Multifunctional and Supramolecular Materials through the Hydrophobic Effect

A DISSERTATION
SUBMITTED ON THE TENTH DAY OF DECEMBER 2014
TO THE DEPARTMENT OF CHEMICAL AND BIOMOLECULAR ENGINEERING
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
OF THE SCHOOL OF SCIENCE AND ENGINEERING
OF TULANE UNIVERSITY
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

BY

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APPROVED:


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Louise B. Lawson, Ph.D.
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ABSTRACT

Ever since the discovery of liposomes by A.D Bangham in 1961, they have attracted significant attention as drug delivery systems because they are biologically inert and weakly immunogenic, have low intrinsic toxicity and possess target delivery properties. Since steric stabilization of liposomes in the biologic milieu is a necessary condition for their utility as drug carriers, significant surface modification methods have been applied to liposomes to increase their stability. In this thesis, we describe a modification of the bilayer surface of liposomes using a hydrophobically modified water-soluble polymer (hydrophobically modified chitosan, hm-chitosan) based on the hydrophobic interaction between the polymer hydrophobes and liposome bilayer. Subsequently, we describe the structure transition of liposome/hm-chitosan systems from hm-chitosan coated liposome solutions at low hm-chitosan concentrations to hm-chitosan/liposome gels at high hm-chitosan concentrations.

Beside the application of liposomes in drug delivery, they also have been investigated intensively as biolubricants. This is due to the fact that they contain surface active phospholipids, which form liposome bilayers, reported as major lubricants in the synovial fluid of human joints. We describe filling the interior void of liposomes with a soft biocompatible material, silk fibroin, to enable the system to perform as rolling lubricants. Since rolling friction is always less than sliding friction, this liposome/silk
fibroin system results in a low coefficient of friction (COF). Additionally this system shows an enhanced property to minimize surface wear.

Based on the projects described above, we developed another lubricant, a biopolymer film with liposomes tethered on the surface. Here, an hm-chitosan film was made and was then used to tether liposomes based on hydrophobic interactions between hm-citosan and liposomes. This method of tethering liposomes leads to a densely packed liposome layer on the film surface. Such liposomal surfaces are effective lubricant, reducing COF values to as low as $10^{-3}$ and minimizing surface wear. Also, the compliancy and robustness of these tethered liposomes allow retention on the film surface upon repeated applications of shear. Because films can be used to cover the damaged area of cartilage to protect the surface and reduce pain, such film lubricants may be better than solution based lubricants in severe joint damage treatment.

Carbon microspheres have been used extensively as supports for nanoscale zerovalent iron (NZVI) in in-situ ground water remediation, as carbon serves as an adsorbent for chlorinated hydrocarbons, bringing the contaminant to the vicinity of the decontaminating agent of NZVI. However, colloidal instability of carbon microspheres limits their application in ground water remediation. Here, we extend the idea of the hydrophobic interaction between hm-chitosan and liposome bilayer to carbon microspheres, and report a strategy of stabilizing carbon microspheres by adding hm-chitosan coatings on carbon surface to create steric repulsions among particles. Such hm-chitosan stabilized carbon microspheres show markedly improved colloidal stability against sedimentation. More importantly, the hm-chitosan stabilized carbon microspheres move more effectively through soil. These results indicate the potential use of such
environmental benign hydrophobically modified biopolymers in groundwater remediation.
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CHAPTER I:

General Introduction

1.1 Liposomes

Phospholipids are a class of amphiphilic molecules containing hydrophilic headgroups and hydrophobic tails. These lipids can spontaneously form bilayer vesicles called liposomes. In nature, phospholipids are the major constituent of cell membranes. Because of bilayer similarity between cell membranes and liposomes, liposomes are taken up by cells through endocytosis. Liposomes may consist of single or multiple bilayers (uni- or multilamellar) of lipids, as shown in Figure 1.1 (a), with sizes varying from as small as 20 nm to around 100 μm. Phospholipids that are usually used to synthesize liposomes include phosphatidylcholines (PC), Phosphatidylglycerol (PG), phosphatidylethanolamines (PE) and phosphatidylserines (PS), [1] as shown in Figure 1.1 (b). Liposomes are usually divided into six major types based on their size and membrane structure: small/large/giant unilamellar vesicles (SUV, LUV and GUV), small/large/giant multilamellar vesicles (SMV, LMV and GMV). GUV and GMV structures have diameters ranging from 500nm to 100 μm. They can be directly observable by optical microscopy and are therefore generally studied as the simplest cell model. [2] LUV and
LMV with diameters ranging from 100 nm to 500 nm and SUV and SMV with diameters from 20 nm to 100 nm are often studied as protective capsules. [3-6] They are efficient drug carriers for a large panel of compounds such as vaccines, diagnostic agents, and proteins, [7-12] with hydrophilic drugs filled in the core of aqueous solution and hydrophobic drugs in the hydrophobic bilayers.
Figure 1.1. (a) Structure of unilamellar liposome and multilamellar liposome composed of phospholipids. (b) Structures of phosphatidylcholines (PC), Phosphatidyglycerol (PG), phosphatidylethanolamines (PE) and phosphatidylserines (PS).
1.2 Liposome Stability as Drug Delivery Vehicles and Stabilization Methods

Due to structure similarity of liposomes to cells, they are able to protect encapsulated molecules from degradation. The phospholipid bilayer structure may also be modified to target specific tissues or organs and also target specific cell types such as cancer cells. [13, 14] As a result, liposome technology for drug delivery has been intensively investigated and has made considerable progress. Several liposome formulations for various disease treatments are now available in the market. [15]

When injected intravenously, liposomes are rapidly captured by the mononuclear phagocyte system (MPS) and removed from the blood circulation.[15] Therefore when the target site is beyond the MPS, efficient liposome uptake by macrophages results in their consequent removal from circulation. This is one of the main disadvantages for possible use of liposomes as drug delivery systems. It has been reported that liposome surface binding of selected opsonins is the first signal for MPS to recognize and remove liposomes, and a limited number of possible opsonizing proteins that serve this purpose have been identified. [16-20] Another reason for liposome instability in plasma is due to their interaction with high and low density phospholipases, and this interaction leads to rapid release of the encapsulated drug into the plasma. [21] For administration of liposome via oral route, which is the most convenient method and carries the lowest cost, liposomes are liable to be destroyed by the pH, bile salts, and pancreatic lipase in the GI tract. [22]

The most widely used method for increasing the stability of liposomes is coating liposome surface with inert molecules or biocompatible materials to form a steric
repulsive barrier. [23, 24] A number of coating materials have been investigated basically involving hydrophilic polymers possessing a flexible chain that occupies the space immediately adjacent to the liposome surface that tends to exclude other macromolecules from this space. Consequently, access and liposome surface binding of molecules which cause instability of liposome are hindered. Among the various polymers investigated to improve the stability of liposomes, poly-(ethylene glycol) (PEG) is the most widely used and well-developed as a polymeric steric stabilizer. 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) covalently linked to poly(2-methyl-2-oxazoline) or to poly(2-ethyl-2-oxazoline) formed liposomes has also been shown to lead to extended blood circulation times and decreased uptake by the liver and spleen.[25] More recent papers describe stealth liposomes prepared using poly [N-(2-hydroxypropyl) methacrylamide], amphiphilic poly-N-vinylpyrrolidones, L-amino-acid-based biodegradable polymer-lipid conjugates, poly(vinyl pyrrolidone) (PVP), poly(acryl amide) (PAA) and polyvinyl alcohol (PVA). [26-30]

1.3 Chitosan to Make Stable and Mucoadhesive Liposomes for Drug Delivery

Chitosan is a linear polysaccharide composed of glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit), and is obtained by treating shrimp and other crustacean shells with the alkali sodium hydroxide. The structure of chitosan is shown in Figure 1.2. It is a hydrophilic, biocompatible and biodegradable polymer of very low toxicity. Also, chitosan exhibits high antimicrobial activity and has been shown to reduce fat adsorption. [31-35]
Figure 1.2. Chemical structure of chitosan.
Since the discovery of polysaccharides on cell surfaces and the high affinity of chitosan to cell membranes, a number of research papers have reported utilizing chitosan and its derivatives as coating materials for liposomes, [36] emulsion droplets and biopolymeric particles.[37, 38] For intravenous injection of chitosan coated liposomes, studies are rare but there are a few reports that show that chitosan coating on liposomes can protect liposomes and increase the circulating time in serum.[39]

More importantly, chitosan is a mucoadhesive polymer with the ability to open epithelial tight junctions. It is reported to act as a permeation enhancer for non-invasive drug delivery leading to an increase in paracellular transport of macromolecular drugs.[31, 39] Several studies have reported the use of chitosan as an absorption-enhancing agent with liposomes.[31, 40] Thereby, by combining characteristics of liposomes and chitosan or its derivatives, specific, prolonged and controlled release formulation for oral administration can be achieved.[41]

1.4 Interaction between hm-Chitosan and Liposomes

As stated above, polymers can be utilized to modify the liposome bilayer surface.[42] Such surface-grafting can lead to steric stabilization of liposomes.[43] While vesicles usually aggregate and fuse due to depletion forces in the presence of hydrophilic polymers, [44, 45] their interactions with water soluble polymers carrying a low fraction of hydrophobic groups are of interest in tuning liposome structure and properties. These hydrophobically modified water soluble polymers can be anchored on the surface of liposome membrane due to the tendency of the hydrophobic groups to insert themselves into the lipid bilayer.[42] The driving force is the change of the free energy associated
with the transfer of the hydrophobic groups from a partially solvent-exposed state to the more ordered hydrophobic environment in the membrane. [46]

The hydrophobic modification of chitosan through random attachment of C12 alkyl groups onto about 2.5% of the amine functionalities does not impact the water solubility of the polymer, but allows the polymer to interact with liposomes through insertion of the alkyl hydrophobes into vesicle bilayers. [47, 48] This property of hm-chitosan can be used to form adhesive coating layers on liposomes for oral drug delivery. [49] Beside coating vesicles, another outcome of such hydrophobe insertion into bilayers of hm-chitosan is the fact that the polymer chains can bridge vesicles, and the vesicles then form nodes in a network of polymer chains to form a “vesicle gel”. [47, 50-52] This ability to gel low viscosity vesicle solutions through the incorporation of hm-chitosan is of significant interest, as the concept of vesicle gelation can be extended to the gelation of erythrocytes thus leading to new applications in hemostasis and cell capture. [47, 53-55]

Therefore, the study of liposome and hydrophobically modified chitosan systems is of great interest and this investigation will contribute to new biomedical applications of liposomes.

1.5 Liposome as Biolubricant for Human Joints

In the human joint, a system of boundary lubrication exists, where layers of lubricant molecules separate the opposing articular cartilage surfaces of the joints. [56-59] The synovial space in the human joint is filled with viscous synovial fluid containing hyaluronic acid and the glycoprotein lubricin, [60-62] typical of the lubricant molecules that participate in this boundary lubrication. Lubricin is composed of proteins,
carbohydrates and surface active phospholipids.[63-65] To understand friction and wear properties for synovial joints with osteoarthritis, chemical components of the synovial fluids have been individually examined for their lubrication properties.[66-68] Most interesting are recent studies that have shown that phospholipids are extremely effective at reducing friction.[69, 70] In fact, phospholipid vesicles (liposomes), either as unilamellar vesicles or as multilamellar vesicles have been reported to reduce the coefficient of friction to physiological levels when coated on surfaces which are allowed to slide past each other.[69, 70]

The mechanism of liposomes lubricating surfaces is based on the hydrophilic head groups of the phospholipids, as described in the literature. [69, 70] The highly-hydrated headgroup of phospholipid can attract up to 12 water molecules around it, as shown in Figure 1.3, which serve as nano ball-bearings between two surfaces to facilitate very low friction motions. Since liposome is such a promising candidate for lubricating human joint due to its highly biocompatible property and ability to reduce friction, the development of various liposome systems for damaged joint conditions is of great interest. My work attempts to address the design of new biolubrication systems.
Figure 1.3. The liposome bilayers have highly hydrated water molecules around hydrophilic headgroups which serve as nano ballbearings, thereby facilitating very low friction motions.
1.6 Carbon Microspheres as Carriers of Nanoparticles in In-situ Remediation and the Using of Biopolymer to Increase Colloidal Stability and Transport Property of the System

Groundwater and soil contamination by dense nonaqueous phase liquids (DNAPLs) is prevalent at numerous hazardous waste sites and contaminated sites.[71-73] Common DNAPLs include chlorinated hydrocarbon compounds such as carbon tetrachloride (CCl₄), chloroform (CHCl₃), trichloroethylene (TCE, C₂HCl₃) and perchloroethylene (PCE, C₂Cl₄). To eliminate the hazardous effect by DNAPLs such as TCE, extensive efforts have been made to develop methods for the remediation of DNAPLs. With growing awareness of the limitations of these traditional processes, the in situ injection remediation technology represents a promising approach for remediation and is getting more and more attention. [74, 75]

Nanoscale zero-valent iron (NZVI) has been found to be very effective in the reductive dechlorination of several halogenated hydrocarbons that are persistent groundwater contaminants, including TCE and TCA. [76-80] However, applications employing NZVI are difficult to realize because the material is not readily dispersible in aqueous solvents and rapidly aggregates in suspension due to the high surface energies of the nanomaterials and their intrinsic magnetic properties when above the superparamagnetic size limit of 15-20 nm. [81-83] Techniques to increase particle mobility include the use of surfactants, starch, modified cellulose, and vegetable oils. [84-89]. Polyelectrolyte (e.g., CMC) coatings are widely used to improve particles or supported particles mobility through soil. [84, 90, 91] Selected shear thinning fluids such as vinyl polymer and gum xanthan have also been employed to enhance colloid emplacement in porous media. [92]
A simple alternative method of stabilizing NZVI particles is to support them on innocuous particles such as silica particles [93] or carbon [86, 90] thus preventing aggregation. Composites with carbon are particularly promising as they introduce a strong adsorptive aspect into remediation technology as the carbons have a strong affinity for chlorinated compounds. [94] Thus, carbon adsorption can be utilized to retain contaminants in close proximity to the supported NZVI and allow adsorptive and reductive reactions to occur simultaneously and efficiently. Such materials have also been used in the development of adsorptive reactive barriers. [95, 96] The use of carbon as a support for NZVI is therefore finding increased application in the environmental remediation of chlorinated compounds. [94, 97, 98] The immobilization of NZVI onto submicron carbon particles may also decrease uptake of NZVI in microorganisms and mitigate the potentially harmful implications of nanoparticles. However, carbon submicron particles sediment out of solution and it becomes necessary to design appropriate strategies to enhance colloidal stability and allow transport through groundwater saturated sediments.

The use of hm-chitosan as a carrier for carbon based supports of NZVI has the potential of leading to colloidal stability of the carbon particles since hm-chitosan can bind to carbon through the hydrophobic effect of water exclusion, thereby form electrosteric repulsion between particles. Additionally, the hydrophobic interaction is relatively insensitive to solution electrolyte concentration, allowing it to be used under a variety of groundwater conditions. My work represents the first use of hm-chitosan to stabilize colloidal particles and to examine the transport characteristics of such particles through groundwater saturated sediments.
CHAPTER II:

Interactions of a Hydrophobically Modified Biopolymer with Phospholipid Vesicles

- Evolution from Vesicle Coatings to Vesicle Gels


2.1 Introduction

Liposomes are small vesicles bounded by lipid bilayer membranes, formed spontaneously when phospholipids are dispersed in aqueous solutions.[99] Since their invention by A.D Bangham in 1961, [99] liposomes have attracted tremendous interest as drug delivery systems because they are biologically inert and weakly immunogenic, have low intrinsic toxicity and possess target delivery properties.[13, 100-102] Their interactions with polymers can be utilized to modify the bilayer surface,[42] and this surface-grafting leads to steric stabilization of liposomes, a necessary condition for their utility as drug carriers for anticancer therapy and targeted drug delivery. [43] Coating of
the external liposome surface with polymers may help resist macrophage attack and phospholipase breakdown and thus increase circulation lifetimes in the body. Existing technology typically uses polyethylene glycol (PEG) functionalized liposomes, which includes PEG-derivatized phospholipids not only in the outer layer of liposome membrane but also in the interior, which leads to reduced drug loading efficiencies. [103]

While vesicles usually aggregate and fuse due to depletion forces in the presence of hydrophilic polymers, [44, 45] their interactions with water soluble polymers carrying a low fraction of hydrophobic groups are of interest in tuning structure and properties. These hydrophobically modified water soluble polymers have the remarkable property of anchoring to the surface of the membrane due to the tendency of the hydrophobic groups (hydrophobes) to insert themselves into the lipid bilayer. [42] The driving force is the free energy change associated with the transfer of the hydrophobic groups from a partially solvent-exposed state to the more ordered hydrophobic environment in the membrane. [46] A very interesting outcome of such hydrophobe insertion into bilayers is the fact that the hydrophobically modified water soluble polymer chains can bridge vesicles; the vesicles then form nodes in a network of polymer chains to form a “vesicle gel”. [47, 50-52]

This ability to gel low viscosity vesicle solutions through the incorporation of hydrophobically modified polymers is of significant interest, as particularly exemplified by the use of hm-chitosan in the recent literature. [47, 53, 55, 104] Chitosan is a linear copolymer composed of glucosamine and N-acetylg glucosamine residues, obtained by the alkaline deacetylation of chitin the main component of the exoskeleton of crustaceans such as shrimp. [105-107] The hydrophobic modification of chitosan to hm-chitosan
through random attachment of C12 alkyl groups onto about 2.5% of the amine functionalities does not impact the water solubility of the polymer, but allows the polymer to form vesicle gels through insertion of the alkyl hydrophobes into vesicle bilayers and bridging between vesicles [47, 48]. The biocompatibility of hm-chitosan is of particular relevance as concept of vesicle gelation can be extended to the gelation of erythrocytes thus leading to new applications in hemostasis and cell capture. [47, 53-55] Beside gelling vesicles, hm-chitosan also has been used as an adhesive coating on liposomes for oral drug delivery. [49]

While gelation of vesicles and cells is certainly of much interest, a second question arises as to the interaction of hm-chitosan with such bilayer systems at sufficiently dilute conditions when mean distances of separation between vesicles makes such direct bridging difficult. In other words, we have asked the question whether the transition to gels would occur through a systematic progress from liposomal coatings to bridging between liposomes. Our hypothesis was that in the dilute system, the polymer would prefer to insert multiple hydrophobes into the bilayer of a single vesicle thus potentially coating the vesicle. Since cryo electron microscopy is very useful in this regard to understand the structure of polymer coatings on liposomes, in this work, we first investigated the coated liposomes using cryo transition electron microscopy (cryo-TEM) and cryo scanning electron microscopy (cryo-SEM). Subsequently, the hm-chitosan/liposome colloid structure evolution from vesicle coatings to vesicle gels was studied using rheological methods, together with gel structure characterization using cryo-SEM. Finally, since hm-chitosan coatings on liposomes can be considered as an
effective way of increasing liposome stability and circulation time in blood, the ability of hm-chitosan coated liposomes to survive in serum was evaluated.

2.2 Experimental Section

2.2.1 Materials

DPPC (1, 2-dipalmitoyl-sn-glycero-3-Phosphocholine, structure shown in Figure 2.1(a), DMPG (1, 2-dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (structure shown in Figure 2.1(b)), and Mini-Extruder were from Avanti Polar Lipids, Alabaster, Alabama. Chitosan of low molecular weight (approximately 50K - 190K Daltons) was obtained from Aldrich. The reported degree of deacetylation was between 75% and 85%. 1% acetic acid solution was used to dissolve chitosan. Dodecyl aldehyde, sodium cyanoborohydride (NaCNBH₃), sodium hydroxide, acetic acid, and ethanol were obtained from Sigma-Aldrich and were used as received without further treatment. De-ionized (DI) water generated with a Barnstead E-pure purifier (IA) was used in all experiments.
Figure 2.1. Structures of (a) DPPC, (b) DMPG and (c) hm-chitosan.
2.2.2 Preparation of Liposomes

The liposomes used in this study were prepared by the thin-film evaporation method as previously described.\[108\] In detail, the phospholipids of DPPC and DMPG were mixed in the ratio 1:1 (w/w, 0.05 g each) and dissolved in 15 mL of chloroform and methanol mixture (2:1 v/v). The solution was evaporated by using a rotary evaporator (BUCHI, Switzerland) for 3 hours to form a dry lipid film. The lipid film was then hydrated for 30 min with 5 mL of DI water at 50 °C and 125 rpm to obtain a 2% (w/v) liposome suspension (except where specified otherwise, the concentrations in the following are always calculated as w/v). The liposome suspension was extruded 11 times through different pore size polycarbonate membranes (Whatman, Mobile, Alabama) at 55 - 65 °C to downsize the liposomes and get uniform liposome suspensions.

2.2.3 Synthesis of hm-Chitosan

hm-Chitosan was derived by reaction the amine groups of chitosan with n-dodecyl aldehyde. The procedure used was identical to that described in the literature. Briefly, 4 g of chitosan was firstly dissolved in 220 mL of 1% (v/v) acetic acid, followed by the addition of 150 mL ethanol to allow the aldehyde used for the alkylation to be in a solvating medium. The pH was adjusted to 5.1 by the addition of sodium hydroxide and then the solution of dodecyl aldehyde in ethanol was added at 2.5% ratio to the chitosan monomole prior to an excess of sodium cyanoborohydride (3 moles per chitosan monomole). The mixture was stirred for 24 hours at room temperature and the final product was firstly precipitated with ethanol and sodium hydroxide solution, and then
washed with ethanol and DI water three times. hm-chitosan structure is shown in Figure 1.1 (c).

2.2.4 hm-Chitosan Coated Liposomes

To prepare hm-chitosan coated liposomes, an appropriate amount of the hm-chitosan polymer was firstly dissolved in 1% (v/w) acetate solution (pH = 2.8) in order to prepare various hm-chitosan solutions that would result in concentration from 0.4 % up to 1.2 %. In each case, an aliquot of the liposome dispersion was mixed with an equal volume of polymer solution, which was added drop by drop to the liposome solution, under continuous stirring. After this, the mixture was incubated for 30 min at room temperature. The resulted hm-chitosan coated liposome suspensions were stored in the refrigerator for further analysis. For gels of liposome and hm-chitosan, higher concentrations of hm-chitosan are used. Liposome dispersion was mixed with an equal volume of hm-chitosan solution, and then the mixture was left at room temperature for 2 hours before test.

2.2.5 Analytical

Cryo-TEM (JEOL 2011) was utilized to image the liposomes or hm-chitosan coated liposomes in their native state. In the process, a 10 μL drop of native liposome or hm-chitosan coated liposome suspension was placed on a Formvar coated copper TEM grid. The grid was blotted to form a thin film and rapidly vitrified in liquid ethane. The sample was then transferred under the protection of liquid nitrogen to a TEM equipped with a Gatan cold stage, and examined under acceleration voltage of 120 kV as the same as in a conventional TEM mode. The temperature of the sample grid was maintained at -
175 °C during imaging. Cryo-SEM was done using a field-emission SEM (Hitachi 4800). Briefly, the procedure involves rapid plunging of the sample into liquid nitrogen, followed by freeze-fracture using the flat edge of a cold knife (-130 0C) and then sublimation for 5 min at -95 0C to etch away surface water and expose internal features. The sample was then sputter coated with platinum at 10 mA for 88 s and imaged on the SEM at a voltage of 3 kV and at a working distance of 6 mm.

Rheological characterizations were done at 25°C using a TA Instruments AR 2000 rheometer with a concentric cylinder geometry and a cone-and-plate geometry with diameter of 40 mm and angle of 1° for analysis.

2.3 Results and Discussion

2.3.1 hm-Chitosan and Liposome Interactions at Low Polymer Concentrations to Generate Coated Liposomes

To test the hypothesis that at low polymer concentration, the hm-chitosan would interact with single liposomes rather than with multiple liposomes, we carried out experiments where hm-chitosan was kept at concentrations less than 0.6 %, based on the visual observation that at 1 %, the system forms a gel. Solutions of higher hm-chitosan concentrations become extremely viscous, and it is extremely difficult to obtain reliable cryo-TEMS of the liposomes at concentrations greater than 0.6% due to the inherent difficulty of blotting highly viscous solutions. The cryo-TEMs of Figures 2.2(a) and (b) indicate an increasing contrast at the rim and the center of the liposomes indicating a greater thickness of the liposomal shell. Figure 2.3 illustrates the cryo-SEM images at hm-chitosan concentration of 0.4 %. We see evidence of a ribbon-like polymer network
forming, but without significant incorporation of liposomes within the strands of the network. We note that at all these concentrations of hm-chitosan, the system is able to flow as visualized by a simple vial inversion test.
Figure 2.2. (a) Cryo-TEM of (A) native DPPC-DMPG liposomes (1%) and (B) hm-chitosan (0.4%) coated DPPC-DMPG liposomes (1%) where a dark layer around liposomes was observed. (b) Cryo-TEM of 1% liposomes (DPPC-DMPG) coated with (A) 0%, (B) 0.2%, (C) 0.4% and (D) 0.6% low molecular weight hm-chitosan showing the increasing thickness of the hm-chitosan coating layer as the concentration of hm-chitosan solution increased.
Further characterization of the hm-chitosan/liposome systems is provided through rheological studies. Figure 2.4 shows the viscosity as a function of shear rate for the systems with increasing hm-chitosan concentrations. For each hm-chitosan concentration the comparison was done for the system containing hm-chitosan alone and for the system containing hm-chitosan and liposomes. We see that even the addition of a small concentration of hm-chitosan (0.2 %) increases the solution viscosity. However, the addition of liposomes does not significantly impact the viscosity implying that the liposomes are just coated; with polymer and do not participate in any network formation. With higher amounts of hm-chitosan this is not true. At 0.4 % hm-chitosan, the viscosity increases significantly with the addition of liposomes. We therefore postulate that at these concentrations, the liposomes generate more connections of the polymer but without reaching the gel threshold. The same observation of an increased viscosity upon addition of liposomes is valid for the higher concentrations of 0.6 % hm-chitosan and 1 % hm-chitosan. At 1 % hm-chitosan, the system is a solution without the addition of liposomes but becomes a rigid gel upon the addition of liposomes.
Figure 2.4. (a) The viscosity as a function of shear rate for different hm-chitosan and liposome systems: 0.4% hm-chitosan+1% DPPC&DMPG liposomes; 0.4% hm-chitosan; 0.2% hm-chitosan+1% DPPC&DMPG liposomes; 0.2% hm-chitosan and 1% DPPC&DMPG liposomes. (b) The apparent viscosity as a function of shear rate for 1% hm-chitosan+1% DPPC&DMPG liposomes; 1% hm-chitosan; 0.6% hm-chitosan+1% DPPC&DMPG liposomes and 0.6% hm-chitosan.
The dynamic oscillatory measurements of the elastic (storage) and viscous (loss) moduli are shown in Figure 2.5. Clearly, it is observed that the dynamic response of liposomes and 1% hm-chitosan satisfies the strict rheological definition of a gel, where \( G' \) is greater than \( G'' \) with no dependence of the moduli on frequency. The system of 0.6% hm-chitosan with liposome is close to the sol-gel transition, while the system with 0.2% hm-chitosan shows solution behavior.
Figure 2.5. Dynamic rheology of liposome and hm-chitosan mixtures (◆: G’ and ◇: G” for 1% hm-chitosan+1% DPPC&DMPG liposome; ▼: G’ and ▽: G” for 0.6% hm-chitosan+1% DPPC&DMPG liposome; ●: G’ and ○: G” for 0.2% hm-chitosan+1% DPPC&DMPG liposome). 0.2% hm-chitosan+1% DPPC&DMPG liposome is a solution, 0.6% hm-chitosan+1% DPPC&DMPG liposome is close to the sol-gel transition, and 1% hm-chitosan+1% DPPC&DMPG liposome is a gel, as shown by its frequency-independent elastic modulus G’ at low frequencies.
2.3.2 Characteristics of the Gel through Cryo-Electron Microscopy

The effect of adding larger amount of hydrophobically modified chitosan on the phase behavior of a 1% solution of liposome is readily observed by vial tests. Figure 2.6(a) shows a photograph of three samples: (A) a control of 1% hm-chitosan solution, (B) a viscous solution containing 1% liposome and 1% native chitosan, and (C) a gel containing 1% liposome and 1% hm-chitosan. In agreement with prior work by Raghavan [47, 53, 55, 104] we observe that the 1% hm-chitosan solution is a clear liquid, whereas a sample containing 1% chitosan and 1% liposome is a viscous fluid. However, upon adding 1% hm-chitosan into 1% liposome solution, the sample is transformed into a gel that is able to hold its own weight under vial inversion, similar with previous results.[47, 109] All systems were dyed with 0.0005 % methylene blue to distinguish them from the background.
Figure 2.6. (a) Photographes of (A) 1% hm-chitosan solution, (B) 1% chitosan +1% DPPC&DMPG liposomes and (C) 1% hm-chitosan +1% DPPC&DMPG liposome solution results an elastic gel that is able to hold its own weight in the inverted vial. Dye used in samples: 0.0005 % methylene blue. Cryo-SEM images of the structure formed by (b) 1% chitosan + 1% DPPC&DMPG liposomes showing liposomes sit on the walls of chitosan network and there is no connections generated by liposomes between the walls, and (c) 1% hm-chitosan + 1% DPPC&DMPG liposomes showing the liposome generate connections between hm-chitosan network walls, which strength the network and results gel formation.
Figure 2.6 (b) and (c) present cryo-SEM images of the structures of chitosan/liposome and hm-chitosan/liposome systems. In Figure 2.6(b), which is the structure of chitosan/liposome solution, we observe pocket-like structures formed by chitosan. Some of the liposomes stay aggregated near the pocket walls with no evident specificity in the aggregation pattern or the aggregation site. On the other hand, for the hm-chitosan/liposome gel shown in Figure 2.6(c), we observe pocket-like structures with tendrils protruding from the cell walls and in the pocket with liposomes attached to the tendrils and dispersed throughout the gel phase in the pocket. More importantly, liposomes are connected with the polymer strings from one wall to another in the pocket.

Our cryo micrographs are the first direct evidence of liposomes contributing to a gel network, and the images correlate with the rheological characterizations. we propose that the addition of liposomes to hm-chitosan solution will generate more connections between the hm-chitosan walls and in the whole system, which increase the network strength and finally facilitate gel formation. While for chitosan solutions, adding liposome does not contribute gel formation because the interaction between liposomes and chitosan is not obvious.

This observation confirms the reported interaction between the hm-chitosan and liposomes in the literature.[47] Although the addition of vesicles to polymer solutions results gel formation also has been reported,[47, 109] the visualized characterization by electron microscopy of the interaction between vesicles and polymers and the gel network structure are seldom reported.

2.3.3 Proposed Structure Evolution of hm-Chitosan/Liposome Systems
**Figure 2.7** provides a schematic illustration of our proposed mechanism of structure evolution from solutions to gels upon adding hm-chitosan to such anionic liposomes. We recognize in agreement with prior work [47, 53, 55, 104] that the hydrophobes of hm-chitosan insert themselves into the liposome bilayer. At low concentrations of hm-chitosan, the free polymer forms loose networks within which liposomes are present. These liposomes are coated and also connect with other liposomes of the polymer network but do not form a robust crosslink with the polymer network. As the hm-chitosan increases, our hypothesis is that the coating of the liposome continues, but additionally free strands on the liposome surface connect up to the polymer network to form the crosslinks shown in Figure 2.7 and resulting in a gel.

At very low concentrations of hm-chitosan, the polymer molecules lie on the liposome surface, as shown in Figure 2.7 (b). With hm-chitosan concentration increased, some of the polymers tends to “standing” on the liposome surface to allow more polymers interact with one liposome, as shown in Figure 2.7 (c). While at sufficiently high concentrations, the hm-chitosan polymer form network pocket-like structure walls and also some of the hm-chitosan molecules outstretched from one wall to bridge another wall. The role of liposomes here in the pocket is firstly based on their inducing more hm-chitosan molecules outstretched from the polymer walls because of the hydrophobic interactions between them, and then serve as junctions to connect hm-chitosan polymer strings on the walls therefore generate more connections to increase network strength, as you can see schemed in Figure 2.7 (c) and (d). Briefly, the liposomes here contribute to the gel formation by inducing more connections between the polymer walls, which will greatly increase the network strength. As schematically shown in Figure 2.7 (d), longer
bridges between two liposomes and two walls formed by several hm-chitosan molecules interact with each other due to hydrophobic interactions.
Figure 2.7. Hydrophobic interaction between hm-chitosan and liposome resulting an hm-chitosan coating layer on liposomes at lower concentration of hm-chitosan, and adding more hm-chitosan results a structure transition from coated liposome suspension to hm-chitosan/liposome gel.
2.3.4 Stability of hm-Chitosan Coated Liposomes in Serum

As we discussed before, liposome surface-grafting of polymers usually leads to steric stabilization of liposomes, an important property for their use as carriers in anticancer therapy and targeted drug delivery.\cite{43} The interactions of liposomes with serum proteins can lead to important effects on liposome stability and in vivo behavior. For example, serum lipoproteins can potentially destabilize bilayer membranes leading to vesicle disruption and loss of encapsulated drugs, as well as result in irregularities in the lipid bilayer.\cite{110} Also, the adsorption of serum proteins, such as bovine serum albumin and ovalbumin, on liposomes may influence the colloidal stability of liposomes and result in liposome aggregation.\cite{111} In our work we tested the stability of liposomes with or without hm-chitosan coatings in fetal bovine serum for one hour, as shown in Figure 2.8.
Figure 2.8. Cryo-TEM image of 1% liposomes (DPPC-DMPG) coated with 0.2% low molecular weight hm-chitosan after incubation in 10% fetal bovine serum for 1 hour showing survival intact liposomes. The inset is Cryo-TEM image of 1% bare liposomes (DPPC-DMPG) after incubation in fetal bovine serum for one hour showing few liposomes.
The cryo-TEM image of hm-chitosan coated liposomes after incubation in 10% fetal bovine serum for 1 hour shows intact liposomes well dispersed in solution. For bare liposomes on the other hand, incubation with serum leads to almost a total loss of liposomes after an hour. This result is in accordance with the observations in the literature [111] and indicates that hm-chitosan stabilized liposomes can resist disruption by serum lipoproteins to a greater degree than the bare liposomes. We also tried to test the releasing profile of coated liposomes in serum using fluorescein isothiocyanate labelled bovine serum albumin (FITC-BSA) as a model drug, however, the separation of the liposomes from serum and the accurate detection of released FITC-BSA in serum are very hard because of the various components in serum.

2.4 Conclusion

In conclusion, the structure evolution of hm-chitosan/liposome systems from solutions of coated liposomes to gels was investigated and the evolution mechanism was proposed. We showed that at low concentrations of hm-chitosan, liposome can be coated with hm-chitosan and the thickness of the coating layer can be tuned. The coated liposomes show higher stability in serum than bare liposomes in serum. At higher concentrations of hm-chitsoan, hm-chitsoan/liposome systems form gels, and the gel structure was visualized by cryo-SEM. The transition to a gel is clearly visualized by the presence of polymer tendrils connecting liposomes. The use of cryo imaging is instrumental to the understanding of structure evolution and this is the first instance of the imaging of liposomes trapped in a gel matrix.
CHAPTER III:

Lubrication Properties of Phospholipid Liposome Coated Silk Microspheres


3.1 Introduction

Under high loads on articular joints, a system of boundary lubrication exists where layers of lubricant molecules separate opposing surfaces of the joints.[56-59] The synovial space is filled with viscous synovial fluid containing hyaluronic acid and the glycoprotein lubricin.[60-62] typical of the lubricant molecules that participate in this boundary lubrication. Lubricin is composed of proteins, carbohydrates and surface active phospholipids.[63-65] To understand friction and wear properties for synovial joints with osteoarthritis, chemical components of the synovial fluids have been individually examined for their lubrication properties.[66-68] Most interesting are recent studies that have shown that phospholipids are extremely effective at reducing friction as the hydration layers around the head groups provide a boundary lubrication effect.[69, 70] In fact, phospholipid vesicles (liposomes), either as unilamellar vesicles or as multilamellar
vesicles have been reported to reduce the coefficient of friction to physiological levels when coated on surfaces which are allowed to slide past each other.[69, 70]

The highly compressible aspect of liposomes brings about the conjecture that filling the interior void with a soft biocompatible material may enable rolling of the liposomes, a better cushioning effect, and the possibility of enhancing wear resistance. Introduction of a rolling element between two contact surfaces can, in addition to reducing friction,[112] also reduce the amount of wear if optimal loads and speeds are not exceeded. The judicious choice of a rolling element may easily give a service life of tens of thousands hours before it wears sufficiently to lose operational efficiency.[113, 114] Earlier studies with hard rolling elements show coefficient of friction (COF) values ranging from 0.01 to 0.1.[115-120] These include micron sized materials such as glass microspheres (40 μm) [115] and stainless steel microballs (285 μm),[116, 117, 119] and also include nanoscale materials such as the fullerenes [121-124] and fulleren-like supramolecules of WS$_2$ and MoS$_2$.[125-128]

The current work specifically address the use of soft rolling elements with potential use in biolubrication. Thus, the intriguing question emerges whether a rolling element consisting of natural and biocompatible biomaterials would have the requisite tribological properties, and eventually benefit long term boundary lubrication properties of synovial joints.

The silk fibroin protein from *Bombyx mori*, the commercialized source of silk for textiles via sericulture, is an extensively studied structural protein used as a biopolymeric material for tissue engineering and drug delivery.[129-134] Further, silks are
mechanically robust due to the extensive physically crosslinked beta sheet crystals and can be programmed to degrade slowly in vivo.[131, 135, 136] Recently, microspheres prepared from silk have been shown to be effective vehicles for controlled drug release.[137]

This paper reports on the translation of these silk microspheres to biolubricants by coating them with a phospholipid liposomal layer. The use of a coating is based on the following reasons: (a) phospholipids, hydrated in their headgroups, are major constituents of synovial fluid surface active materials [64] and are promising natural candidates to facilitate low friction in articular cartilage for the proper mobility of synovial joints,[63, 65, 69, 70, 138] (b) inserting a “lubricant” between rolling elements may avoid jams caused by sticking between the rolling elements, and improve the rolling properties of the silk microspheres. [112] Rolling elements that stick and subsequently jam tend to slide rather than roll upon application of shear, resulting in a sliding wear. [114] The phospholipid coating was added with the hypothesis that motion under the hydration layer would also decrease sliding wear. A schematic illustration of an aqueous liposome coated silk microsphere suspension as a lubricant is shown in Figure 3.1.
Figure 3.1: An aqueous liposome coated silk microsphere suspension as a ball bearing lubricant rolling in between two shearing surfaces.
3.2 Experimental Section

3.2.1 Friction Measurements

The experiments were performed with silicon surfaces (rms = 0.54 nm, test grade, University Wafer, Boston, MA) as the bottom shearing surfaces and a curved optically polished glass surface (radius of curvature = 3 cm, Anchor Optics, Barrington, NJ) as the probe (top surface). All surfaces were cleaned by sonication in ethanol for 5 min, followed by rinsing with water and a subsequent plasma-cleaning step (Harrick Plasma, Ithaca, NY). In a typical experiment, a drop (~50 μL) of the 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) liposome coated silk microspheres (12.5 mg mL$^{-1}$ DOPC, 5 mg mL$^{-1}$ silk microspheres) suspension was placed between the bottom surface and the probe, wetting both surfaces. Surface tension forces ensure that the aqueous suspension remains in the contact region. The probe, attached to a force sensor (DFM-0.5, CETR, Campbell, CA) with a cantilever (spring constant $k_{DFM} = 4113$ N/m), was then brought into contact with the bottom surface at a predetermined preload. A universal materials tester (CETR, Campbell, CA) was used to measure the friction force between the shearing surfaces as the load was either held constant or increased stepwise with each shear cycle. The coefficient of friction was determined by taking the slope of the average friction force versus the average load for each shear cycle. Longer runs, in which the evaporation of the aqueous-based lubricant was significant, were performed in a home-built enclosure with excess water in the surroundings to maintain constant humidity.

3.2.2 Preparation of Silk Microspheres Encapsulated in DOPC Liposomes

DOPC was dissolved in a chloroform and methanol mixture (2:1 v/v) and dried under low pressure in a standard rotary evaporator to obtain a lipid film. The dried lipid
film was hydrated with the silk microspheres suspension at room temperature for 30 min, and the suspension was then sonicated (VWR B2500A-MTH, a bath sonicator) at low power for 15 minutes. For washing DOPC liposome coated silk microspheres, DI water (50 mL) was added to disperse the suspension (0.5 mL), and then the sample was centrifuged (4000 rpm, 10 minutes) to collect silk microspheres. The procedure was repeated multiple times (typically three times) as needed. The coated silk microspheres were re-dispersed in DI water (0.5 mL) after washing away the excess liposomes.

3.2.3 Confocal Laser Scanning Microscopy for Characterization of Silk Microspheres Coated with DOPC Liposomes

We first labeled silk microspheres with rhodamine B (excitation 553 nm, emission 627 nm) which has a strong binding affinity to silk fibroin, resulting in a high loading efficiency and a slow release profile. [139] Rhodamine-B (0.05 mg) was first added to silk microspheres suspensions (1 mL, 5 mg mL\(^{-1}\)), then stirred at room temperature for 2 hours to let the rhodamine B penetrate and adsorb to the silk microspheres. Centrifugation of the suspension led to the precipitation of the microspheres with adsorbed rhodamine B. The supernatant, which contained the excess label in solution, was then removed and DI water was added. The washing procedure was repeated three times to remove all excess label. The rhodamine B labeled microspheres were then coated with DOPC liposomes as described above, with the notable difference that a fluorescein labeled phospholipid (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(carboxyfluorescein), excitation 490 nm, emission 515 nm) was doped into the system at a level of 1 %. Digital overlaying of the emission from the liposome layer (green) and the microsphere (red) was then done to obtain the composite emission from the coated
microsphere. In a system with both filled and empty liposomes, the empty liposomes are indicated by the green color and the silk filled liposomes by a yellow color.

### 3.3 Results and Discussion

A dispersion of DOPC liposome coated silk microspheres was prepared and used in the lubrication tests. A typical concentration of the silk microspheres used was 5 mg mL$^{-1}$ while the DOPC concentration was 12.5 mg mL$^{-1}$. **Figure 3.2** illustrates the native silk microsphere (a) and a microsphere coated with DOPC liposomes (b). The diameter of silk microspheres ranges from 3 μm to 10 μm, and most of them has a diameter around 5μm. **Figure 3.2(c)** to **Figure 3.2(f)** provides the verification of the DOPC liposomal coatings on the silk microspheres. Emission from the DOPC containing fluorescein-labeled DOPE (green) is shown in **Figure 3.2(c)**, while emission from the silk microspheres containing adsorbed rhodamine B (red) is shown in **Figure 3.2(d)**. The merging of the two images results in the fluorescence spectra shown in **Figure 3.2(e)**, where the small offsets are a result of the fact that the particles move slightly during the time period for collection of the fluorescence images. The direct merging of the two images of **Figure 3.2(c)** and 3.2(d) in **Figure 3.2(e)** is an indication that the microspheres are indeed coated with liposomes. **Figure 3.2(f)** shows a corresponding image where the empty DOPC liposomes have not been washed out. As indicated, these free liposomes can be seen (as shown by the arrows of **Figure 3.2(f)**) and are typically much smaller than the silk microspheres which are the load bearing elements. There is a wide distribution of sizes of the liposomes but they do not exceed 3μm and can be easily identified in the fluorescence micrograph.
Figure 3.2. (a) SEM image of silk microsphere and (b) DOPC liposome coated silk microsphere. The insets are low magnification images of silk microspheres and DOPC liposome coated silk microspheres. Scale bars in the insets are 10 µm. Figure 1 (c-f) show confocal laser scanning micrographs of silk microspheres coated with DOPC liposomes after removal of empty DOPC liposomes. Images of (c) fluorescein labeled DOPC liposomes and (d) rhodamine-B loaded silk microspheres are digitally merged into image (e) to show that the liposomes coat the silk microspheres. Image (f) is the same system without removal of empty DOPC liposomes, where the arrows point to the liposomes. The offsets in the images are based on the fact that the particles move during the data collection period.
A commercial universal materials tester (UMT) with a spherical glass probe and a silicon wafer substrate was used to measure frictional properties of the sample placed between the probe and substrate. A schematic of the instrument is shown in Figure 3.3. The probe is attached to a sensor through a cantilever, and movement in the x and z direction is controlled by a motion actuator. A typical measurement consisted of applying an initial preload, shearing the surfaces at a fixed velocity \(v_x\) and distance, and reversing the path. After a preset delay, the load is then increased and the shear cycle repeated. The force required to move the probe in the x direction \(F_x\) is collected as a function of the load \(L\) and data analysis is done digitally. A magnified view of the confined region in Figure 3.3 illustrates the lubrication mechanism of DOPC liposome coated silk microspheres. The motion of the top surface causes the microspheres in the contact region to roll, while the confined region is continuously replenished with silk microspheres from the surrounding medium.
Figure 3.3. Schematic of the universal materials tester used to measure the friction force showing the contact region between the probe and the lubricant material.
The friction coefficients between a spherical glass tip and silicon wafers lubricated by various samples of interest are shown in Figure 3.4(a). The spherical glass and silicon wafer were first sheared with 5 mg mL\(^{-1}\) silk microspheres suspended as the lubricant, resulting in a COF of 0.167. We attribute the higher COF to the possibility of “jamming” [140-143] where the friction between the particles hinders rotational mobility and inhibits smooth rolling. One way to avoid jams is to insert a “lubricant” between the spherical bearings. [128, 144, 145] With this objective, the silk microspheres were coated with DOPC liposomes. This coating was achieved by encapsulating the microspheres with DOPC liposomes during liposomal preparation, and washing off the excess free DOPC liposomes. A suspension of de-ionized (DI) water washed silk microspheres with DOPC liposome coating layers was used as a lubricant. This system provides good lubrication with a COF of 0.026, as shown in Figure 3.3a. Analysis of the phospholipid content in the silk microspheres was estimated by the phosphorus content after acid digestion, [146, 147] with a remnant level of 1.4 mg mL\(^{-1}\) of DOPC liposomes in 5 mg mL\(^{-1}\) silk microspheres after repeated washing. Figure 3a illustrates that the control DOPC liposomes at a concentration of 1.4 mg mL\(^{-1}\) lipid, exhibit a COF of 0.137.
Figure 3.4. (a) Plot of friction force $F_x$ versus applied load $L$ while shearing a spherical glass probe versus a silica surface using the following lubricant candidates, (▲) an aqueous silk microspheres suspension (5 mg mL$^{-1}$) (■) an aqueous DOPC liposomal solution (1.4 mg mL$^{-1}$) and (●) an aqueous DOPC liposome (12.5 mg mL$^{-1}$) coated silk microspheres (5 mg mL$^{-1}$) suspension after removal of empty liposomes (1.4 mg mL$^{-1}$ DOPC left). (b) Plot of friction force $F_x$ versus applied load $L$ with the following lubricant candidates (■) an aqueous DOPC liposomal solution (12.5 mg mL$^{-1}$) and (●) an aqueous DOPC liposome coated silk microspheres suspension without removal of empty liposomes (12.5 mg mL$^{-1}$ DOPC, 5 mg mL$^{-1}$ silk microspheres). The inset is the friction coefficient at varying concentrations of DOPC liposomes alone. (c) Plot of a typical friction force $F_x$ and applied load $L$ versus time over the load 294 mN to 980 mN in the presence of an aqueous DOPC liposomal solution (12.5 mg mL$^{-1}$) as the lubricant. (d) The corresponding plot of friction force $F_x$ and applied load $L$ versus time, when using a suspension of DOPC liposome (12.5 mg mL$^{-1}$) coated silk microspheres (5 mg mL$^{-1}$). All measurements were performed with an increasing load from 294 mN to 980 mN and a sliding velocity of 1 mm s$^{-1}$. 
These results indicate that at the same DOPC liposome concentration of 1.4 mg mL\(^{-1}\), DOPC liposome coated silk microspheres suspensions are more effective than DOPC liposomes alone to reduce friction. The synergistic, high lubrication performance of DOPC liposome coated silk microspheres may result from the rolling behavior of silk microspheres between the two surfaces upon shear application, where the DOPC liposome coating layers act as a lubricant between the surfaces of the microspheres and reduce the tendency of the microspheres to jam.

Figure 3.4(b) further illustrates the comparison of the lubrication properties of empty liposomes to liposomes filled with silk microspheres. The inset to Figure 3.4(b) illustrates that at a liposome concentration of 12.5 mg mL\(^{-1}\), the COF decreases to a value of 0.058, which is reasonably close to that of the coated silk microspheres and in agreement with literature values. [70] The lubrication property of DOPC liposomes is attributed primarily to the hydration layers surrounding the phosphocholine groups at the outer surface of the vesicle bilayer that prevents the two surfaces from achieving true contact, thus leading to the term “hydration lubrication”.[48, 148] Phospholipid based surfactants as boundary lubricants require a close packed layer of the head groups to provide the hydration layer between surfaces in contact and this typically leads to high phospholipid concentrations to induce good lubrication properties.[70, 149] Figure 3b also illustrates that the COF of silk microspheres at high levels of liposomal coating maintain a low COF (0.025), and the COF is not measurably reduced upon washing away excess DOPC present in the form of empty liposomes (filled circles of Figure 3.4(a)). Thus the empty liposomes have a negligible impact on the coefficient of friction of the system.
The system incorporating a combination of empty liposomes and liposomes filled with silk microspheres exhibits additional advantages over the system of liposomes alone. Figure 3.4(c) and 3.4(d) illustrates segments of typical friction and load traces of the two systems. A single shear cycle involved an increase in the applied load followed by shearing in one direction (the -x direction) and then a reversal in the shearing direction over the same distance. As shown in Figure 3.4(c) and 3.4(d), “stiction spikes” due to stick-slip characteristics occur frequently in the force traces at the initiation of shear in either direction. The stick-slip behavior for empty DOPC liposomes alone illustrates stiction effects that are significantly greater than those of the combined system and the magnitude of the stiction spikes is considerably reduced by including the coated silk microspheres in the system. The reduced stiction is important in applications requiring frequent stop and go motion, and has potential relevance to articular joints. Typically, rolling systems have lower stiction spikes than sliding systems [150] and the possibility of using soft rolling systems for biolubrication may be validated. A second observation from Figure 3.4(c) and 3.4(d) is the stick slip behavior for the system of DOPC liposomes when the load is increased. Each step in the load tends to squeeze out the lubricant from the confined region, generating an initial high friction [69] that may also contribute to the stiction. Any possible addition to stiction at the beginning of each cycle may also be dampened by using soft rolling liposomes filled with silk microspheres.

Finally, the effectiveness of a lubricant is measured not just by its ability to provide a low friction coefficient but also by its ability to reduce surface wear. AFM images of the wear pattern were obtained after shearing a spherical glass probe against a silicon surface, in the contrasting systems of: (a) empty liposomes alone, and (b) empty
liposomes together with liposomes filled with silk microspheres. In both systems, the level of DOPC was kept constant at 12.5 mg mL\(^{-1}\). The wear test was conducted for 500 shearing cycles with a constant load of 490 mN. In the absence of the silk microspheres, surface damage occurs throughout the contact region (**Figure 3.5(a)**). The AFM images (Figure 3.5(a) and 3.5(b)) reveal that the addition of silk microsphere filled liposomes reduces surface wear and provides protection against long term surface damage.
Figure 3.5. AFM images (10 μm x 10 μm) of the wear pattern formed after shearing a glass probe against a silicon surface at 490 mN load for 500 cycles in the presence of (a) an aqueous DOPC liposomal solution (12.5 mg mL$^{-1}$) as lubricant and (b) an aqueous DOPC liposome (12.5 mg mL$^{-1}$) coated silk microsphere (5 mg mL$^{-1}$) suspension as lubricant. The coated silk microspheres system show significantly reduced wear signatures.
3.4 Conclusion

In summary, we have shown that an aqueous DOPC liposome (12.5 mg mL\(^{-1}\)) coated silk microsphere (5 mg mL\(^{-1}\)) suspension is an effective lubricant, providing a low COF on the order of 0.025, minimizing surface wear and avoiding degradation even over relatively prolonged cycling conditions. We propose that the lubricating properties of the silk microspheres are a result of an efficient rolling mechanism, and the liposomal coating layer acts as a lubricant in the slip step of the rolling process between the two shear surfaces, avoiding jamming of silk microspheres while rolling. These results may have implications for biomedical applications especially in joint lubrication. With current therapeutic options for improving joint comfort being limited, new biomaterials with the appropriate biological and tribological properties offer an intriguing opportunity. Since silk is a biocompatible and slowly degradable biomaterial, the data reported here suggest a path forward toward such a goal.

3.5 Supporting Information

The Supporting Information contains details of the calculation of the phospholipid level necessary to form a unilamellar coating on silk microspheres.

3.5.1 Calculation of the DOPC Phospholipid Level that is needed to Coat Silk Microspheres

The data used for calculation is based on a system containing 0.5 % silk microspheres with average diameter 5 \(\mu\)m.

Density of silk fibroin = 1.4g·cm\(^{-3}\) [151]
The number of silk microspheres \((N_{SM})\) in 1 mL of solution is

\[
N_{SM} = \frac{1 \text{ mL} \cdot (1 \text{ g/mL}) \cdot 0.5\%}{\left(\frac{4}{3} \pi r^3 \text{ cm}^3\right) \cdot (1.4 \text{ g/cm}^3)}
\]

where \(r\) is the average radius of silk microspheres (2.5 \(\mu\)m).

If we assume that there is a unilamellar phospholipid bilayer coating on the silk microspheres, the number of lipid molecules needed for coating one silk microsphere \((N_{lipid})\) is calculated as:

\[
N_{lipid} = 2 \times \frac{4\pi r^2}{a}
\]

where \(4\pi r^2\) is the surface area of one of the "monolayers" of the the coating layer with ‘\(a\)’ being the lipid head group area. The headgroup area of phosphatidylcholine is about 0.72 nm². [152] The thickness of the bilayer is negligible (5 nm) compared to the diameter of silk microspheres (5000nm).

In the above equation the surface area of both monolayers in a unilamellar coating layer are added together. Then the total lipid area is divided by the head group area of one lipid molecule.

The total DOPC needed for coating 1 mL 0.5 % silk microspheres suspension \((m_{lipids})\) is calculated as following:

\[
m_{lipids} = \frac{N_{SM} a?? N_{lipid} a?? M_{DOPC}}{N_A}
\]
where $N_A$ is the Avogadro number, $M_{DOPC}$ is the molar mass of DOPC (786.11 g/mol).

$$m_{lipids} = 1.55 \times 10^{-2} \, g$$

Then the concentration of lipids needed is 1.55 mg/mL for a unilamellar vesicle coating.

The concentration of lipids we have used is 12.5 mg/mL, significantly greater than that needed for unilamellar coating. However, after washing out the excess lipids after coating the microspheres, the amount of lipids measured was 1.4 mg/mL, well in agreement with that required for unilamellar coating. Hence we feel we have designed a unilamellar coating to the silk microspheres.
CHAPTER IV:

Liposomes Tethered to a Biopolymer Film through the Hydrophobic Effect Create a Highly Effective Lubricating Surface


4.1 Introduction

Phospholipid vesicles known as liposomes have been studied extensively as biomembrane mimics,[153, 154] and are of much interest in applications related to drug and biomolecule delivery. [5, 102] In the recent literature, liposomes, either in solution [70] or adsorbed onto surface, [69, 155] have been reported to be efficient boundary lubricants at physiologically relevant conditions, exhibiting very low coefficients of friction (COF). Such low COF values are attributed to the lubrication ability of the highly hydrated phospholipid head groups exposed at the vesicle outer surfaces.[70, 155] For example, the phosphocholine head group moiety can attach up to 15 rapidly relaxing water molecules[148, 156-158] leading to the concept of a hydration based lubrication.[159, 160] In this context, the water of hydration can sustain large compression without being squeezed out from the gap between surfaces in shear, while at
the same time allowing the hydration shells to relax rapidly, ensuring a fluid like response on shear. [159]

In this paper, we report a novel concept of fabricating films of a specific biopolymer (hydrophobically modified chitosan, hm-chitosan) that interact with liposomes through the hydrophobic effect of hydrophobe insertion into liposomal bilayers,[47, 55] to tether liposomes on the film surface. We show that such tethered liposomal surfaces are robust and exhibit excellent lubrication properties reducing the COF values to between $10^{-2}$ and $10^{-3}$, at pressures up to 158 MPa, significantly higher than the contact pressures reported in the human hip joint (up to 18 MPa).[161, 162] The biocompatibility and antimicrobial properties of chitosan [163-165] additionally make these systems potentially applicable as materials for synovial joint lubrication.

4.2 Results and Discussion

Figure 4.1 illustrates the concepts of this paper. Figure 4.1(a) shows the structure of hm-chitosan used in this work where about 2.5% of the amine groups on the chitosan backbone are substituted with C-12 alkyl groups. The synthesis procedure follows that reported in the literature, [47, 166] and involves the addition of aldehyde to an acidic chitosan solution in a water-ethanol mixture, followed by the addition of sodium cyanoborohydride. The detailed procedure can be found in the Supporting Information section. $^1$H NMR (Supporting Information Figure S4.1) confirms the presence of alkyl groups on the chitosan backbone. Films of hm-chitosan were prepared by evaporating aqueous solutions of hm-chitosan in 1% acetic acid (to sustain solubility) containing glutaraldehyde as a crosslinking agent. Briefly, 1 mL of 0.5% hm-chitosan in 1% (v/v)
Acetic acid solution was mixed with 0.0015 mL 10% glutaraldehyde, and the solution was mechanically stirred for 30 s in order to be homogeneous; then the mixture was dropped on a 22 mm × 22 mm cover glass for drying at room temperature for at least 24 hours. Our hypothesis as shown in Figure 4.1(b), was that upon formation of the film, there would be a sufficient number of exposed hydrophobes on the surface of the film that are able to attach to liposomes through insertion into the lipid bilayer, in accordance with earlier studies from Raghavan’s laboratory. [47, 54, 104] While native chitosan crosslinked films are hydrophilic (contact angle 47.2º) the use of hm-chitosan increases the contact angle to 56.8º possibly indicating an exposure of hydrophobic groups on the surface (data in Supporting Information Figure S4.2). Figure 4.1(c) shows the next length scale of lubrication characterization, where immobilized liposomes are placed in the contact zone between the hm-chitosan/liposome film and a glass probe surface.
Figure 4.1. (a) hm-Chitosan molecular structure; (b) schematic of hm-chitosan film tethering liposomes by inserting its alkyl groups into the liposomal bilayer (c) schematic illustrations of the contact region between a glass probe with one flat and one outward curved face and an hm-chitosan/liposome film. $v_x$ is the fixed probe velocity.
The details of the study follow. Subsequent to the preparation of hm-chitosan films, they were placed in an aqueous suspension of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) liposomes. To prepare DPPC liposome, 0.1 g DPPC (Avanti Polar Lipids, Inc) was dissolved in a chloroform and methanol mixture (2:1 v/v) and dried under low pressure in a rotary evaporator (Buchi R210) to obtain a thin lipid film. The dried lipid film was hydrated with 5 mL of a PBS buffer solution at 50 °C for 30 min, and the suspension was then extruded through polycarbonate membranes, first with a 400 nm membrane and then with a 100 nm pore size membrane at a temperature between 55 °C and 65 °C, using an Avanti-Extruder (Avanti Polar Lipids, Inc). The cryogenic transmission electron microscopy (cryo-TEM) image of the liposome solution is shown in supporting information Figure S4.3. The hm-chitosan films were incubated in DPPC liposome solution for 30 min, and then washed with phosphate buffered saline (PBS, pH=7.4) 3 times to remove the free and loosely attached liposomes on the film. The results of this exposure of the hm-chitosan film to liposomes are shown in Figure 4.2. Figure 4.2(a) shows a bare hm-chitosan film with a smooth surface prior to incubation with the liposome solution. Figures 4.2(b) and 4.2(c) illustrate the film after 30 min incubation with liposomes where it is clear that the liposomes are intact and densely packed. The dense packing of the tethered layer leads to distortions from sphericity as also observed by Klein and coworkers for liposomes physically adsorbed on mica. [69] We note that the liposomes on hm-chitosan are tethered through hydrophobe insertion as extensive washing of the surface film has no effect on the integrity of liposome packing on the surface. Additionally, as Figure 4.2(d) indicates, films of native chitosan without
the alkyl hydrophobes are unable to capture liposomes and only a few liposomes are
adsorbed to the chitosan surface after washing. Thus we attribute the tethering of
liposomes to the hm-chitosan film surface as due to the hydrophobic effect wherein the
alkyl groups on the polymer backbone insert into the lipid bilayer.
Figure 4.2. Cryogenic scanning electron microscopy (cryo-SEM) images of (a) the hm-chitosan film, (b) and (c) hm-chitosan/liposome film after being washed by PBS buffer, showing a layer of close-packed liposomes on the film surface, and (d) chitosan/liposomes film after being washed by PBS buffer showing only a few liposomes on the film surface.
In order to demonstrate the functional lubrication properties of these tethered films, a commercial universal materials tester (UMT, CETR, Campbell, CA) was used to measure the friction force between the hm-chitosan/liposome film and a glass probe (radius of curvature=3 cm, Anchor Optics, Barrington, NJ) with a curved optically polished surface (Figure 4.3(a)). The hm-chitosan/liposome film was fixed on the bottom holder. A glass probe was attached to a force sensor (DFM-0.5, CETR, Campbell, CA) through a cantilever (spring constant $k_{DFM} = 4113 \text{ N/m}$) and the movement in $x$ and $z$ direction was controlled by a motion actuator. The film and the probe were immersed in a PBS buffer solution for testing. A typical measurement consisted of applying an initial preload, shearing the surfaces at a fixed velocity $v_x$ and distance, increasing the normal load, and repeating the shear cycle. The data was collected and analyzed digitally. Details of the experiment and the complete data from this equipment can be found in the Supporting Information.

Figure 4.3(b) shows plots of the friction forces $F_x$ between two shearing surfaces as a function of the normal loads $L$, the slope of which is the COF. Details of a complete experiment showing the $F_x$, $L$ and COF values as a function of time for each cycle are in the supporting information section (Figure S4.4). It is seen from Figure 4.3(b) that the hm-chitosan film coated with a close packed liposome layer provides a very low COF of 0.0076 in the range of COF values observed for synovial fluids. [63, 167] The data indicates that over the range of loads applied, Amonton’s first law of friction where the frictional force is directly proportional to the applied load is applicable. To confirm the reproducibility of the COF value of this system, three liposome/hm-chitosan samples were fabricated and evaluated. In all cases the COF was highly reproducible with a COF
value of 0.0076 ± 0.0003. As a control experiment, the friction experiment was performed with a chitosan film after incubation in a liposome solution. This system provided a COF of 0.024. The higher COF is attributed to the relatively few liposomes adsorbed on the chitosan film surface. As another control experiment, a liposomal solution was used as the lubricant between two shearing glass surfaces, resulting in a COF of 0.048; in the absence of liposomes in solution the COF was 0.099. Finally, when the hm-chitosan film and the glass probe were sheared in the absence of liposomes, a high COF (0.074) was generated. These experimental results confirmed that close-packed tethered liposomes play a major role as lubricants to reduce the COF. As an additional experiment we added liposomes in solution to the system of immobilized liposomes on hm-chitosan film, but did not observe any appreciable reduction in the COF (data in Supporting Information Figure S4.5).
Figure 4.3. (a) Schematic of the universal materials tester used to measure the friction force showing the contact region between the probe and the lubricant material, (b) plot of the friction force $F_x$ versus the applied load $L$ while shearing a glass probe versus a bare hm-chitosan film (▼ triangle down), chitosan/liposome film (▲ triangle up) and hm-chitosan/liposome film (● circle) in PBS buffer solution; and plot of the friction force $F_x$ versus the applied load $L$ while shearing a glass probe versus a flat glass surface using a DPPC liposome solution (2%) (♦ diamond) or DI water (■ square) as a lubricant. The measurements were performed with an increasing load from 196 mN (20 g) to 784 mN (80 g), equivalent to pressures from 30 to 120 MPa, sliding velocity of 1mm/s, and dwell time of 5s. (c) Cryo-SEM image of an hm-chitosan/liposome film after shearing a glass probe against the film at 980 mN (100 g) load for 50 cycles in PBS buffer solution show no significant wear on either the film or the liposomes.
Thus, the tethering of liposomes to hm-chitosan through the hydrophobic effect leads to a dense packing of liposomes on an hm-chitosan film and exhibits lubrication properties that are in the biologically-relevant range. However, the effectiveness of a lubricant is not only measured by its ability to provide a low COF but also by its ability to reduce surface wear. [48, 168] In other words, it is important that the densely packed liposomal layer is retained on the surface upon repeated applications of a shear force. Figure 4.3(c) illustrates the cryo-SEM images of the hm-chitosan/liposome film surface after shearing a glass probe against the hm-chitosan/liposome film for 50 shearing cycles at a constant high load of 980 mN (equivalent to pressure of 158 MPa, higher than the physiological pressures in joints which are up to 18 MPa). [161, 162] We observed that the liposomes remained intact and closely packed on the hm-chitosan film surface indicating a robustness of the tethering process. Throughout the cycles studied in this experiment, the COF remains at 0.0076 (data in supporting information Figure S4.6).

### 4.3 Conclusion

In summary, we have shown that an hm-chitosan film can tether a close-packed liposome layer on the film surface via hydrophobic interactions between hm-chitosan and liposomes. The tethering of liposomes to hm-chitosan is easily accomplished and represents a facile method to capture and immobilize liposomes. The hm-chitosan/liposome film significantly reduces the COF and minimizes surface wear. The consistent lubrication properties of the hm-chitosan/liposome film are attributed to resilient hydrophobic interactions between the hm-chitosan film and liposomes. These interactions maintain a robust close-packed liposomal layer on the film surface allowing hydration lubrication over extended wear cycles. In addition to the effective lubrication
properties shown here, the ease of liposome immobilization through such tethering based on the hydrophobic effect, leads to several applications in drug delivery and in fundamental investigations of biomembranes using captured liposomes and other vesicular entities.

4.4 Supporting Information

Supplementary Information available: Details on NMR spectroscopy study of hm-chitosan structure, contact angles of water droplet on hm-chitosan film, cryo-TEM images of DPPC liposomes, complete data from the tribometer showing the friction force, coefficient of friction as a function of increasing load and the plot of the coefficient of friction when shearing a glass probe versus hm-chitosan/liposome film in 2% DPPC liposome solution.

Synthesis of hm-Chitosan: 4 g of chitosan was dissolved in 220 mL of 1% (v/v) acetic acid, and then 150 mL ethanol was added to allow the aldehyde used in the alkylation step to be in a solvating medium. The pH was adjusted to 5.1 by the addition of sodium hydroxide, and then a solution of dodecyl aldehyde in ethanol was added at a concentration that 2.5% of the monomer concentration. This is followed by the addition of an excess of sodium cyanoborohydride (3 mol/mol sodium cyanoborohydride/chitosan-monomer). The mixture was stirred for 24 hours at room temperature and the final product was first precipitated with ethanol and sodium hydroxide solution, and then was washed with ethanol and deionized (DI) water three times. The molecular structure of hm-chitosan was characterized through $^1$H NMR spectroscopy to verify the attachment of the hydrophobes.
The experiments were conducted in deuterium oxide using a Bruker Avance 500 MHz NMR spectrometer, and the results are shown in Figure S4.1.

Figure S4.1. $^1$H NMR spectroscopy of 0.50% (a) hydrophobically modified chitosan (hm-chitosan) and (b) chitosan samples indicates the successful addition of alkyl groups to the chitosan backbone.
Figure S4.2. The contact angles of a water droplet on (a) hm-chitosan film and (b) chitosan film. The increase in contact angle with hm-chitosan is representative of the increase in hydrophobicity with exposure of alkyl groups.
**Figure S4.3.** Cryo-TEM image of prepared DPPC liposomes.
Friction Measurements: The experiments were performed with a cover glass (Fisherfinest Premium Cover Glass, Fisher Scientific), chitosan/liposome film and hm-chitosan/liposome film as the bottom shearing surfaces and a curved optically polished glass surface (radius of curvature = 3 cm, Anchor Optics, Barrington, NJ) as the probe (top surface). The glass surface was cleaned by sonication in ethanol for 5 min, followed by rinsing with DI water and a subsequent plasma-cleaning step (Harrick Plasma, Ithaca, NY). In a typical experiment, the bottom surface, either a cover glass or a prepared film, was glued onto a holder and a drop (∼50 μL) of the DPPC liposome (2%) suspension or PBS buffer solution was placed between the bottom surface and the probe. The probe, attached to a force sensor (DFM-0.5, CETR, Campbell, CA) with a cantilever (spring constant $k_{DFM} = 4113$ N/m), was then brought into contact with the bottom surface at a predetermined preload. A universal materials tester (CETR, Campbell, CA) was used to measure the friction force between the shearing surfaces as the load was either held constant or increased stepwise with each shear cycle. The COF was determined by taking the slope of the average friction force versus the average load for each shear cycle.
Figure S4.4. Complete data from the universal materials tester (tribometer) showing the friction force (gold), and the COF (pink) as a function of increasing load (blue). The measurements were performed with a stepwise increasing load from 196 mN (20 g) to 784 mN (80g), a sliding velocity of 1mm/s, and a dwell time of 5s. The specific measurement shown is for the system of hm-chitosan film containing tethered liposomes in contact with a glass probe. We note that the COF remains constant at 0.0076.
Figure S4.5. The plot of the friction force $F_x$ versus the applied load $L$ while shearing a spherical glass probe versus hm-chitosan/liposome film in 2 % DPPC liposome solution. The measurements were performed with an increasing load from 196 mN (20 g) to 784 mN (80g), sliding velocity of 1mm/s, and dwell time of 5s. The data indicates that the addition of liposomes in solution does not affect the lubrication properties of the film.
Figure S4.6. Complete data from the universal materials tester (tribometer) showing the friction force (gold, $F_x$, g), and the COF (pink) as a function of increasing load (blue, $F_z$, g). The measurements were performed with a constant load of 980 mN (100g), a sliding velocity of 1 mm/s. The specific measurement shown is for the system of hm-chitosan film containing tethered liposomes in contact with a glass probe. The COF value remains stable during the test.
CHAPTER V:

Using the Hydrophobic Effect to Attach Modified Biopolymers onto Carbon Based Carriers of Nanoparticles to Enhance Colloidal Stability and Transport in In-situ Remediation


5.1 Introduction

Nanoscale zero-valent iron (NZVI) has been found to be very effective in the reductive dechlorination of several halogenated hydrocarbons that are persistent groundwater contaminants, including trichloroethylene (TCE), trichloroethane (TCA), and carbon tetrachloride. [76-80] However, applications employing NZVI are difficult to realize because the material is not readily dispersible in aqueous solvents and rapidly aggregates in suspension due to the high surface energies of the nanomaterials and their intrinsic magnetic properties when above the superparamagnetic size limit of 15-20 nm. [81-83] NZVI particles stabilized by organic species such as surfactants, vegetable oils, starches, or polyelectrolytes such as carboxymethyl cellulose (CMC) and poly (acrylic
acid) (PAA), or triblock copolymers have been reported to exhibit increased colloidal suspension stability. [85, 89, 169-173] Techniques to increase particle mobility include the use of surfactants, starch, modified cellulose, and vegetable oils. [84-89]. Polyelectrolyte (e.g., CMC) coatings are widely used to improve particles or supported particles mobility through soil. [84, 90, 91] And selected shear thinning fluids such as vinyl polymer and gum xanthan have also been employed to enhance colloid emplacement in porous media. [92]

A simple alternative method of stabilizing NZVI particles is to support them on innocuous particles such as silica particles [93] or carbon [86, 90] thus preventing aggregation. Composites with carbon are particularly promising as they introduce a strong adsorptive aspect into remediation technology as the carbons have a strong affinity for chlorinated compounds. [94] Thus, carbon adsorption can be utilized to retain contaminants in close proximity to the supported NZVI and allow adsorptive and reductive reactions to occur simultaneously and efficiently. Such materials have also been used in the development of adsorptive reactive barriers. [95, 96] The use of carbon as a support for NZVI is therefore finding increased application in the environmental remediation of chlorinated compounds. [94, 97, 98] The immobilization of NZVI onto submicron carbon particles may also decrease uptake of NZVI in microorganisms and mitigate the potentially harmful implications of nanoparticles. However, carbon submicron particles sediment out of solution and it becomes necessary to design appropriate strategies to enhance colloidal stability and allow transport through groundwater saturated sediments.
We therefore describe in this paper, the development of a biopolymer carrier for carbon based supports of NZVI that leads to colloidal stability of the carbon particles and allows transport through porous media. The design of the biopolymer is such that it binds to the carbon through the hydrophobic effect of water exclusion, and is relatively insensitive to solution electrolyte concentration, allowing it to be used under a variety of groundwater conditions. Specifically, we use hydrophobically modified chitosan (hm-chitosan) where about 2.5% of the amine functionalities are modified through the attachment of a long chain alkyl group (Figure 5.1(a)). The tendency of the alkyl groups to attach to the hydrophobic carbon through the hydrophobic effect is the premise behind the proposed use of the modified biopolymer. Figure 5.1(b) is a schematic of the proposed multifunctional system of carbon microspheres containing zerovalent iron (the pink cubes). The contaminant (shown as red dots) adsorbs strongly to the carbon, [90] while the polymer (green curves) has hydrophobes (yellow lines) that attach to the carbon.
Figure 5.1. (a) Molecular structure of hm-chitosan, illustrating the alkyl group incorporation (the typical ratio x:y is 97.5% : 2.5%). (b) Schematic of a carbon particle with attachment of hm-chitosan (the green lines signify the backbone of chitosan, the yellow lines the alkyl groups grafted on the backbone of chitosan) containing nanoscale zerovalent iron (red cubes). The contaminant, trichloroethylene (red dots) strongly adsorbs to carbon. (c) Scanning electron microscopy (SEM) image of the relatively monodisperse carbon microspheres (600±72 nm) used in this study.
Chitosan is a common biocompatible and biodegradable polymer. It is a linear copolymer composed of glucosamine and N-acetylglucosamine residues, obtained by the alkaline deacetylation of chitin the main component of the exoskeleton of crustaceans such as shrimp. [105-107] The hydrophobic modification of chitosan to hm-chitosan has been shown to confer additional properties to the biopolymer. For example, the hydrophobes insert themselves into the bilayer of liposomes (phospholipid vesicles) and link them to form liposomal gels. [47, 48] In recent work from our laboratory, we have shown the same principle can be applied to prepare carbon gels in solution. [174] Thus, the hydrophobes of hm-chitosan attach to carbon microspheres and at appropriate concentrations link across carbon microspheres such that a gel is formed with the carbon microspheres as nodes in the gel framework. However, with sufficient dilution and with low molecular weight hm-chitosan, it is our hypothesis that the polymer chains will wrap around a particle rather than bridge particles leading to a polymer coating on the hydrophobic carbon and rendering the material colloidally stable.

While we have established the concepts of TCE adsorption and reaction in our earlier work with these systems containing NZVI on carbon, [90] the focus of the current paper is the concept behind the development of the polymer carrier. Accordingly, we use the model system of relatively monodisperse carbon microspheres synthesized through a hydrothermal process [175] to understand clearly the nature of polymer binding and colloidal stability. Figure 5.1(c) is a scanning electron micrograph of the carbon particles used as the model system, showing a relative monodispersity with a mean particle size of 600 nm (std. deviation 72 nm).

5.2 Experimental Section
5.2.1 Materials

Chitosan of low molecular weight (approximately 50K - 190K Dalton) was obtained from Sigma-Aldrich. The reported degree of deacetylation was between 75% and 85%. Other reagents include sucrose (C_{12}H_{22}O_{11}, ACS reagent), CMC (mean molecular weight 90K, low viscosity), dodecyl aldehyde, sodium cyanoborohydride (NaCNBH₃), sodium hydroxide, acetic acid, and ethanol. All regents were obtained from Sigma-Aldrich and were used as received. Fluorescein isothiocyanate (FITC)-Labeled hm-chitosan used in Confocal Laser Microscope characterization was provided by the Raghava laboratory at the University of Maryland.

5.2.2 Preparation of Monodispersed Carbon Microspheres

Monodispersed hydrophobic carbon microspheres were prepared by hydrothermal dehydration and pyrolysis as reported in the literature. [91, 175] Briefly, a total of 45 mL of 0.15 M sucrose water solution was introduced into a 50 mL stainless steel autoclave vessel, which was then closed with a stainless steel cap. The vessel was heated at 190 °C for 5 h, subjecting the sucrose to hydrothermal treatment. The resulting solid suspension was centrifuged and washed three times with ethanol and air-dried. The dry particles were pyrolyzed in a tube furnace at 1000 °C for 5 h with continuous argon flow. The morphology of these carbon microspheres is shown in Figure 1c.

5.2.3 hm-Chitosan Synthesis

hm-Chitosan was synthesized by reaction of the amine groups of chitosan with n-dodecyl aldehyde (hm-chitosan structure is shown in Figure 5.1a). The procedure follows that previously reported in the literature. [166] Briefly, 4 g of chitosan was first dissolved
in 220 mL of 1% (v/v) acetic acid, and then 150 mL ethanol was added to allow the aldehyde used for the alkylation to be in a solvating medium. The pH was adjusted to 5.1 by the addition of sodium hydroxide, followed by a solution of dodecyl aldehyde in ethanol containing a 2.5% molar ratio of the aldehyde to the amine groups in chitosan. This was followed by the addition of an excess of sodium cyanoborohydride. The mixture was stirred for 24 hours at room temperature and the final product was firstly precipitated with ethanol and sodium hydroxide solution, and then washed with ethanol and deionized (DI) water three times. The hm-chitosan solutions were stored in 1% acetic acid to maintain polymer solubility and minimize self-aggregation.

5.2.4 Preparation of hm-Chitosan Stabilized Carbon Microsphere Suspensions

To prepare the hm-chitosan stabilized carbon microspheres, the carbon microspheres were first ultrasonicated in DI water to minimize pre-aggregation and settling of these hydrophobic particles prior to addition of the polymer. The desired amount of hm-chitosan solution was added to the microsphere suspension and held at 50°C for 2 h. The hm-chitosan coated carbon microspheres was stored and analyzed at room temperature.

5.2.5 Analytical

Field-emission SEM (Hitachi S-4800, operated at 20 kV) was used to characterize particle size and morphology of the carbon microspheres. A UV-Vis spectrophotometer (Shimadzu 1700) was used to analyze the sedimentation kinetics, where 0.53 mL of the sample was inserted in tight glass cells (35mm in height, 10mm×4mm in plane) and the transmittance at 532 nm was measured hourly. [170]
Confocal Laser Scanning Microscopy was used to confirm the adsorption of hm-chitosan on carbon microsphere surface. Fluorescently tagged samples of hm-chitosan stabilized carbon microspheres were prepared by adding the FITC-labeled hm-chitosan solution to the carbon microsphere suspension, sonicating the sample to ensure mixing followed by incubation for 2 hours. A 1 ml aliquot of the suspension was centrifuged to collect the stabilized carbon microspheres followed by washing with 15 ml DI water 3 times to remove any free hm-chitosan. The carbon microspheres were re-dispersed into 1 mL DI water and imaged. The control experiments were done without any carbon microspheres and no precipitate was observed after centrifugation.

\(^1\)H NMR (Bruker Avance 500 MHz NMR spectrometer) was used to characterize the interaction between carbon microspheres and the hm-chitosan alkyl groups. Zeta potential experiments were performed with a Malvern Zetasizer particle analyzer Nano Series with Multi Purpose Titrator (MPT-2).

5.2.6 Transport Characterizations

To characterize the mobility of these particulate systems, we conducted experiments on transport through capillaries following the particles through optical microscopy. The capillary transport experiment is a simple and effective method to study particle transport through porous media requiring small amounts of sample. [90, 91, 93, 175] In this experiment, glass melting point tubes with both ends open (1.5-1.8 mm i.d. × 100 mm length, Corning, NY) were used as capillaries. In all cases, the capillary tubes were placed horizontally to simulate groundwater flow, after a 3 cm length was packed with wet Ottawa sand (EMD, CAS 14808-60-7, Fisher Scientific). A continuous water
flow at a flow rate of 0.1 mL/min (superficial velocity of 5 cm/min) was provided by a syringe pump, and the exit point of the capillary was capped with a small glass wool plug. After 30 μL of the particle suspension (0.3%) was injected into the inlet of the capillary, water flushing was initiated and an inverted optical microscope was used to observe the pore-scale transport of the particles. Particles are recovered at the capillary outlet and any remnant particles on the glass wool plug are also recovered by washing the plug extensively.

5.3 Results and Discussion

5.3.1 Characterization of Polymer-Particle Interactions

The binding of hm-chitosan to the carbon particles through the hydrophobic interaction is shown through the fluorescent microscopy images of Figure 5.2(a). The fluorescently localized signals in Figure 5.2(a) indicate attachment of the polymer to carbon particles. We note that in the absence of the carbon microspheres, a dull green fluorescence of hm-chitosan in solution is observed as expected.
Figure 5.2. (a) Confocal laser scanning microscopy image of carbon microspheres coated with FITC-labeled hm-chitosan. (b) $^1$H NMR spectroscopy of 0.5% hm-chitosan samples with increasing carbon microsphere concentrations. The spectra indicate a line broadening of the CH$_2$ and CH$_3$ on the alkyl group of hm-chitosan with increasing carbon concentration.
Figure 5.2(b) shows the interaction of the hydrophobes on hm-chitosan with carbon through the $^1\text{H}$ nuclear magnetic resonance (NMR) resonances of the alkyl groups. The spectra indicate subtle but observable changes in the hm-chitosan alkyl group resonances with increasing concentration of the carbon microspheres. A clear line broadening of the $\text{CH}_2$ and $\text{CH}_3$ proton resonances is observed as the concentration of the carbon microspheres increased. The $\text{CH}_2$ and $\text{CH}_3$ peaks of the carbon microsphere/hm-chitosan samples are obviously broader than the control sample of 0.5% hm-chitosan. The line broadening indicates motional restrictions of the alkyl side chains upon introduction of the hydrophobic carbon particles. Small increases in the line-width for the entire spectrum with 3% carbon may indicate motional restrictions on the entire polymer upon strong adsorption of the alkyl group on carbon.

Other indicators of polymer adsorption to the particles lie in zeta potential values which change from -8.4 mV for the bare carbon particles to 24.5 mV with addition of the cationic hm-chitosan.

5.3.2 Sedimentation Characteristics

The colloidal stability of carbon microspheres supported by hm-chitosan was characterized through traditional sedimentation experiments. Here, we carried out studies to compare hm-chitosan with unmodified chitosan, and a standard particle stabilizing polymer used in earlier studies, carboxymethyl cellulose (CMC). [84, 90, 91] Figure 5.3(a) illustrates sedimentation curves obtained by plotting the transmittance vs time, with the transmission monitored at 532 nm at ambient temperature. All polymer stabilized systems show significantly slower sedimentation compared to native carbon.
microspheres. The extremely slow sedimentation of hm-chitosan stabilized carbon over a period of 2 days appears to be superior to the other systems for the same polymer loading, although we note that small differences in solution viscosity due to remnant polymer may contribute to differences in sedimentation rates. Over the 48 hour period of monitoring the sedimentation, there is virtually no change in the transmittance of the hm-chitosan system. The photographs in Figure 5.3(b) illustrate the sedimentation rates of the various systems, and we note significant retention of stabilized particles in solution even after a period of 4 days. The same trend of enhanced stabilization is also seen at higher salinities representative of groundwater, [176] and the results are shown in the Supporting Information (Figure S5.1). We note that the stability of hm-chitosan/carbon microsphere suspension is not influenced by salt concentration. In most cases, the hydrophobic effect is entropically enhanced by electrolytes that bind the water, reducing hydrogen bonding around nonpolar solutes (clathration), thus allowing an interaction between the nonpolar solutes, typically referred to as salting-out of such solutes. [177] We propose that electrolyte binding of water at higher salt concentrations favor hydrophobe-carbon interactions enhancing the stability of the hm-chitosan coating on the carbon.
Figure 5.3. (a) Sedimentation curves of carbon microsphere (0.025%) suspension (filled circle), 0.5% CMC (filled triangle up), 0.5% chitosan (filled square) and 0.5% hm-chitosan (filled triangle down) stabilized carbon microsphere suspension (0.025%) in water by UV vis. (b) Photograph of (i) none, (ii) 0.5% CMC, (iii) 0.5% chitosan and (iv) 0.5% hm-chitosan stabilized carbon microspheres (0.025%) suspension in water after sedimentation for several hours.
5.3.3 Transport Properties

The capillary experiment is an easy visualization technique to characterize particle transport through porous media. [90, 91, 93, 175] Figure 5.4(a) is a schematic of the experimental setup. Figure 5.4(b) is a photograph of the capillary showing the zone of initial particle placement ahead of the water saturated sediment packing, prior to initiation of water flushing. In Figure 5.4(c), panel (i) depicts the capillary transport experiment using bare carbon microspheres after 5 min of water flushing, and panel (ii) depicts the identical capillary transport experiment using hm-chitosan stabilized carbon microspheres. Aggregates of the dark carbon particles can be relatively easily visualized with the naked eye. Both visual and microscopic observations indicate a significant enhancement of transport when the carbon is stabilized with hm-chitosan. The mobility of these carbon particles with or without hm-chitosan through sediments was also tested using column transport experiments, and the results indicate that hm-chitosan stabilized carbon microspheres transport significantly better through model soils than carbon microspheres alone (Supporting Information Figure S5.2).
Figure 5.4. (a) Experiment setup to study transport in horizontal (flow rate: 0.1 mL/min, sand length: 3 cm and injected suspension volume: 0.03 mL). (b) Photograph of capillary before water flushing. (c) Optical micrographs of the capillaries after 5 mins of water flushing showing sediments and the particles: (i) carbon microspheres are retained primarily at the capillary entrance, (ii) carbon microspheres with hm-chitosan stabilized readily transport through the packed capillaries. (All scale bars are 100 um). The arrows indicate aggregates of the carbon microspheres.
In quantifying the transport characteristics, we have used the classical colloid filtration theory (CFT) originally developed by Yao et al. [178]. Here, Brownian diffusion, interception and gravitational sedimentation are the mechanisms that govern the mobility of colloidal particles through porous media such as soil. The Tufenkji-Elimelech (T-E) model is a comprehensive model to describe these effects in the presence of inter-particle interactions. [179] In this model, the mobility of particles through porous media is quantified through the single collector efficiency $\eta_0$, which is simply defined as the ability of the sediment to collect migrating particles, thus limiting transport through the porous media. The single collector efficiency is written as

$$\eta_0 = 2.4A_S^{2/3}N_K^{-0.081}N_{pe}^{-0.715}N_{vdw}^{0.652} + 0.5A_3 N_K^{1.675}N_A^{0.125} + 0.22N_K^{-0.24}N_G^{1.11}N_{vdw}^{0.053}$$

(1)

where the three terms on the right are contributions due to particle diffusion, particle interception by the collector and particle sedimentation (the values of each variable in equation (1) are presented in the Supporting Information section). Optimal mobilities through sediment are obtained at the minimal collector efficiency, depending on the particle physical properties and groundwater flow characteristics. With carbon based particles, low collector efficiencies below 0.01 span a broad particle size range of 0.1 $\mu$m to 2 $\mu$m with a minimum value of 0.0036 at 0.5 $\mu$m (Supporting Information Figure S3). The carbon particles used here (0.6 $\mu$m) fall in this minimum. Details of the single collector efficiency calculations are in the Supporting Information, together with the calculations of attachment efficiency or sticking coefficient to reflect particle capture over an entire packed bed, as obtained from breakthrough data. [179]

5.3.4 Reversal of hm-Chitosan Attachment on Carbon Microsphere Surfaces
As discussed above, hm-chitosan attachment on carbon microsphere surfaces can stabilize carbon microsphere suspensions and facilitate carbon microsphere transport through porous media. The interaction between carbon microspheres and hm-chitosan is driven by the hydrophobic affinity between the alkyl groups on the polymer and the hydrophobic surface of carbon microspheres. This also suggests a mechanism to reverse the attachment by introducing species that competitively and preferentially bind to the hm-chitosan alkyl groups and thereby disengage hm-chitosan from the carbon microsphere surface. The possibility of such reversal has been demonstrated through the use of α-cyclodextrin (α-CD) which is able to release hm-chitosan from the bilayer of liposomes through preferential binding of the alkyl groups within the hydrophobic cavities of the cyclodextrin molecules.[53] We have adopted the same approach to determine if α-CD can displace hm-chitosan from the carbon microspheres allowing the microspheres to subsequently sediment out of solution.

**Figure 5.5** shows the result of adding 3% of α-CD to an hm-chitosan stabilized carbon microsphere suspension. From the sedimentation experiments, it is clear that the α-CD is able to destabilize the hm-chitosan coating from the carbon microspheres and accelerate the sedimentation process. α-CD is entirely innocuous and relatively inexpensive, and these experiments indicate a method to control the fate of the carbon particles. In other words, in actual practice, if it is necessary to deposit NZVI containing carbon particles after the NZVI has been exhausted through oxidation, or if it is desirable to precipitate NZVI containing carbon onto subsurface pools of chlorinated solvents, the use of cyclodextrins to destabilize colloidal suspensions of hm-chitosan containing carbons becomes an additional tool in remediation technologies.
Figure 5.5. (a) Sedimentation curves of hm-chitosan stabilized carbon microsphere suspension (0.025%) in water (filled circle) and in 3% α-CD solution (filled triangle up) by UV vis. (b) Photograph of (i) 0.5% hm-chitosan and (ii) 0.5% hm-chitosan+3% α-CD with carbon microspheres (0.025%) suspensions after sedimentation for several hours.
5.4 Conclusion

In summary, the use of hydrophobically modified chitosan as a polymeric coating for carbon microspheres has significant applications in the colloidal stabilization and transport properties of such particles. Carbon based particles are specifically susceptible to attachment by such polymers through the hydrophobic effect, which can be exploited to provide stable suspensions of such particles. While the current work has focused on hm-chitosan as an example of a biocompatible and water soluble polymer, there are a number of other water soluble naturally occurring polymers that could be modified with pendent alkyl groups, including the series of polysaccharides such as the anionic alginates. Additionally, once the base polymer is attached to the particle surface through the hydrophobic effect, oppositely charged polymers could be added on through layer-by-layer deposition. The generality to carbon particles can be extended to the more novel carbon based materials such as carbon nanotubes and graphene, if the objective is to impose colloidal stability on such materials. While we have shown the applicability of hm-chitosan stabilized carbon microspheres to the environmental problem of chlorinated hydrocarbon remediation, the use of the hydrophobic effect to coat polymers onto carbon is one of general interest to a variety of health, environment and energy technologies. Of interest is the use of such hydrophobically modified polymers to adsorb to the oil-water interface through insertion of the alkyl hydrophobes into the oil. [180] We also note that the attachment of such polymers to hydrophobic surfaces is reversible through the addition of competitively binding additives such as the cyclodextrins.

5.5 Supporting Information
Supporting Information includes the sedimentation characterizations of hm-chitosan/carbon microspheres in electrolyte solutions, results of column transport, and calculations for the single collector efficiency ($\eta_0$) and the attachment efficiency (sticking coefficient) $\alpha$. 
Suspension Stability in the Presence of Electrolytes:

Figure S5.1. (a) Sedimentation curves of 0.5% CMC and 0.5% hm-chitosan stabilized carbon microsphere suspension (0.025%) in 1 mM NaCl solution. (b) Photograph of (i) 0.5% CMC, (ii) 0.5% chitosan and (iii) 0.5% hm-chitosan stabilized carbon microspheres (0.025%) suspension in 1 mM NaCl solution at selected time intervals. The hm-chitosan coated suspensions of carbon show improved stability.
**Column Transport Experiments:**

Column tests were based on a 50 mL glass buret packed with standard Ottawa sand (EMD, CAS 14808-60-7, Fisher Scientific). The sand was packed to a volume of 10 mL, and the measured porosity of the packing was 0.32 as measured by comparing the weight of the dry column with that of the water-saturated column. [14] A small glass wool plug at each column bottom prevented the loss of the sand. The column was saturated with water prior to addition of the carbon microspheres suspension. After introducing a 10 mL carbon microsphere suspension, the column was flushed with 100 mL of DI water at a flow rate of 20 mL/min (22.22 cm/min). The concentration of carbon microspheres in the bulk suspension was 0.3% with 0 (control) or 0.5% hm-chitosan, considered a threshold for economical application in the field. [21] Continuous water flow was provided by a large water-filled separatory funnel connected to the top of the burette.

Figure S5.2 (a) and (b) illustrates the setup where the carbon microspheres are first introduced as a plug over the sand packing. Figure S5.2 (c) shows the results of flushing with 100 mL of DI water indicating that much of the carbon microspheres remain trapped in the column. In contrast to this poor transportability, the carbon microspheres stabilized by hm-chitosan eluted efficiently with collection of the particles in the conical flask as shown in Figure S5.2 (d). These results are quantified by the elution profiles. Figure S5.2 (e) shows that when the ratio of the volume eluted to volume column is 6, over 99% of the carbon microspheres stabilized with hm-chitosan have eluted through the column, while the carbon microspheres without hm-chitosan stabilization only have less than 50% being eluted through the column. This indicates that
hm-chitosan (0.5%) stabilized carbon microspheres move far more effectively through model soils than carbon microspheres alone.
Figure S5.2. Elution characteristics of carbon microspheres with a flow rate of 20 mL/min and concentration of 0.3% with 0 (control) or 0.5% hm-chitosan. (a): Experimental setup showing the column packed with sand and glass wool; (b): column after addition of the carbon microsphere suspension; (c): column after flushing with 100 mL of DI water. Sample: carbon microsphere suspension without hm-chitosan (control sample). The image indicates retention of the carbon in the column; (d): column after flushing with 100 mL of DI water. Sample: carbon microsphere suspension with 0.5% hm-chitosan. The image indicates elution of the microspheres; (e) Elution Profiles of carbon microspheres in sand packed vertical columns stabilized with and without hm-chitosan (0.5%). M/M_0 represents the fraction of particles that are eluted.
Calculation of the Single Collector Efficiency and the Attachment Efficiency
(Sticking Coefficient)

The T-E formulation for the single collector efficiency is

\[ \eta_0 = 2.4A_s^{1/3}N_R^{-0.081}N_{pe}^{-0.715}N_{vdw}^{-0.052} + 0.55A_sN_R^{1.675}N_A^{0.125} + 0.22N_R^{-0.24}N_G^{-1.11}N_{vdw}^{0.053} \]

Here, all the dimensionless parameters are defined as following: [179]

\[ A_s = \frac{2(1 - \gamma^5)}{2 - 3\gamma + 3\gamma^5 - 2\gamma^6} \]

where \( \gamma = (1 - f)^{1/3} \), and \( f \) is the porosity of the medium (0.32).

\[ N_R = \frac{d_p}{d_c} \]

the ratio of the diameters of the particle \( (d_p=600 \text{ nm}) \) to the sediment grain (collector) \( (d_c=300\mu m) \)

\[ N_{pe} = \frac{Ud_c}{D_\infty} \]

the Peclet number, where \( U \) is the approach velocity \( (8.3 \times 10^{-4} \text{ m/s for capillary experiment}) \), and \( D_\infty = kT/(3\pi\mu d_p) \) where \( k \) is the Boltzmann constant \( (1.3805 \times 10^{-23} \text{ J/K}) \), \( T \) is the absolute temperature \( (298.15 \text{ K}) \), \( \mu \) is the viscosity of the eluting solution \( (0.001 \text{ Pa}\cdot\text{s}) \).

\[ N_{vdw} = A/kT \]

with \( A \) being the Hamaker constant[181] \( (A=3.27 \times 10^{19} \text{ J}) \).
\[ N_A = \frac{A}{3U\pi\mu d_p^2} \]

the attraction number.

\[ N_G = \frac{d_p^2 (\rho_p - \rho_f) g}{18\mu U} \]

the gravity number, where \( \rho_p \) is the density of particle, \( 2.26 \times 10^3 \text{ kg/m}^3 \) for carbon microspheres, \( \rho_f \) is the density of density of the eluting solution, \( 1.0 \times 10^3 \text{ kg/m}^3 \).

We have calculated the single collector efficiency \( \eta_0 \) for 600 nm size carbon microspheres following the procedure of Zhan and co-workers. [182, 183]

Figure S3 illustrates the effect of particle size on single collector attachment efficiency \( \eta_0 \) using a collector size of 300 \( \mu \text{m} \). With a particle size of 600 nm, \( \eta_0 \) is 0.0036.
Figure S5.3. Collector efficiency dependency on the particle size of carbon microspheres as predicted by the Tufenkji-Elimelech filtration model. The calculation based on the parameters given above.
In the T-E formulation, the single collector efficiency is modified by an attachment efficiency or sticking coefficient ($\alpha$) estimated from breakthrough data of transport experiments.\(^3\)

$$\alpha = -\frac{2d_c \ln \left( \frac{C}{C_0} \right)}{3(1 - f) \eta_0 L}$$

Where $d_c$ is the average diameter of sand grains, $C$ and $C_0$ are effluent and influent particle concentration, $f$ the porosity of the sand grains, and $L$ the length of the column. In both the capillary and column experiments conducted in this work, virtually all the particles were eluted, implying a sticking coefficient close to zero.
CHAPTER VI:

CONCLUSIONS AND FUTURE WORK

6.1 Conclusions

The works in this thesis are investigations of multifunctional and supramolecular materials composed of liposomes, carbon microspheres, silk microspheres and hm-chitosan based on hydrophobic effects.

In chapter 2, the structure evolution of hm-chitosan/liposome systems from solutions of coated liposomes to gels was investigated and the evolution mechanism was proposed. We showed that at low concentrations of hm-chitosan, liposome can be coated with hm-chitosan and the thickness of the coating layer can be tuned. The coated liposomes show higher stability in serum than bare liposomes in serum. At higher concentrations of hm-chitosan, hm-chitosan/liposome systems form gels, and the gel structure was visualized by cryo-SEM. The transition to a gel is clearly visualized by the presence of polymer tendrils connecting liposomes. The use of cryo imaging is instrumental to the understanding of structure evolution and this is the first instance of the imaging of liposomes trapped in a gel matrix.
Chapter 3 is about another application of liposomes in lubrication. Based on the application of liposome solution in lubrication reported in the literature, we enhanced this property of liposomes by incorporation of a biopolymer, silk fibroin. In this work, silk fibroin microspheres were coated with liposomes. We have shown that an aqueous DOPC liposome (12.5 mg mL\(^{-1}\)) coated silk microsphere (5 mg mL\(^{-1}\)) suspension is an effective lubricant, providing a low COF on the order of 0.025, minimizing surface wear and avoiding degradation even over relatively prolonged cycling conditions. We propose that the lubricating properties of the silk microspheres are a result of an efficient rolling mechanism, and the liposomal coating layer acts as a lubricant in the slip step of the rolling process between the two shear surfaces, avoiding jamming of silk microspheres while rolling. These results may have implications for biomedical applications especially in joint lubrication. With current therapeutic options for improving joint comfort being limited, new biomaterials with the appropriate biological and tribological properties offer an intriguing opportunity. Since silk is a biocompatible and slowly degradable biomaterial, the data reported here suggest a path forward toward such a goal.

In Chapter 4, the investigation of liposomes as lubricant continued. In this chapter, we were looking for new liposome based lubrication systems for human joints that can be attached on damaged cartilages. The system we designed is an hm-chitosan film with a liposome layer tethered on the film surface based on the hydrophobic interaction between liposome and hm-chitosan. We have shown that an hm-chitosan film can tether a close-packed liposome layer on the film surface via hydrophobic interactions. The tethering of liposomes to hm-chitosan is easily accomplished and represents a facile method to capture and immobilize liposomes. The hm-chitosan/liposome film significantly reduces
the COF and minimizes surface wear. The consistent lubrication properties of the hm-chitosan/liposome film are attributed to resilient hydrophobic interactions between the hm-chitosan film and liposomes. These interactions maintain a robust close-packed liposomal layer on the film surface allowing hydration lubrication over extended wear cycles. In addition to the effective lubrication properties shown here, the ease of liposome immobilization through such tethering based on the hydrophobic effect, leads to several applications in drug delivery and in fundamental investigations of biomembranes using captured liposomes and other vesicular entities.

In Chapter 5, we extended the hydrophobic interaction between hm-chitosan and liposomes to carbon microspheres which are widely used as NZVI support for groundwater remediation. Because carbon microsphere surface is hydrophobic, it is specifically susceptible to attachment by hm-chitosan through the hydrophobic effect. The use of hm-chitosan as a polymeric coating for carbon microspheres has significant applications in the colloidal stabilization and transport properties of such particles. The generality to carbon particles can be extended to the more novel carbon based materials such as carbon nanotubes and graphene, if the objective is to impose colloidal stability on such materials. While we have shown the applicability of hm-chitosan stabilized carbon microspheres to the environmental problem of chlorinated hydrocarbon remediation, the use of the hydrophobic effect to coat polymers onto carbon is one of general interest to a variety of health, environment and energy technologies. We also note that the attachment of such polymers to hydrophobic surfaces is reversible through the addition of competitively binding additives such as the cyclodextrins.
6.2 Future Work

For the application of using hm-chitosan to coat or form gel with liposomes, more structure characterization methods are needed to investigate the intermediate structures of hm-chitosan/liposome between the solution and gel. For example, the liposome distribution and 3D hm-chitosan network can be investigated by confocal microscopy with larger liposome and fluorescent labeled liposomes and hm-chitosan. This structure study can be utilized to achieve slow release purpose of the system.

For the application of using liposome with silk fibroin microspheres as lubricant, the effects of the silk microspheres size, mechanical properties can be tested to get a better lubrication result.

For the investigation of the film lubricant of hm-chitosan with liposomes tethered on the surface, the biopolymer film quality, such as the mechanical properties and duration lifetime, can be further improved by blending hm-chitosan with other biocompatible polymers.

For the application of using hm-citosan in groundwater remediation, the concept of using hm-chitosan can be extended to other biopolymers with neutral or negatively charges and higher solubility in aqueous solutions, such as carboxymethyl cellulose. Those neutral biopolymers are more eco-friendly.
LIST OF REFERENCES


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1. Preparation of alendronate liposomes

Alendronate liposomes were prepared using lipid film hydration method. During the preparation procedure, [184] 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) (Avanti Polar Lipids), 1,2-dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DMPG) (Avanti Polar Lipids) and cholesterol (Sigma-Aldrich), (or DSPC, DSPG and cholesterol), at a molar ratio of 3:1:2, were dissolved in a 2:1 (v/v) chloroform-methanol mixture (total weight of the three lipids is 0.1 g for 5 ml liposomes). The solution was completely dried on a rotary evaporator, and the dried film was hydrated with 1X PBS containing sodium alendronate at 50 °C for 1 hour to obtain a liposome suspension. The liposome suspension was subsequently extruded through a series of 400 and 200 nm pore size polycarbonate membranes (Whatman) at 65°C to downsize the liposomes (11 times extrusion through each).

The obtained liposomes were purified on a Sephadex G-25 column (GE Healthcare life sciences) and eluted with 1X PBS buffer pH 7.4 to remove free alendronate. The final alendronate concentration in the liposomal solution is approx. 5.1 mg/mL, which was measured by UV-vis spectrophotometry at a wavelength of 300 nm. [185] The value varies a little for different batches of alendronate liposomes.
Drug-free liposomes were prepared by the same procedure, excluding the alendronate.

2. Calibration procedure for alendronate to calculate encapsulation efficiency.

Five standard alendronate solutions were freshly prepared with concentrations of 8.1, 40.5, 81, 121.5, 162.5 g/mL alendronate in 2 M perchloric acid solution. Iron (III) chloride standard solution (5 mM) was prepared by dissolving ferric chloride hexahydrate in 2 M perchloric acid. 2 mL of each standard solution of alendronate was mixed with 0.4 mL ferric chloride solution and the absorbance of the complex formed was measured at 300 nm immediately after mixing. All measurements were performed at room temperature against a reagent blank of 2 M perchloric acid solution. The absorbances were plotted verse alendronate concentrations and linearly fitted to get a relationship equation.

3. Measurement of alendronate in liposomes

For measuring the alendronate in liposomes, the liposomes were broke by adding 0.1 mL Triton X-100 to 1 mL alendronate liposome solution and sonicate the mixture for 15 mins. Then 0.055 mL of the mixture (contains 0.005 mL Triton X-100 and 0.05 mL sample) was diluted with 2 M perchloric acid to a volume of 2 mL. 2 mL of this diluted mixture was mixed with 0.4 mL ferric chloride solution and the absorbance of the complex formed was measured at 300 nm immediately after mixing against a reagent blank (0.05 mL drug free liposomes and 0.005 mL Triton X-100 were added to 2 M perchloric acid to a total volume of 2.4 mL). The concentration of alendronate in liposomes was calculated according to the standard curve.
Organic solvent can also be used to break liposomes, such as ethanol. 0.1 ml ethanol will be needed for every 0.05 ml alendronate liposomes, and then the mixture of liposomes and ethanol is diluted with 2 M perchloric acid to a volume of 2 mL. 2 mL of this diluted mixture is then mixed with 0.4 mL ferric chloride solution and the absorbance of the complex formed was measured at 300 nm immediately after mixing against a reagent blank (for reagent blank, 0.05 mL drug free liposomes, 0.1 mL ethanol and 0.4 ml ferric chloride solution were added to 2 M perchloric acid to a total volume of 2.4 mL). The concentration of alendronate in liposomes was calculated according to the standard curve.

4. Clodronate liposomes

The preparation method of clodronate liposomes is the same as that of alendronate liposomes, and the measurement method is the same as alendronate liposomes too, since they have similar structures.

5. The use of alendronate/clodronate liposomes in HIV/SIV therapy investigation

Appendix B:

LIPOSOME PREPARATION METHOD FOR VACCINE DELIVERY AND INFLUENZA THERAPY

1. The procedure for preparing DPPC/DMPG/cholesterol liposomes, coated liposomes, and liposome gels.

   The DPPC/DMPG/cholesterol liposomes are composed of dipalmitoyl-phosphocholine (DPPC), dimyristoyl-phosphoglycerol (DMPG) and cholesterol, the molar ratio of the three lipids is 3:1:2. For each liposome preparation, the lipids are hydrated in the presence of an aqueous solution containing Flufirvite-3 (FF3) peptide or antigen (Polio Vaccine, IPV) to facilitate the drug encapsulation within the aqueous core of the vesicles. To prepare the hydrophobically modified chitosan (hm-chitosan) coated liposomes, hm-chitosan is added drop by drop to the liposome dispersion with continuous stirring for 2 hours to ensure hm-chitosan has been evenly attached on the liposome surface, the final hm-chitosan concentration will be 0.2%.

   To prepare DPPC/DMPG/cholesterol liposome/hm-chitosan gel, hm-chitosan solution (2%) and the liposome solution were mixed at equal volume. The mixture will need to be mixed well and leave it at room temperature for at least 30 mins for gelling.

2. The procedure for preparing cationic (1,2-dioleoyl-3-trimethylammonium-propane, DOTAP) liposomes and liposome gels.
L-α-phosphatidylcholine (PC)/DOTAP (10:1 w/w) were dissolved in a 2:1 (v/v) chloroform-methanol mixture. The dissolved lipid was completely dried on a rotary evaporator, and the dried lipid film was hydrated with 1X PBS at 50 °C to obtain a 1.25% (w/v) liposome suspension. The liposome suspension was extruded through a series of 400 and 100 nm pore size polycarbonate membranes at a temperature between 55-65 °C to downsize the liposomes.

For DOTAP liposomes with antigen (Polio Vaccine, IPV): The only difference with the preparation of control DOTAP liposomes is that, during the hydration step, antigen solution (IPV) solution was used instead of 1X PBS. The concentration of lipid is 1.25 % (w/v).

To prepare DOTAP liposome/hm-chitosan gel, hm-chitosan solution (2%) and the IPV liposome solution were mixed at equal volume. The mixture was mixed well and left at room temperature for at least 30 mins for gelling.

3. hm-Chitosan coated liposome encapsulating FF3 peptide promotes the efficient delivery of FF3 peptide for viral inhibition.

The DPPC/DMPG/cholesterol liposomes are composed of dipalmitoyl-phosphocholine, dimyristoyl-phosphoglycerol and cholesterol. For each liposome preparation, the lipids are hydrated in the presence of an aqueous solution containing FF3 peptide to facilitate the peptide’s encapsulation within the aqueous core of the vesicles. To prepare the hm-chitosan coated liposomes, hm-chitosan is added drop by drop to the liposome dispersion with continuous stirring for 2 hours to ensure chitosan has been evenly attached on the liposome surface. We expect that encapsulation of the peptide
within lipid vesicles will promote more efficient delivery of the FF3 peptide and that hydrophobic coating of the vesicles will increase its effectiveness for delivery of the peptide.

Using an immunoplaque assay with Influenza A/PR/8 and MDCK cells, we were able to compare viral inhibition in the presence of FF3 peptide encapsulated in the formulations described above. Virus was pre-treated with peptide alone, peptide encapsulated in a microemulsion, liposomes, or hm-chitosan coated liposomes. MDCK cells were then infected with the virus +/- peptide for one hour after which the inoculum was removed and an Avicel overlay was added to the cell monolayer. After a 40-hour incubation, cells were stained for the presence of influenza virus and plaque numbers quantified. All treatments were performed in triplicate.

In initial studies, we compared the viral plaque-forming unit concentration following incubation with peptide formulations at a constant peptide concentration of 10 µM. Results are shown in Figure B1, indicating that oil-in-water microemulsions and hm-chitosan coated liposome preparations encapsulating Flufirvitide-3 peptide at a concentration of 10 µM resulted in reduced numbers of viral plaques as compared to either peptide alone or virus alone. However, viral inhibition with the DPPC/DMPG/cholesterol non-coated liposomes was similar to the results seen with peptide alone. Inhibition was also noted with the blank microemulsion.
Figure B1. Immunoplaque assay with formulations encapsulating FF3 peptide.
In subsequent studies we have compared the lowest peptide concentration in the various formulations that completely inhibited viral plaque formation at a constant virus concentration of 100 pfu/ml. These results indicated that the peptide alone showed viral inhibition at concentrations as low as 1.56 µM, as did FF3 encapsulated within coated liposomes, as shown in Figure B2. FF3 peptide encapsulated within non-coated liposomes required a higher concentration of FF3 to induce complete viral inhibition.
Figure B2. Minimum inhibitory concentration of FF3 peptide encapsulated in liposome formulations.
The lipid content of the coated vesicle preparations at the inhibitory peptide concentration was 0.0672 percent (wt/vol). Viral plaque formation with blank coated vesicles was noted at lipid concentrations of 0.26 percent or more, indicating some non-specific interaction of the lipid vesicles with the virus and/or the epithelial cells. There was no detection of disruption to the cell monolayer with any of the vesicle preparations.

These results support our hypothesis that hm-chitosan coated liposome preparations encapsulating FF3 peptide will promote the efficient delivery of FF3 peptide for viral inhibition. We expect that further in vivo experiments with these formulations will demonstrate that the particulate nature of the liposome carriers will improve delivery to the respiratory tract and that the interaction of hm-chitosan with the mucosal epithelium will enhance the efficacy of FF3 peptide.

4. Immunization study on IPV-liposome formulations.

Female Balb/c mice were immunized twice one month apart with 12.5 µL with the inactivated polio vaccine (IPV, Sanofi Pasteur) encapsulated within the indicated liposome formulations with five animals per group. One-tenth the human clinical dose, or 8 total D-antigen units, was administered in a total volume of 12.5 µL per animal. One month post-final immunization, serum was collected from individual animals and analyzed for anti-IPV antibodies by ELISA. As shown in the Figure B3, Animals immunized with IPV DPPC/DMPG/cholesterol liposomes and IPV cationic liposomes showed an increase in the number of responders and in the magnitude of the response as compared to animals immunized with IPV in an aqueous solution containing a mucosal adjuvant, dmLT.
Figure B3. Anti-IPV serum antibodies induced by different formulations. Liposomes are DMPG/DPPC/cholesterol IPV liposomes, coated liposomes are DMPG/DPPC/cholesterol IPV liposomes coated with hm-chitosan, liposome gel is DMPG/DPPC/cholesterol IPV liposomes and hm-chitosan gel, cationic liposomes are PC/DOTAP IPV liposomes as described in the above procedure section.
Appendix C:

PREPARATION OF MULTIPLE POLYMER-LIPOsome FILm FOR
POTENTIAL APPLICATIONS IN DRUG DELIVERY AND VESICLE CAPTURE

1. The procedure for preparing hm-chitosan and DPPC liposome multiple layer films.

   Step 1: The DPPC liposomes and hm-chitosan films were prepared as described in Chapter IV.

   Step 2: A few drops of DPPC liposome solution were put onto an hm-chitosan film surface and the solution was spread well on the film surface. The film was left under room temperature for 15 mins and then was washed by DI water for 3 times. Basically this process is similar as preparation method of the lubrication film in Chapter IV, and this is the base layer of the multiple polymer-liposome film.

   Step 3: After that, a few drops of hm-chitosan solution (0.4%) were spread on the surface of the base polymer-liposome film prepared above. After another 15 mins, the film was washed again to remove free hm-chitosan on the surface. By now, polymer-liposome-polymer film was generated.

   Step 4: A second layer of liposome was formed on the film formed in Step 3 by adding DPPC liposome solution again on the top of the film and the whole film was left under room temperature for another 15 min to allow liposomes being tethered on the film.
All those steps can be repeated again and again to generate multiple layer of polymer-liposome film. And this film can be used for drug delivery since either liposomes or the first film layer can be used to encapsulate drugs to achieve slow release. And Figure C1 (a-c) shows the structure of a multiple layer polymer-liposome film. Also, as shown in Figure C1 (d), the top layer of the whole film can be another polymer with negative charges, which can interact with hm-chitosan through electrostatic interactions because hm-chitosan is positively charged.
Figure C1. (a)-(c) show the structure of a multiple layer polymer-liposome film. Image (d) shows using another polymer with negative charges as the top layer of the whole film, the negatively charged polymer can interact with hm-chitosan through electrostatic interaction because hm-chitosan is positively charged.
The following is a 4 layer film sample prepared by the method shown above, the first layer is hm-chitosan film, the second layer is DPPC liposomes, the third layer is hm-chitosan film again and the final layer (on the top of the whole film) is DPPC liposome layer. And all the layers were characterized by cryo-SEM and the images shown in the following Figure C2.
Figure C2. Cryo-SEM images of layers of a layer by layer hm-chitosan-liposome film, the first layer is hm-chitosan film, the second layer is DPPC liposomes, the third layer is hm-chitosan film again and the final layer (on the top of the whole film) is DPPC liposome layer.
Biography

Rubo Zheng was born in Yexian, Henan Province, China on September 24th, 1984. She graduated with a B.S. in Chemical Engineering from Zhengzhou University in 2006 and received her M.S. in Chemical Engineering from Dalian University of Technology in 2009. After graduated from Dalian University of Technology, Rubo Zheng made a decision to pursue her Ph.D. degree in the department of Chemical and Biomolecular Engineering at Tulane University under the supervision of Prof. Vijay T. John in 2009. Rubo’s research focuses on the development of novel applications of colloidal systems in the areas of drug delivery, biolubrication, and so on. She is also working on using various biopolymers to enhance application performance of various colloidal systems. During her study in Tulane, Rubo has authored or co-authored 7 peer-reviewed publications, three U.S. patents and presented his work at 8 professional conferences. Her honors from Tulane University include Distinguished Graduate Student Award (2012) and Outstanding Graduate Student Research Award (2012) from Department of Chemical & Biomolecular Engineering.