

A key challenge in drug delivery revolves around the mechanisms employed for triggering the delivery of the drug from the vehicle. Changes in the chemical, physical and biological environment have been exploited to trigger vehicles to release drugs.[4] In this vein, it is of interest to design "mothership" capsules so that they respond to external triggers. For example, a trigger could cause the capsule shell to open up, thereby releasing the internalized vesicles for delivery to a target. Alternately, another concept is to design self-destructing vehicles. These would offer the advantage of being immune to external stimuli while relying on an internal mechanism to trigger the release of drugs.

The latter concept is explored here and we make self-destructing mothership capsules by including within the capsule an enzyme that is capable of degrading the capsule shell, as shown in figure 1.2. In this way, we can design a mothership that self-destructs within a given time, thus releasing babyships into the surrounding environment, each carrying its own distinct payload. The degradation time of motherships is shown to be controllable by the concentration of encapsulated enzyme. The capsules can be stored as a dry powder, and when they are hydrated, the internal clock for destruction is begun. If the capsules have targeting moieties such as antibodies on their surface, they can reach a specific location before the self-destruction is initiated. This can ensure release of the babyships at the desired location.

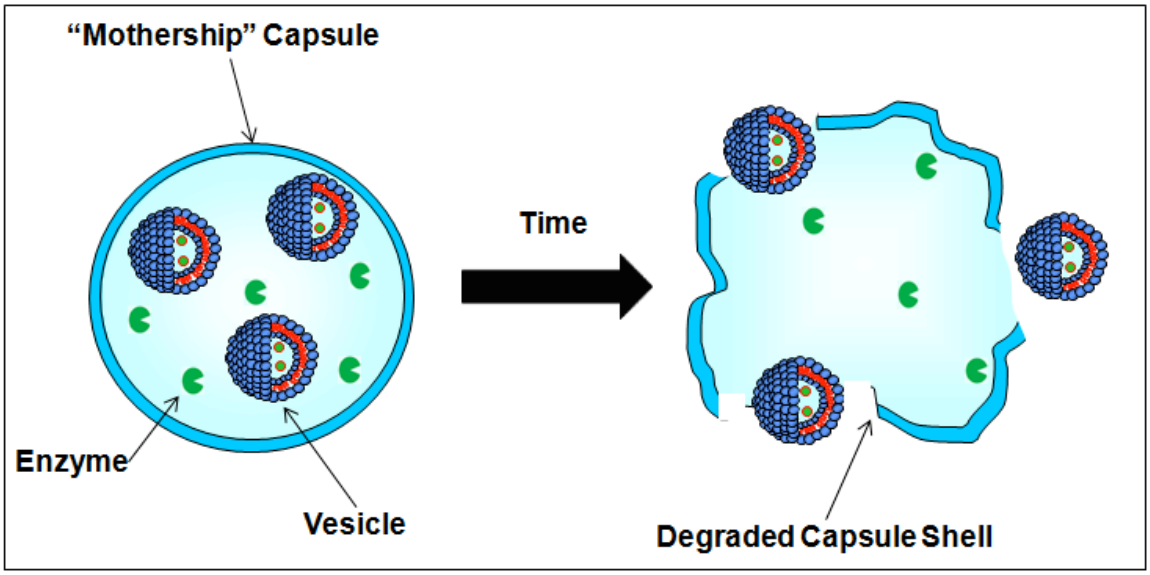

Figure 1.2. Self destruction of mothership capsule with internalized enzyme for timed release of babyship vesicles

We envision self-destructing mothership capsules as being analogous to "ticking time bombs" with an internal trigger to deliver encapsulated payload at a pre-determined

time. This concept of timed release of drug-loaded nano-vector from a larger container can be useful to a variety of applications such as delivering not only drugs and biological therapeutics, but also cosmetics, fertilizers, chemicals and flavor ingredients.

Chapter 2: BACKGROUND

In this chapter, we discuss basic aspects pertaining to the components of the mothership capsules to be studied in Chapter 3. The main components of the capsules are the biopolymers, chitosan and gellan gum, and the structures encapsulated in the capsules include vesicles and enzymes. We also provide a description of the process for forming capsules and specifically the mothership capsules.

2.1. BIOPOLYMERS

Polymers are macromolecules formed from repeating structural units. Polymers having biological origin are called biopolymers.[5] For the purposes of this study, we will focus on biopolymers that have specific charge and render viscosity to water by forming entangled networks or gels.

Chitosan

Chitosan is a linear cationic polysaccharide obtained from the N-deacetylation of chitin.[6] Chitin is derived from the exoskeleton of crustaceans and is abundantly available in nature. The chitosan backbone is mostly composed of D-glucosamine (β-(1,4)-2-deoxy-2-amino-D-glucopyranose) sugars, with a small fraction of N-acetyl-D-glucosamine (β-(1,4)-2-deoxy-2-acetamido-D-glucopyranose) sugars from the parent chitin. Chitosan is soluble in water under acidic conditions of pH < 6.5, as the amine groups on the parent chain are ionized and the structure acts as a cationic polymer. The

structures of these sugars are shown in Figure 2.1. Chitosan is biocompatible, biodegradable and non-toxic making it useful for drug delivery applications.

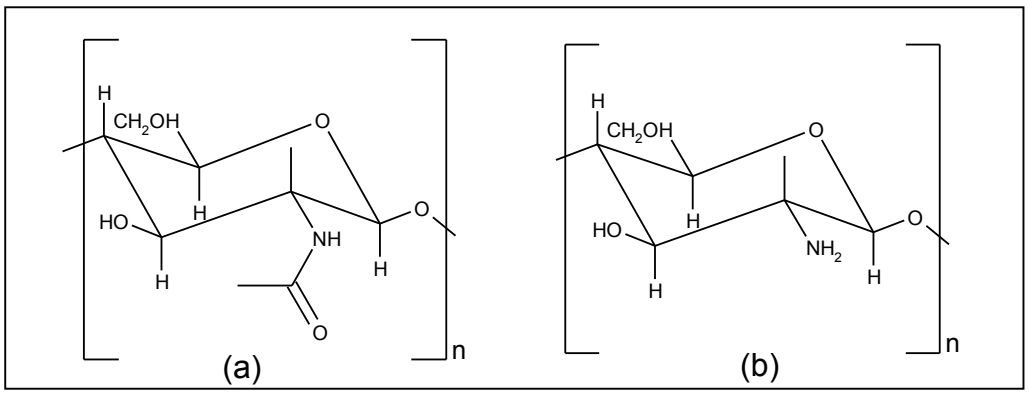

Figure 2.1. Structures of the parent sugars in (a) chitin and (b) chitosan. The N-acetyl-D-glucosamine sugar in chitin is deacetylated to give the D-glucosamine sugar in chitosan.

Gellan Gum

Gellan gum is an anionic polymer that is derived from the bacterium pseudomonas elodea.[7] It is a water soluble polymer and has a repeat unit of the tetrasaccharide D-glucose, D-glucuronic acid, D-glucose, and L-rhamnose. These units are connected by α–(1,3) glycosidic bonds. Because of the carboxylate groups, gellan gum is an anionic polymer. Gellan gum is biocompatible, biodegradable and non-toxic, which makes it suitable for drug delivery applications.

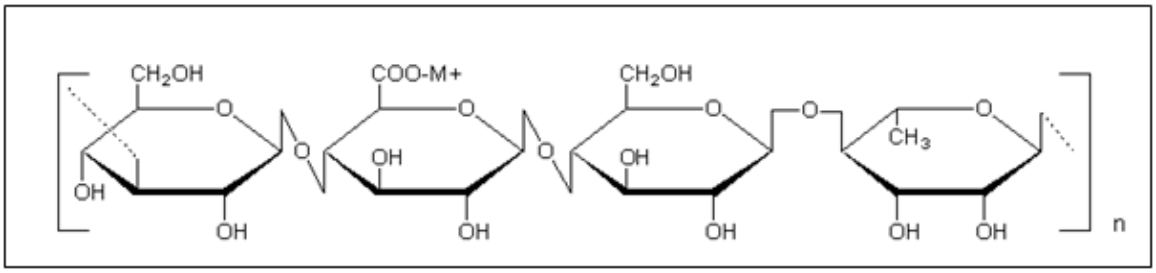

Figure 2.2. Structure of the recurring tetrasaccharide unit in gellan gum.

2.2. VESICLES

Vesicles are containers formed by the self assembly of amphiphilic molecules in water.[1] Amphiphilic molecules such as phospholipids or surfactants are composed of a hydrophilic head and hydrophobic tails. These molecules self assemble in water to form bilayers with hydrophilic heads exposed to water protecting the hydrophobic moiety as shown in figure 2.3. Folding of the bilayer gives vesicle structures having diameters in the range of 30 nm to 30 μm. Vesicles are known to encapsulate a variety of compounds and are useful as delivery agents for drugs and cosmetics.

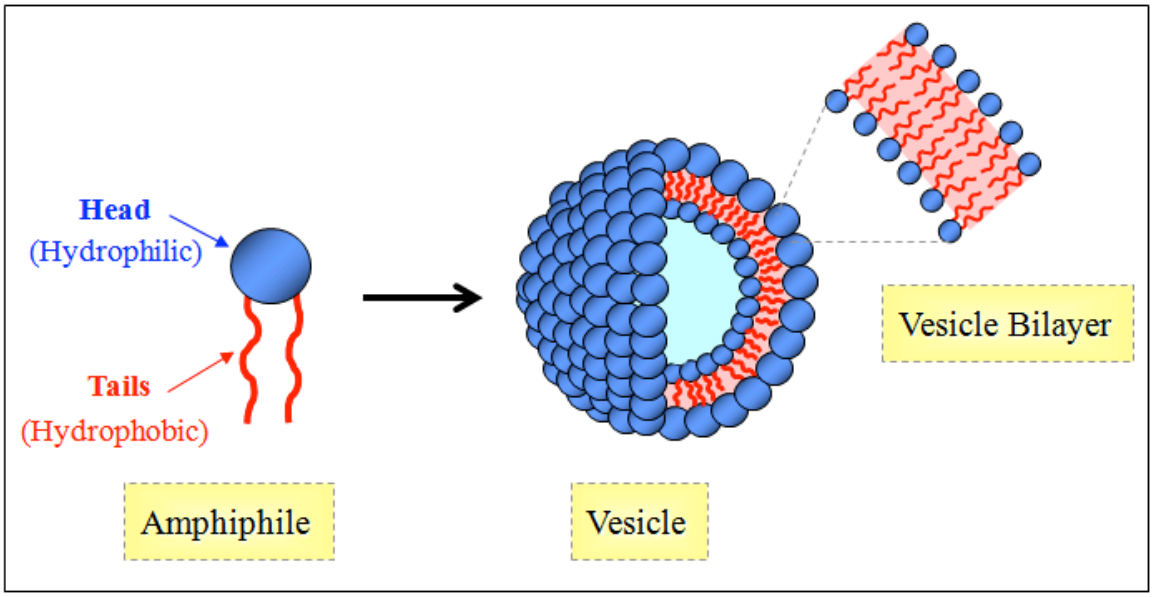

Figure 2.3. Structure of vesicles formed by the self-assembly of amphiphiles. The vesicle is formed by the folding of an amphiphilic bilayer 5-10 nm in thickness.

Lipids are amphiphiles of biological origin and typically have two hydrophobic tails and one hydrophilic head.[2] Vesicles formed from lipids are known as liposomes. Liposomes have been known to be taken up by tumor cells and are useful drug delivery

agents. Research in this field has involved chemically modifying liposomes making them sterically stabilized to improve their targetability and functionality. Recently, cationic liposomes have found applications in gene delivery application.[8]

2.3. POLYMER CAPSULES

Capsules may be defined as structures having a liquid core enclosed within a solid shell. They have sizes ranging from a few microns to several millimeters and can be prepared using various techniques such as emulsification, layer-by-layer assembly, internal phase separation and coacervation.[4] The shell thickness determines the mechanical integrity of capsules.

2.3.1. CAPSULE FORMATION

The method we use for capsule formation involves the coacervation process. This method utilizes polyelectrolyte complexation of oppositely charged polymers. We use chitosan as the cationic polymer and gellan gum as the anionic polymer in the coacervation process.[9] Chitosan solution is added drop-wise to a bath containing a solution of gellan gum (or vice versa) to form capsules, as shown in Figure 2.4. This forms a poly-ion network at the capsule shell which traps the liquid within. The size of the capsules is equal to the size of the generating drop. Smaller capsule sizes of the order of microns can be generated by employing techniques such as atomization or microfluidics. Note also that any payload included with the chitosan solution becomes encapsulated in the capsules. Thus capsules with suitable payload can be generated in a simple, single step under mild conditions.

2.3.2. MOTHERSHIP CAPSULES

Mothership capsules are hierarchical structures with babyship vesicles internalized in them. Encapsulation of vesicles within larger capsules has been studied before and proven useful for delayed drug delivery.[3, 10] For our application, we create mothership capsules by modifying the coacervation technique. Vesicles are mixed with the anionic gellan gum solution to form the feeder solution as the first step. Feeder solution is then added drop wise to a bath of cationic chitosan solution. After a fixed incubation time, mothership capsules are formed that contain internalized babyship vesicles. Electrostatic interactions of oppositely charged polymers stabilize the capsule shell. Capsules are stable under mechanical stirring and are stored in buffer solutions.

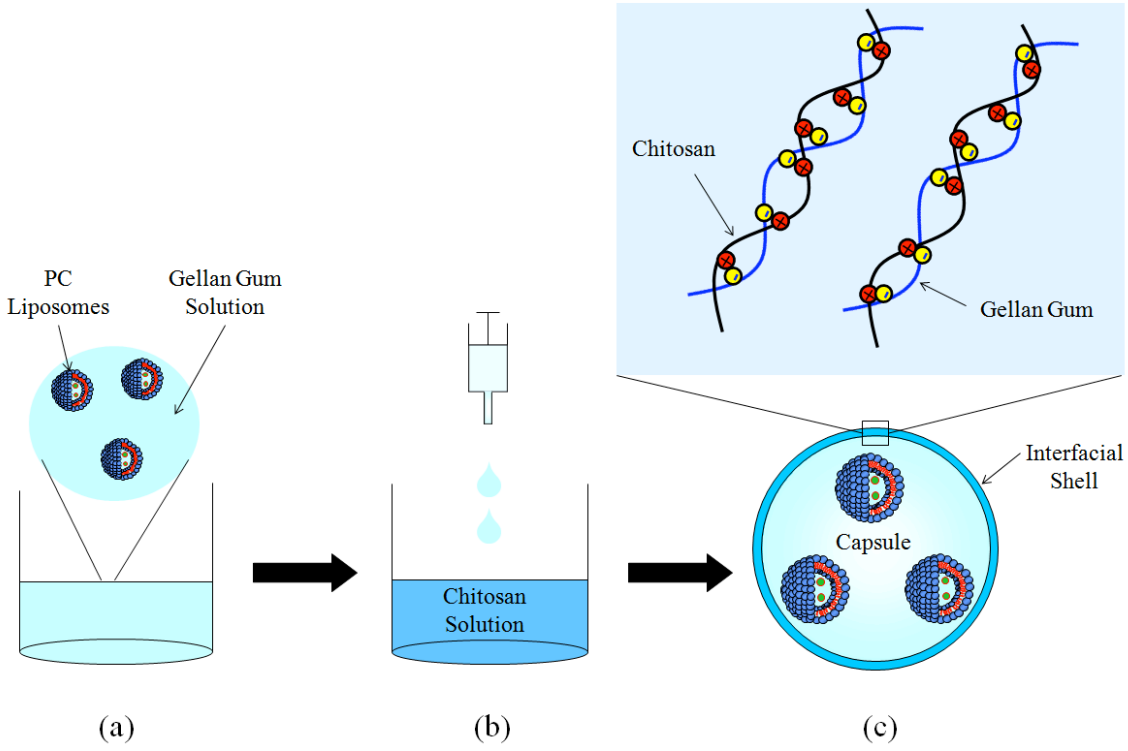

Figure 2.4. Preparation of mothership capsules (a) PC liposomes are mixed with anionic gellan gum solution to form feeder; (b) Feeder solution is added drop wise to cationic chitosan solution; (c) Mothership capsules are formed by electrostatic complexation of oppositely charged polymers at drop interface with internalized liposomes.

2.3.3. TRIGGERED CAPSULE DEGRADATION

Capsules can be induced to break open to release internalized contents in response to physical, chemical or biological stimuli. Previously, light activated capsules have been reported which degrade on exposure to light of specific wavelength.[11] Capsules have also been designed to degrade in response to changes in pH or ionic strength.[12, 13] Ultrasound and magnetic fields have also been used as stimuli to rupture capsules.[14, 15] For our application, we employ a biological stimulus to rupture capsules. More specifically, we utilize enzymes to degrade our capsules and deliver payload.

2.4. ENZYMATIC DEGRADATION OF BIOPOLYMERS

Enzymes are biocatalysts that perform a precise reaction on a specific substrate.[16] They offer the advantages of specificity and control under mild conditions. We employ enzymatic degradation to break down our mothership capsules. As mentioned earlier, we utilize biopolymers to form our capsules. By choosing an appropriate enzyme, we can selectively degrade the polymers in the capsule shell. In particular, we use chitosanase, an enzyme that catalyzes the degradation of polymeric chitosan into oligomers.[17] Chitosanase is derived from various sources such as bacteria, fungi and plants. Some other enzymes capable of degrading chitosan are chitinase, hemicellulase and lysozymes. However, chitosanases have high specificity and activity in cleaving the β-(1,4) linkages between the D-glucosamine units in chitosan, as shown in Figure 2.5b, making them the optimal choice for our application. The final products obtained from chitosan hydrolysis are dimers and trimers, accompanied by a very limited quantity of monomers.[18] Enzyme activity is defined in term of units where one unit of chitosanase will liberate 1.0

micromole of reducing sugar (measured as D-glucosamine equivalents) from chitosan per minute at pH 5.5 and 37°C.

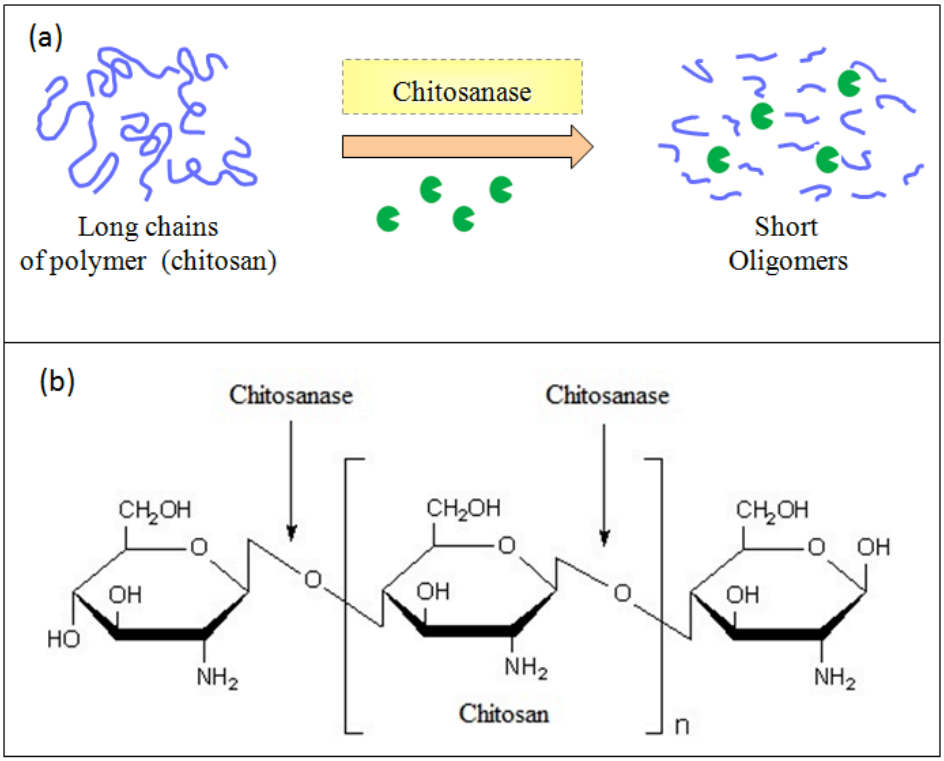

Figure 2.5. Enzymatic degradation of biopolymers: (a) Enzymatic degradation of polymeric chitosan to form oligomers; (b) Chitosanase cleaving the β-(1,4) linkages in chitosan.

Chapter 3: SELF-DESTRUCTING MOTHERSHIP CAPSULES: SYNTHESIS AND CHARACTERIZATION

3.1. INTRODUCTION

In Chapter 3, we explore the synthesis of "mothership" capsules, which are microscale (or larger) container structures that contain much smaller (nanoscale) secondary containers. The nanoscale containers of interest here are vesicles, which are well-known for their ability to encapsulate hydrophilic solutes in their aqueous core. The synthesis of polymeric capsules containing nanoscale vesicles has been explored by a few research groups over the past two decades.[3, 10] An advantage of this type of structure is that it can prolong the release of solute. That is, solutes entrapped in the vesicles will have to first leak out through the bilayer of the vesicles into the lumen of the capsules and thereafter out to the external solution through the capsule shell. The presence of two transport resistances – from the vesicle bilayer and the capsule shell – serves to extend or prolong the release of solute.

A second and more important aspect of our work is that we seek to make our mothership capsules self-destruct after a period of time due to encapsulated enzyme. To our knowledge, there have been only a few studies that have attempted to create self-destructing capsules.[4, 19] Geest *et al*. designed self-rupturing microcapsules composed of a dextran-based microgel coated with a membrane of polyelectrolyes by layer-by-layer (LbL) deposition.[20, 21] The microgel was engineered such that it underwent degradation on hydration, which increased the internal osmotic pressure, leading to rupture of the

capsule membrane and release of internalized contents. Self-degrading capsules were also designed by Borodina *et al*. by incorporating enzymes (proteases) within the lumen of polymer microcapsules prepared by the LbL technique.[22] The enzymes were loaded into calcium carbonate particles, and these were embedded within the capsules. A calcium-chelating agent was then used to dissolve the particles and thereby release the enzymes within the capsule lumen. In turn, the enzymes digested the capsule shell, thus releasing the contents in a time-dependent manner. The above schemes for creating self-destructing capsules are elegant, but rather complex. For example, LbL requires a series of labor-intensive incubation and washing steps to form the capsule shell. The use of multi-step or aggressive conditions for capsule preparation can also be problematic for applications involving the delivery of sensitive biomolecules or drugs.

In our work, we demonstrate a simple, mild process to generate self-destructing mothership capsules. Our method uses two commercially available biopolymers, chitosan and gellan gum, as the precursors for our capsules. Previous studies have been carried out on capsules involving these polymers though none have involved encapsulating vesicles.[23-25] We show that the capsules can be created and loaded with both enzymes and vesicles in a single step. The enzyme used is chitosanase, which is capable of hydrolyzing the chitosan and thus degrading the shell of our capsules, which leads to release of the internalized vesicles en masse. We also demonstrate release experiments with other payloads such as fluorescent microparticles. The capsules can be stored as a dry powder, and when they are hydrated, the internal clock is started for degradation of their shells.

The degradation time of motherships is shown to be controllable by the concentration of encapsulated enzyme and can be varied from minutes to hours.

3.2. EXPERIMENTAL SECTION

Polymers

Chitosan was obtained from Wako Pure Chemicals (product name Chitosan 1000). It is expected to have an average molecular weight of 1,800,000.[26] As chitosan is soluble only under acidic conditions, 0.2 M acetic acid was used to dissolve it. Gelrite® gellan gum was obtained from Sigma Aldrich and its molecular weight was specified by the manufacturer to be 1,000,000. Gellan gum solutions were prepared in deionized (DI) water by heating up to 60°C.

Vesicles

The phospholipid L-α-phosphatidylcholine (PC) was purchased from Avanti Polar Lipids Inc. Liposomes were prepared by an extrusion method, as recommended by the manufacturer. Briefly, dried films of the lipids were hydrated under moderate stirring. They were freeze-thawed five times, and then passed through two double-stacked polycarbonate membrane filters (100 nm pores) using a Lipex pressurized extrusion system. This ensured liposomes of size approximately 100 nm, which was confirmed by dynamic light scattering experiments. The fluorescent lipid, 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) was obtained from Sigma Aldrich.

Enzymes and Other Chemicals

Chitosanase from Streptomyces species was obtained from Sigma Aldrich with activity of 18 units/mg and stored in 50% glycerol solution containing 100 mM sodium acetate at pH 5. Chitosanase from streptomyces species have a reported molecular mass in the

range of 30-35 KDa.[17] Carboxylate-modified polystyrene beads (2 μm, fluorescent green) were obtained from Sigma Aldrich.

Dynamic Light Scattering

A Photocor-FC instrument with a 5 mW laser light source at 633 nm was used to analyze the size of vesicles. Studies were done at 25°C with the scattering angle being 90°. A logarithmic correlator was used to obtain the autocorrelation function, which was analyzed by the method of cumulants to yield a diffusion coefficient for the vesicles analyzed. The apparent hydrodynamic radius of the vesicles was obtained from the diffusion coefficient through the Stokes-Einstein relationship.

Microscopy

The Zeiss Axiovert 135 TV inverted microscope equipped with Motic Image Plus imaging system has been used for optical microscopy. Capsules were imaged with a 2.5X objective. Fluorescence microscopy was carried out with excitation wavelength set at 480 nm and emission filter at 510 nm.

3.3. Results and Discussion

3.3.1. Mothership Capsules

We prepared mothership capsules containing vesicles as babyships within their lumen by the following procedure. The method involves electrostatic complexation of the cationic biopolymer, chitosan and the anionic biopolymer, gellan gum.[23-25] Motherships can be prepared with either a chitosan core and gellan shell or a gellan core and chitosan shell. The latter system is preferred for the self-destructing motherships and so we will focus on that.

First, PC vesicles were prepared in DI water using the zwitterionic phospholipid, PC at a concentration of 1 wt%. The vesicles are expected to be unilamellar structures and their diameter was measured to be ~ 100 nm by DLS. Then, a gellan gum solution was mixed with the PC vesicles to form the feeder solution, with the gellan and PC concentrations in the overall mixture being 1 wt% and 0.5 wt% respectively. The feeder solution was then loaded into a syringe and dispensed as droplets via the tip of a 22G needle into a solution of chitosan (1 wt%), as shown in Figure 3.1. The droplets were allowed to incubate for 3 min during which the droplet was converted into a capsule, i.e., a shell was formed on the surface of the droplet by the electrostatic complexation of chitosan and gellan gum into a poly-ion network (Figure 3.1). The resulting capsules are the motherships and they contain the vesicles inside. They were scooped out from the chitosan solution and washed 3 times with DI water. They were then stored in phosphate buffer (pH 6) solution, where they remained stable under mechanical stirring. Note that

the size of the capsules can be controlled by adjusting the size of the generating drop. For our purposes here, we mostly have prepared relatively large capsules with sizes > 1 mm. Smaller capsules can be created by reducing the drop size by employing techniques such as microfluidics and atomization.

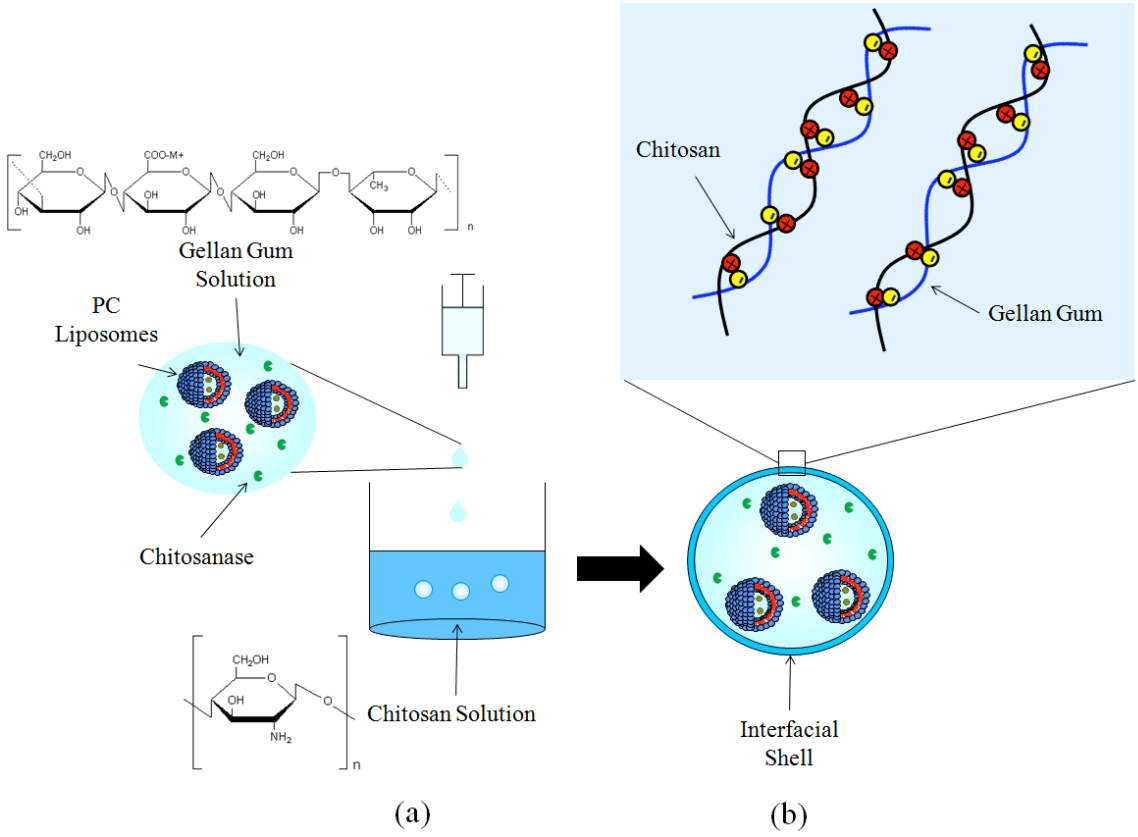

Figure 3.1. Preparation of self destructing mothership capsules: (a) anionic gellan gum mixed with PC liposomes and chitosanase forms feeder solution which is added drop wise to cationic chitosan; (b) spherical capsules formed by electrostatic complexation of oppositely charged polymers resulting in a polyion network at capsule shell.

3.3.2. Self-Destructing Mothership Capsules

To generate self destructing "mothership" capsules, we modified the preparation method, as shown in figure 3.1. In this case, the feeder solution contained gellan gum (1 wt%), PC liposomes (0.5 wt%), and the enzyme chitosanase (1.5 units/mL). This was again dispensed into a 1 wt% chitosan solution and incubated for 3 min. Thus, mothership capsules containing vesicles and chitosanase were prepared, and these were again washed and placed in pH 6 buffer for observation.

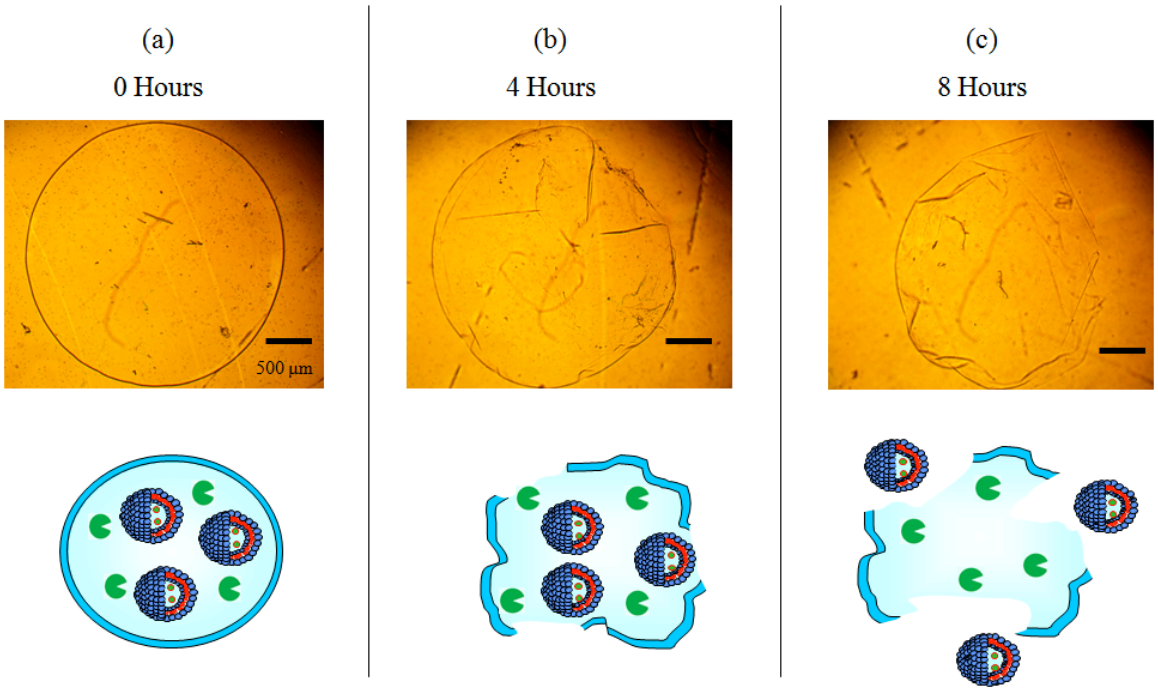

Figure 3.2. Self-destruction of a "mothership" capsule (gellan core, chitosan shell) by internalized chitosanase enzyme (1.5 units/mL) at various times after placing in buffer: (a) initial, intact capsule shell; (b) after 4 h, showing partial degradation of the shell; (c) after 8 h, showing complete degradation. As the capsule degrades, the capsule releases its payload of vesicles into the external solution as illustrated in the cartoons below each micrograph. The scale bar in each image equals 500 µm.

The self-destructing property of the capsules could be seen both visually as well as by optical microscopy. Figure 3.2 presents optical micrographs showing the capsule at different times. Initially (Figure 3.2a), the capsule is intact and its shell uncompromised. After 4 h (Figure 3.2b), the shell is seen to be partially degraded. After 8 h (Figure 3.2c), the shell is sufficiently degraded that the capsule no longer holds its shape. The shell looks shriveled and by this point, the capsule has released its internal content. As mentioned earlier, control capsules with no encapsulated enzyme show no degradation or loss of mechanical integrity. Thus, it is clear that the changes in the capsule structure are due to the enzymatic action of chitosanase. It is well-known that chitosanase degrades polymeric chitosan into short oligomers. Since the capsule shell is formed by complexation of gellan gum chains and chitosan chains, the conversion of chitosan into short oligomers will result in the disruption of the shell and thereby the breakdown of the capsule.

A key aspect of our self-destructing motherships is that the babyships, i.e., vesicles, are released intact into the surroundings. How can we test if the vesicles are released intact? For this, we show the experiment in Figure 3.3. Here, the vesicles inside the motherships have been formulated with the fluorescent lipid DiI. This lipid incorporates into the vesicle bilayer and is thus a part of each vesicle. Note that the vesicles have a pink color due to the DiI, and in turn, the capsules also take on this pink color, which shows the presence of encapsulated vesicles. Figure 3.3a shows a number of intact capsules in the buffer solution at time zero. Note that the pink color is confined to the capsules. Figure 3.3b shows the solution after 4 h. In this case, the capsules are

beginning to lose their integrity and in turn some of the pink color is transferred to the solution. Figure 3.3c shows the solution after 8 h. By this time, the capsules are fully degraded releasing all the vesicles to the surrounding buffer solution. We have a homogenous pink solution, and a DLS measurement on this solution gives an average diameter of structures in solution of about 100 nm, which matches the size of the original vesicles. This is indirect proof for the motherships being able to release intact vesicles.

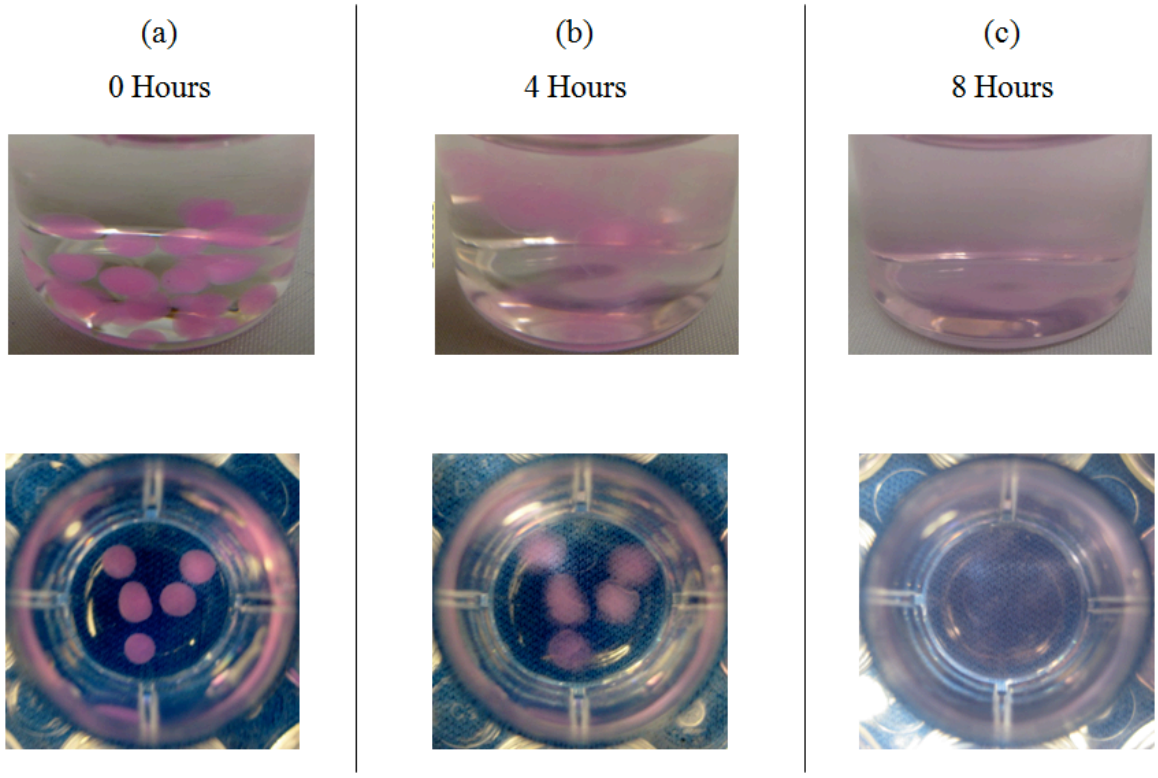

Figure 3.3. Release of DiI tagged liposomes from self destructing mothership capsules (chitosanase: 1.5 units/mL): (a) initially pink vesicles contained in intact capsule; (b) after 4 h, partial degradation of capsule with some release of pink vesicles; (c) after 8 h, complete degradation of capsule, pink liposomes uniformly dispersed in external buffer. Capsules are shown degrading in a vial (side view) and in well plates (top view).

The degradation time of the capsules can be tuned by varying the concentration of encapsulated enzyme. Figure 3.4 shows the results of experiments with different enzyme concentrations. The degradation time was assessed visually from experiments as shown in Figure 3.3 and it corresponds to complete degradation of capsules and uniform dispersion of pink liposomes in solution. (akin to Figure 3.3c).

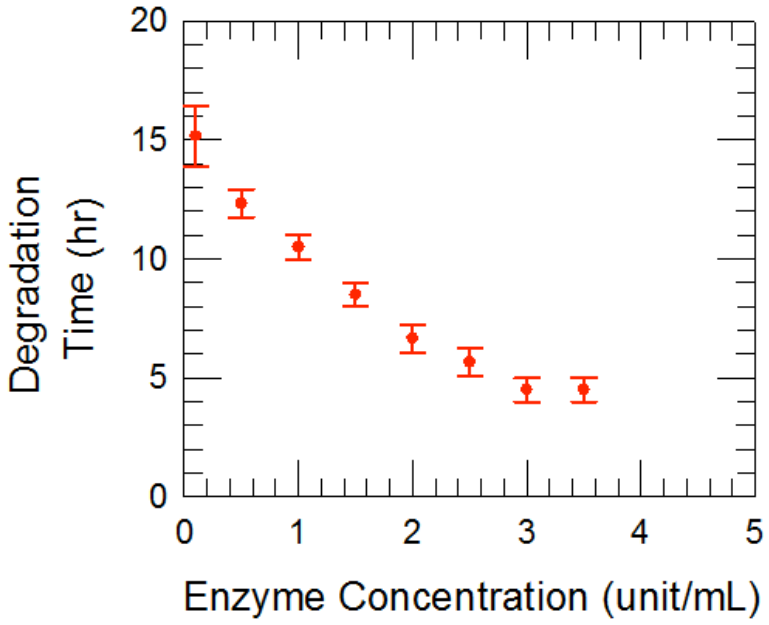

Figure 3.4. Degradation time of self-destructing "mothership" capsules as a function of enzyme concentration. Degradation time was observed to decrease almost exponentially with increase in enzyme concentration.

As shown by Figure 3.4, the lowest enzyme concentration for which degradation was observed was 0.1 units/mL, and in this case, complete capsule degradation took approximately 15 h. Below this enzyme concentration, capsule degradation was not seen even over a period of several days. As the enzyme concentration was increased, the degradation time was observed to decrease almost exponentially. For 3 units/mL of enzyme, degradation was completed in 4 h. This may be a saturation concentration of

chitosanase for capsule degradation, i.e., higher enzyme concentrations did not lead to faster degradation. We should point out that there are a number of factors that go into determining degradation time apart from the kinetics of the enzymatic reaction. These include the rate at which enzymes diffuse through the gellan core to get to the gellan-chitosan shell, and the rate at which pores are formed in the shell. In any case, we are able to tune the degradation time by about 5-fold by simply altering the enzyme concentration.

Another experiment to monitor release of encapsulated contents was conducted with fluorescent polystyrene microparticles (2 μm diameter) as the payload. In this case, the microparticles (0.5 wt%) were mixed with chitosanase (1.5 units/mL) and gellan gum (1 wt%) to form the feeder solution, which was added drop wise to a chitosan solution (1 wt%). Capsules were formed with encapsulated particles and their self-destruction in buffer was monitored by fluorescence microscopy. Figure 3.5a shows that initially the particles are localized in the interior of the capsule. A shell region having a thickness of a few microns surrounds the particle-filled lumen. The fluorescence signal was confined to the capsules; no fluorescence could be seen in the background. At the 3 h mark (Figure 3.5b), some fluorescence could be observed in the background, indicating that the enzyme had partially degraded the capsule shell, allowing some particles to leak out. With increasing time (Figure 3.5c, d), the particles are seen to diffuse out radially and they extend beyond the disrupted shell. At the same time, an increasing fluorescence signal is observed in the background. The particles seem to be still locally concentrated even after near-complete degradation of the capsule. This is because the particles are an

order-of-magnitude larger compared to the liposomes, which ensures that their diffusion will be considerably slower.

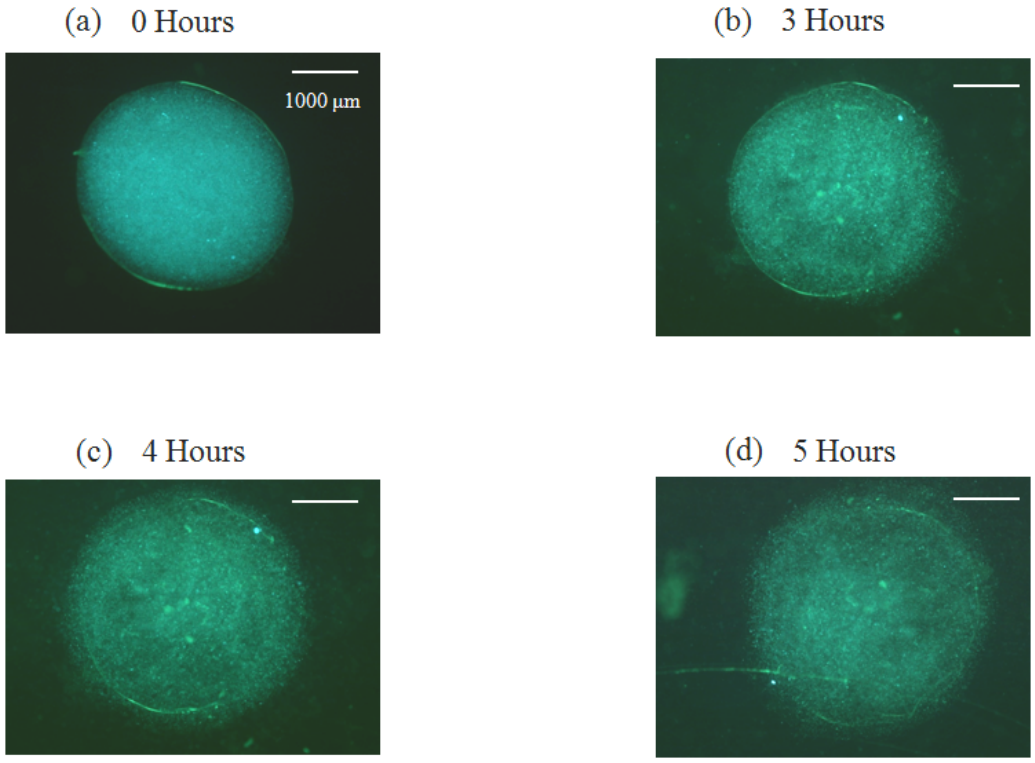

Figure 3.5. Release of fluorescent microparticles from self destructing "motherships" (chitosanase:1.5 units/mL): (a) Initial, fluorescent particles contained within capsule; (b) after 3 h, capsule showing partial degradation with release of a few microsparticles; (c) after 4 h, increasing background fluorescence; (d) after 5 hours, fluorescent particles diffusing out radially. The scale bar in each image equals 1000 µm.

3.3.3. Storage Of Self Destructing Motherships

Our self-destructing mothership capsules start degrading as soon as they are placed in buffer solution. For use in various applications, it would be preferable if we could delay the "clock" for starting the enzymatic self-destruction sequence. Towards this end, we explored whether these capsules could be freeze-dried and stored, and whether they would still be active when re-hydrated. Figure 3.6 shows that this is indeed the case. We took freshly prepared mothership capsules (Figure 3.6a) and immediately subjected them to freeze drying under mild conditions. Dehydrated capsules collapsed to a dry powder (Figure 3.6b), allowing for convenient storage. Subsequently, the freeze-dried capsules were reconstituted by hydrating with a pH 6 buffer solution. Hydration caused the capsules to regain their original form, as shown in Figure 3.6c. Additionally, capsules retained their self-destructing nature and underwent degradation as expected (Figure 3.6d). Control capsules without enzyme could also be rehydrated after freeze-drying, but did not undergo any degradation. Thus, by employing a simple freeze-drying method, we have demonstrated that self-destructing motherships can be stored for future application.

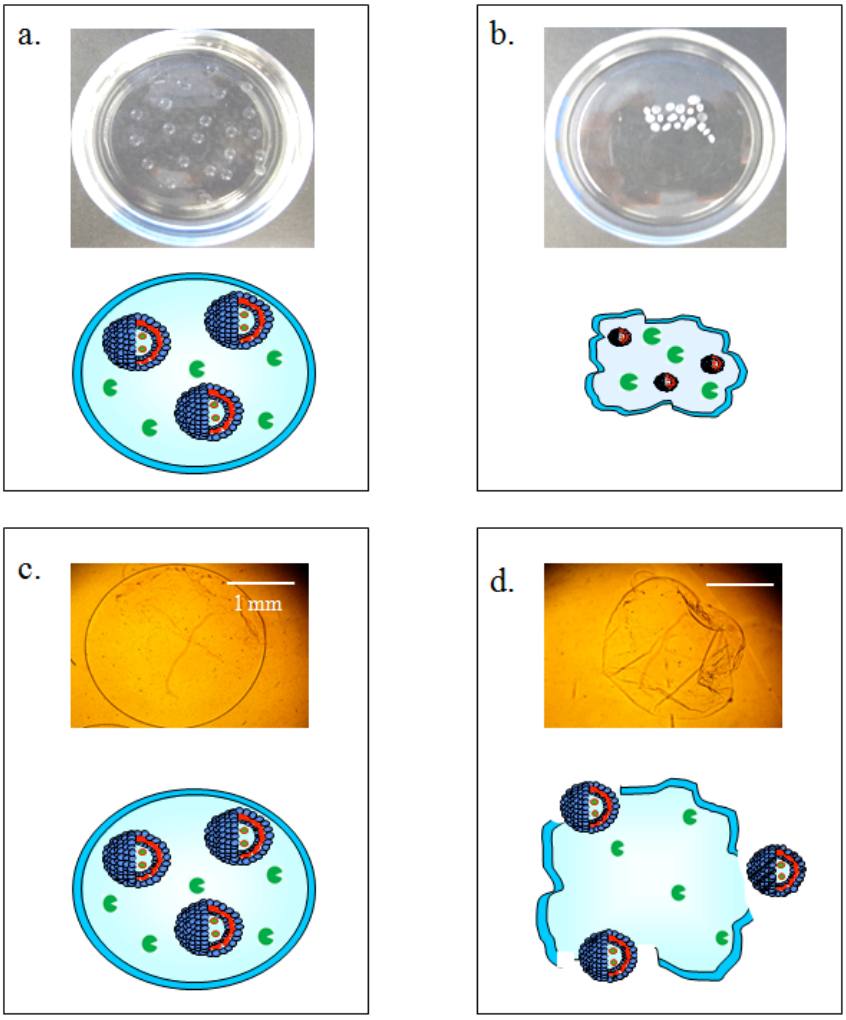

Figure 3.6. Storage of mothership capsules (a) Freshly prepared mothership capsules; (b) Capsules in powdery form after freeze drying; (c) Micrograph of reconsituted capsule in buffer; (d) Reconstituted capsule undergoing degradation**.** The scale bar in images (c) & (d) equal 1 mm.

3.3.4. External Degradation of Motherships by Enzymes

Finally, it is worth pointing out that our mothership capsules can also be degraded using enzymes from the outside. That is, if instead of packaging these capsules with enzymes, we added enzyme to the external solution, the added enzyme would indeed trigger the degradation of the capsules. Such enzyme-induced breakdown can be made to

occur for capsules with either a gellan or a chitosan core. We should point out that the self-destructing capsules could not be made with a chitosan core, i.e., the enzyme was not active if placed in a chitosan core. That is why we packaged the enzymes in a gellan gum core and with chitosan forming the shell. However, consider the results in Figure 3.7, which are for vesicle-bearing mothership capsules with a chitosan core and a gellan shell (note the bluish color of the capsules in Figure 3.7a, which arises due to light scattering from the vesicles). These capsules were placed in a buffer solution and to this solution we introduced 3 units/mL of chitosanase. Initially, the capsule remained intact but started to degrade after 1 day. Over the next 2 days, the capsule continued to degrade, as can be seen from the photo at the end of 3 days (Figure 3.7b). The capsules were completely broken at the end of 5 days (Figure 3.7c), and the debris from the broken capsules was mostly dissolved, leaving behind a clear solution.

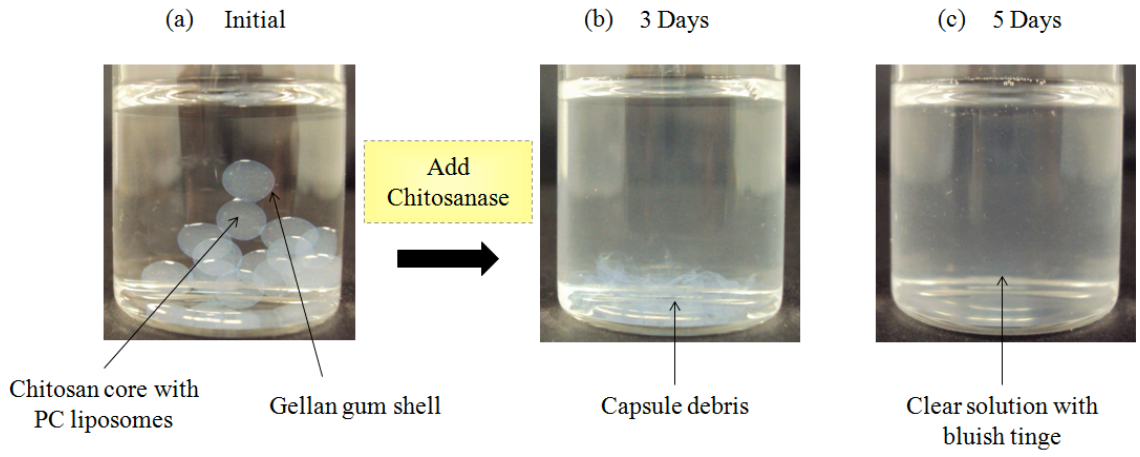

Figure 3.7. Capsules (chitosan core, gellan shell) under attack of chitosanase (3 units/mL) placed in external buffer solution. (a) initial, intact capsules with internalized vesicles, (b) 3 days, debris from degraded capsules (c) 5 days, note the bluish tinge in the clear solution due to intact vesicles scattering light.

Note that in this case, the chitosanase enzyme is degrading not just the shell, but presumably also the chitosan in the core of the capsules. For this reason, the degradation process takes a longer time (days) than in the case of gellan-core capsules, where the chitosan is present only in the shell. Also, when DLS was performed on this solution, structures approximately 100 nm in size were detected, which are evidently intact vesicles released from the capsules. Overall, we have thus established that mothership capsules can not only self-destruct due to internal enzyme, but also be induced to open up due to external enzymes.

3.4. CONCLUSIONS

We have synthesized mothership capsules, which are polymeric container structures that encapsulate much smaller babyships, i.e., vesicles. A simple, single-step method is used to generate these capsules under mild conditions. Additionally, we designed motherships that can self-destruct due to an encapsulated enzyme. The enzyme breaks down the capsule shell and thereby allows the vesicles to escape intact. Capsule degradation time can be tuned by the concentration of encapsulated enzyme. These motherships can be stored as an inert, freeze-dried powder until required; when reconstituted with buffer, they continue to have their self-destructing property. We believe that such capsules have great potential for delivery of a variety of payloads including drugs, biological therapeutics, cosmetics, or flavor ingredients.

Chapter 4: CONCLUSIONS & RECOMMENDATIONS

4.1. CONCLUSIONS

In this thesis, we have assembled hierarchical "mothership" structures encapsulating "babyship" vesicles inside them. We have developed a single step method to form such hybrid structures by employing a simple coacervation technique. We have internalized babyship vesicles within the lumen of the mothership capsules by employing an easy encapsulation technique. An advantage of our system is that we have utilized mild settings, avoiding the use of extreme conditions or harsh solvents for capsule preparation.

We have then demonstrated the destruction of these mothership capsules by employing enzymes as stimuli and ensured the delivery of intact vesicles to the target. By internalizing the enzyme within the capsule lumen, we confer self destructing properties to these "mothership" capsules. Finally, we have demonstrated a simple method to store these self destructing capsules for future applications. We visualize self destructing "mothership" capsules analogous to ticking time bombs with an internal trigger to deliver encapsulated drugs at a targeted time. We believe that such structures have significant advantages as well as potential uses in drug delivery systems. Such structures can be useful to a variety of applications such as delivering biological therapeutics, cosmetics, fertilizers, chemicals and flavor ingredients in addition to drugs

4.2. FUTURE DIRECTIONS

For future studies we could load vesicles with model drugs and monitor release profiles as a function of enzyme concentration. This would give us a better understanding of degradation kinetics of the capsule. The ultimate goal would be to develop an empirical equation which would enable us to calculate the degradation time. Investigation of other factors affecting degradation time such as molecular weights of polymers, different strains of enzymes and reaction conditions would be helpful in this direction.

Mothership capsules could also be tested for in-vivo applications. We could study the uptake of drug laden liposomes by cells when released by "mothership" capsules. Another direction to take in the future would be to develop capsules at the microscale. This can be done by employing processes such as microfluidics or atomization. This would enhance the utility of the mothership capsules.

We could also attempt make modification to the current system to bring about changes in degradation kinetics. For example, hydrophobically modified chitosan could be used as the cationic polymer which would offer stearic hindrance to enzyme attack and thereby increase degradation time. Alternatively, we could use two enzymes within the system, degrading both polymers to hasten the delivery of vesicles. It would also be beneficial to extend this concept and explore other systems and polymers to prepare and degrade mothership capsules. This would increase their versatility.

References

[1] Discher, D. E.;Eisenberg, A. “Polymer vesicles.” *Science* **2002**, *297*, 967-73.

[2] Lasic, D. D.;Papahadjopoulos, D. “Liposomes revisited.” *Science* **1995**, *267*, 1275-1276.

[3] Kibat, P. G.;Igari, Y.;Wheatley, M. A.;Eisen, H. N.;Langer, R. “Enzymatically activated microencapsulated liposomes can provide pulsatile drug release.” *Faseb Journal* **1990**, *4*, 2533-2539.

[4] Esser-Kahn, A. P.;Odom, S. A.;Sottos, N. R.;White, S. R.;Moore, J. S. “Triggered Release from Polymer Capsules.” *Macromolecules* **2011**, *44*, 5539-5553.

[5] Chandra, R.;Rustgi, R. “Biodegradable polymers.” *Progress in Polymer Science* **1998**, *23*, 1273-1335.

[6] Kumar, M. “A review of chitin and chitosan applications.” *Reactive & Functional Polymers* **2000**, *46*, 1-27.

[7] Wen, Y. L.;Yang, X. D.;Hu, G. H.;Chen, S. H.;Jia, N. Q. “Direct electrochemistry and biocatalytic activity of hemoglobin entrapped into gellan gum and room temperature ionic liquid composite system.” *Electrochimica Acta* **2008**, *54*, 744-748.

[8] Balazs, D. A.;Godbey, W. “Liposomes for use in gene delivery.” **2011**, *2011*, 326497.

[9] Amaike, M.;Senoo, Y.;Yamamoto, H. “Sphere, honeycomb, regularly spaced droplet and fiber structures of polyion complexes of chitosan and gellan.” *Macromol. Rapid Commun.* **1998**, *19*, 287-289.

[10] Machluf, M.;Regev, O.;Peled, Y.;Kost, J.;Cohen, S. “Characterization of microencapsulated liposome systems for the controlled delivery of liposome-associated macromolecules.” *Journal of Controlled Release* **1997**, *43*, 35-45.

[11] Angelatos, A. S.;Radt, B.;Caruso, F. “Light-responsive polyelectrolyte/gold nanoparticle microcapsules.” *Journal of Physical Chemistry B* **2005**, *109*, 3071-3076.

[12] Broaders, K. E.;Pastine, S. J.;Grandhe, S.;Frechet, J. M. J. “Acid-degradable solid-walled microcapsules for pH-responsive burst-release drug delivery.” *Chemical Communications* **2011**, *47*, 665-667.

[13] Antipov, A. A.;Sukhorukov, G. B. “Polyelectrolyte multilayer capsules as vehicles with tunable permeability.” *Advances in Colloid and Interface Science* **2004**, *111*, 49-61.

[14] De Geest, B. G.;Skirtach, A. G.;Mamedov, A. A.;Antipov, A. A.;Kotov, N. A.;De Smedt, S. C.;Sukhorukov, G. B. “Ultrasound-triggered release from multilayered capsules.” *Small* **2007**, *3*, 804-808.

[15] Hu, S. H.;Liu, T. Y.;Huang, H. Y.;Liu, D. M.;Chen, S. Y. “Magnetic-sensitive silica nanospheres for controlled drug release.” *Langmuir* **2008**, *24*, 239-244.

[16] Chaplin, M. F.;Bucke, C. *Enzyme Technology*; Cambridge University Press: New York, New York, USA, 1990.

[17] Somashekar, D.;Joseph, R. “Chitosanases - Properties and applications: A review.” *Bioresource Technology* **1996**, *55*, 35-45.

[18] Boucher, I.;Dupuy, A.;Vidal, P.;Neugebauer, W. A.;Brzezinski, R. “Purification and characterization of a chitosanase from Streptomyces N174.” *Applied Microbiology and Biotechnology* **1992**, *38*, 188-193.

[19] Johnston, A. P. R.;Lee, L.;Wang, Y. J.;Caruso, F. “Controlled Degradation of DNA Capsules with Engineered Restriction-Enzyme Cut Sites.” *Small* **2009**, *5*, 1418-1421.

[20] De Geest, B. G.;Dejugnat, C.;Sukhorukov, G. B.;Braeckmans, K.;De Smedt, S. C.;Demeester, J. “Self-rupturing microcapsules.” *Advanced Materials* **2005**, *17*, 2357-+.

[21] De Geest, B. G.;Dejugnat, C.;Prevot, M.;Sukhorukov, G. B.;Demeester, J.;De Smedt, S. C. “Self-rupturing and hollow microcapsules prepared from bio-polyelectrolyte-coated microgels.” *Advanced Functional Materials* **2007**, *17*, 531-537.

[22] Borodina, T.;Markvicheva, E.;Kunizhev, S.;Moehwald, H.;Sukhorukov, G. B.;Kreft, O. “Controlled release of DNA from self-degrading microcapsules.” *Macromolecular Rapid Communications* **2007**, *28*, 1894-1899.

[23] Yamamoto, H.;Senoo, Y. “Polyion complex fiber and capsule formed by self-assembly of chitosan and gellan at solution interfaces.” *Macromolecular Chemistry and Physics* **2000**, *201*, 84-92.

[24] Yamamoto, H.;Ohkawa, K.;Nakamura, E.;Miyamoto, K.;Komai, T. “Preparation of polyion complex capsule and fiber of chitosan and gellan-sulfate at aqueous interface.” *Bulletin of the Chemical Society of Japan* **2003**, *76*, 2053-2057.

[25] Ohkawa, K.;Kitagawa, T.;Yamamoto, H. “Preparation and characterization of chitosan-gellan hybrid capsules formed by self-assembly at an aqueous solution interface.” *Macromolecular Materials and Engineering* **2004**, *289*, 33-40.

[26] Fujii, T.;Ogiwara, D.;Ohkawa, K.;Yamamoto, H. “Alkaline phosphatase encapsulated in gellan-chitosan hybrid capsules.” *Macromolecular Bioscience* **2005**, *5*, 394-400.