ABSTRACT

Title of Document: CHARACTERIZATION AND STABILITY STUDIES OF CATANIONIC SURFACTANT VESICLES

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Catanioinic surfactant vesicles have been explored as suitable drug carriers due to their similarities to phospholipids. Great strides have already been made in developing phospholipids for drug delivery and several liposomal drugs are already on the market. However, due to their inherent instability suitable alternatives have been explored. Herein six methods for the preparation of catanioinic surfactant vesicles containing sodium dodecylbenzylsulfonate (SDBS), cetyltrimethylammonium tosylate (CTAT), and glycoconjugate is explored. Four of the six methods were determined to produce vesicles with an average hydrodynamic radius of 76 nm. These vesicles have been shown to be to environmental changes to pH and ionic strength with no discernible difference based on preparation method.

Also discussed is work down towards the development of a multilamellar vesicle system based on the functionalized catanionic surfactant system. Larger vesicles were able to be obtained, however were unable to systematically and reproducibly create multilamellar vesicles.

CHARACTERIZATION AND STABILITY STUDIES OF CATANIONIC SURFACTANT VESICLES.

By

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Dedication

To those I love.

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Chapter 1: History of the Field

Introduction

Advancements in technology and methodology have produced a wide array of medicinal chemicals previously unattainable. Many of these compounds demonstrate much promise during the initial trial phases only to be abandoned due to poor solubility, high toxicity, or inadequate selectivity. Attempts to circumvent these hindrances usually involve additional structural modifications. However, such changes often alter the lead molecules desired pharmacological effect. For example a hydroxy group may be added in order to decrease the lipophilic character of a drug; this change could however decrease circulation time through elimination by a new metabolic pathway. This small change also introduces a new site for hydrogen bonding which could interfere with binding in the active site.[1]

The use of nanoparticles for drug delivery has been found to overcome many of these shortcomings. Nanoparticle formulations are known to improve the solubility of lipophilic drugs, improve the pharmokinetics, and improve the selectivity.[2] Further enhancements of nanoparticles have also permitted environmentally stimulated release, sustained release, and combinatorial delivery of synergistic drugs.[2]

Liposomal based nanoparticles were the first to obtain the approval from the Federal Drug Administration (FDA) for clinical use. Doxil, a polyethylene glycosylated (PEGylated) liposome carrier loaded with the cytotoxic anti- cancer drug doxorubicin, was approved in 1995. Free doxorubicin indiscriminately diffuses into healthy and diseased tissues alike, thus having a high toxicity. However, Doxil

exhibits a half-life 100 times longer and a cardiotoxicity seven fold lower when compared to doxorubicin.[2,3]

Due in part to this early discovery, liposomes are perhaps the most widely and well-studied of all therapeutic nanoparticles. Liposomes make suitable drug carriers since they are effectively able to encapsulate both hydrophilic and hydrophobic drugs. Several examples within literature demonstrate the ability of liposomes to act as drug carriers with 11 such drugs currently on the market(Table 1).[2–4] Liposomes have also been shown to function as imaging agents and biosensors.[5,6] Despite their obvious advantages the non-spontaneous formation and long term instability are major shortcomings that have remained unresolved. Conversely, ionic surfactant vesicles are known to spontaneously form unilamellar vesicles which demonstrate long term stability.

Table 1: List of liposomal drugs that have been approved or are currently in development

Name	**Indication**
Abelcet	Fungal infections
AmBisome	Fungal and protozoal infections
DepoCyt	Malignant lymphomatous meningitis
DaunoXome	HIV-related Kaposi's sarcoma
Myocet	Breast cancer (in combination with cyclophosphamide)
Epaxal	Hepatitis A
Inflexal V	Influenza
DepoDur	Postsurgical analgesia
Visudyne	Age-related macular degeneration, pathologic myopia, ocular histoplasmosis
Doxil/ Caelyx	HIV-related Kaposi's sarcoma, metastatic breast

	cancer, metastatic ovarian cancer
Estrasorb	Menopausal therapy
MCC-465	Metastatic stomach cancer (Phase 1)
MBP-426	Advanced or metastatic solid tumors(Phase 1/ 2)
SGT-53	Solid Tumors (Phase 1)

Liposome Based Drug Delivery

The discovery of liposomes and the methodology for their development is credited to Dr. Bangham. In 1963 Dr. Bangham discovered that when dispersed in water, phospholipids formed closed, multilayered aggregates. Phospholipids are comprised of a charged hydrophilic head and two aliphatic hydrophobic tails. When placed in an aqueous medium they orient themselves in a bilayer fashion so that the polar heads interact with the water while the tails are hidden from the water. Due to their remarkable similarity to the cell membrane, liposomes were initially studied as artificial cell membranes. However, later research demonstrated their ability to encapsulate materials and their utility for drug encapsulation was studied.

As aforementioned, the basic structure of liposomes consists of a closed phospholipid bilayer which creates a spherical entity. The formation of such vesicles *in vivo* is quite common as part of many cellular processes such as the transport of molecules or sequestering of nutrients. However, *in vitro* this formation is not spontaneous and thus requires some form of mechanical energy input for formation (Figure 1).[7] The original method described by Bangham involved dissolving the phospholipids in an aqueous media followed by sonication to induce liposome formation. The majority of modern liposome synthesis methods involve drying lipids from organic solvents followed by dispersion into an aqueous media. This dispersion

is then followed by one of a variety of purification techniques such as extrusion, freeze-thaw, and reverse-phase evaporation. This leads to the creation of liposomes with vast differences in size, lamellar formation, and encapsulation efficiency.

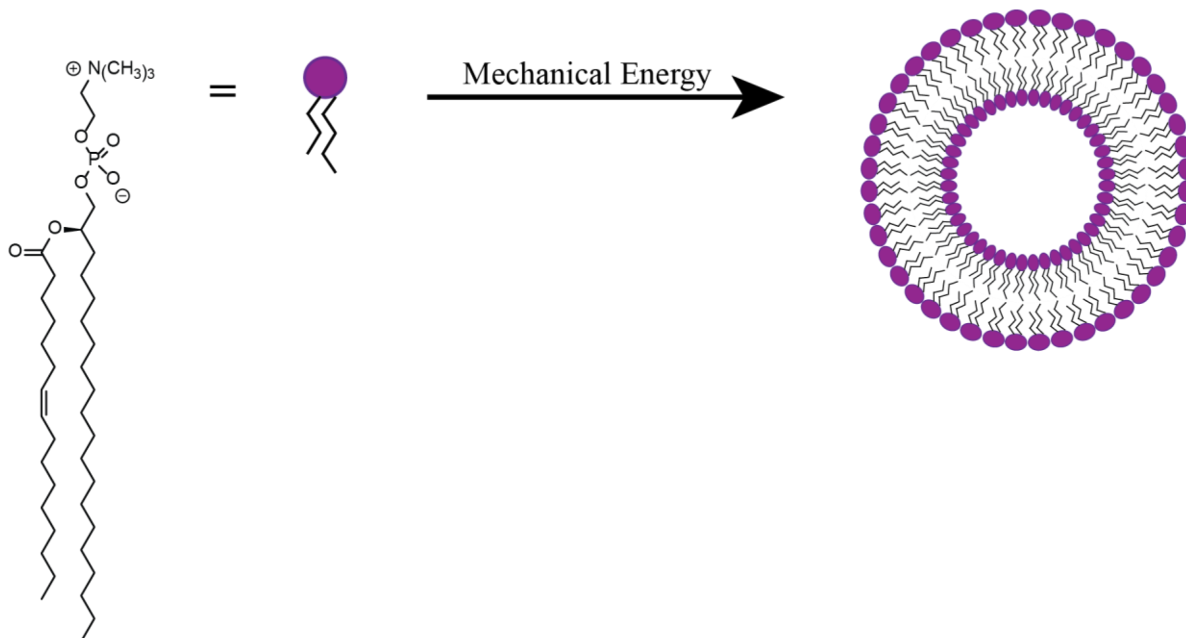


Figure 1: Synthetic scheme of phosphatidylcholine (PC) based liposome. Phospholipid is dispersed in aqueous media and the treated with mechanical energy (i.e. extrusion) in order to induce spherical bilayer formation

Given the wide array of preparation methods and encapsulation efficiencies of liposomes, a great deal of time and effort has gone into their development for therapeutic use. As previously mentioned, Doxil was the first liposomal drug carrier approved by the FDA. Doxil was developed as a treatment for Kaposi's Sarcoma, which usually afflicts AIDS patients. It is the pegylated liposome encapsulated form of doxorubicin. The liposomal encapsulation of doxorubicin has been found to change its pharmacological profile. First, the circulation time of Doxil is increased due to the lowered renal clearance of liposomes. Secondly, due to the size of the liposomes the ability of doxorubicin to enter tissues with smaller gap junctions i.e. cardiac tissue is

decreased resulting in a lowered cardiotoxicity. The, encapsulation also slowed the release of free doxorubicin into the body resulting in a lower overall toxicity. Finally, due mainly to the enhanced permeability and retention effect (EPR) there was a greater distribution of Doxil into tumor sites when compared to free doxorubicin.[8–11] Since 1995 many more liposomal drug systems have made it through FDA approval and even more are in current clinical trials.[6]

While liposomes have demonstrated the ability to effectively encapsulate and deliver drugs they still suffer from major drawbacks. These drawbacks include their inherent long-term instability, tendency to aggregate, expense, and complicated formation methods. The latter two of these drawbacks are inherent to the materials used for liposomal formation (phospholipids) and thus cannot be easily changed. However, the first two of these drawbacks are directly correlated to the metastable nature of liposomes. Liposomes are thought to contain an excess of energy which leads to breakdown into more stable bilayers over time.[7,12,13] Attempts to minimize these shortcomings include the addition of stabilizing additives to the bilayer and structural changes to the phospholipid components; however, a universal solution has not been seen.

An alternative liposomal technology is niosomes. Niosomes are comprised of single-tailed uncharged surfactant molecules which are capable of arranging themselves in a bilayer when prepared under the right conditions.[14] It has been demonstrated that niosomes are capable of behaving like liposomes including the entrapment of solutes, bilayer formation, and controlled drug delivery.[6,14,15] However, unilike liposomes, they are resistant to aggregation, inexpensive, and have a wider

array of principle components. Yet, since their preparation method is analogous to that of liposomes, niosomes suffer from the same metastable thermodynamics that make long term shelf-life a problem.

Despite the ubiquitous usage of liposomal technology for drug delivery, there remains a great need to improve upon their inherent disadvantages. Many have focused on improving or attempting to eliminate these problems; whereas others have sought suitable replacements such as niosomes. One such suitable alternative that will be explored here is the use of catanionc surfactant vesicles.

Catanionic Surfactant Vesicles

In 1989, Kaler and co-workers presented a simple alternative to liposomal technology. The method presented the formation of vesicles from single-tailed charged surfactants. When mixed in defined proportions these surfactants formed bilayers analogous to those formed by phospholipids. The size of the vesicles varied (radius between 30-80 nm) according to the molar ratios of the surfactants to each other as well as the %wt of water.[16] The novelty of this system derives from the inexpensive components, ease of preparation, and remarkable similarity to lipisomal bilayers. Following the work of Kaler, other groups divulged the various capabilities and properties of the surfactant vesicle system.

The formation of the bilayer from surfactants was found to require that one of the charged surfactants be present in an excess molar amount to the other. Further inquiry into vesicle formation elucidated properties which controlled vesicle formation such as the alkyl chain length and area of the head group.[13,17–21] Unlike the formation of liposomes, surfacant vesicle formation is simple and spontaneous.

Addition of water to the solid surfactants followed by gentle mixing causes vesicle formation. (Figure 2) The lack of a need to input energy in order induce vesicle formation is believed to increase the stability of the system when compared to the metastable liposomal systems. Though whether or not theses vesicle formations are truly thermodynamically stable is an area of contention.

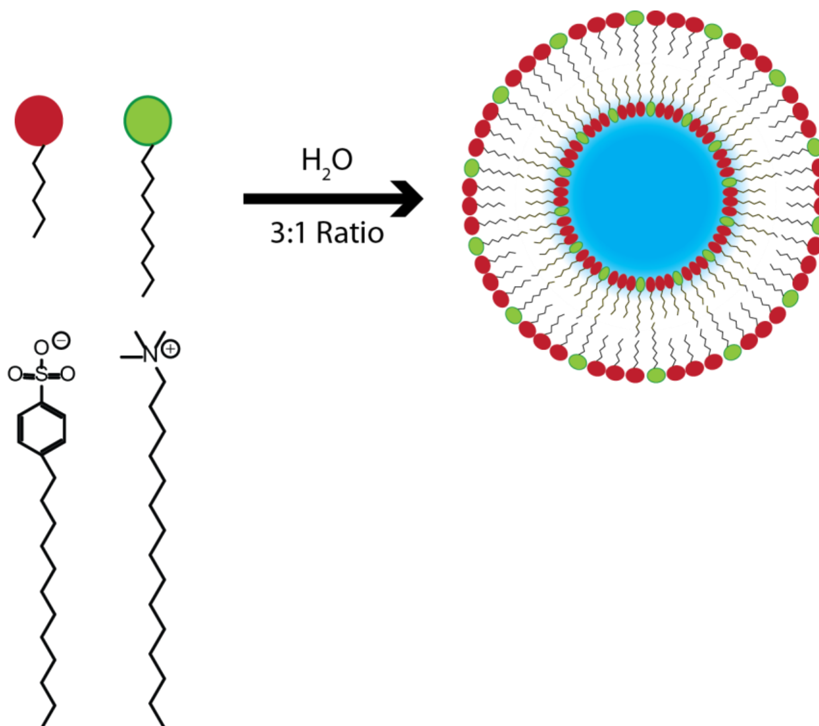
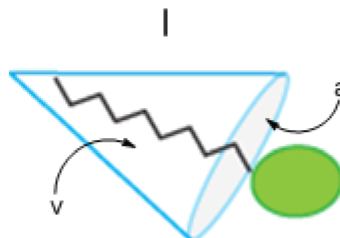


Figure 2: : Spontaneous formation of catanionic surfactant vesicles. Water is added to solid surfactants followed by gentle stirring resulting in spontaneous vesicle formation

Aforementioned, was the requirement for definite proportions needed for the vesicle formation. Kaler found that vesicle formation was constrained by; surfactant ratios to each other, asymmetry of the aliphatic tails, and total wt % of the surfactants in solution. Vesicle formation is believed to be the result of unique pairing of the ionic surfactants. Studies performed by Kaler, Regev, and others have elucidated the geometric constraints of the vesicle formation.[20,22–24] They found that formation of vesicles could be explained through the packing parameter (Figure 3). Packing

parameter is defined as the ratio between the volume of the hydrophobic region to area of the polar head group combined with the optimal chain length of the aliphatic chain.



$$P = \frac{v}{a\,l}$$

Figure 3 Packing parameter equation. Where P is the packing parameter v is the hydrophobic volume, a_0 is the polar head group area and l is the optimal chain length.

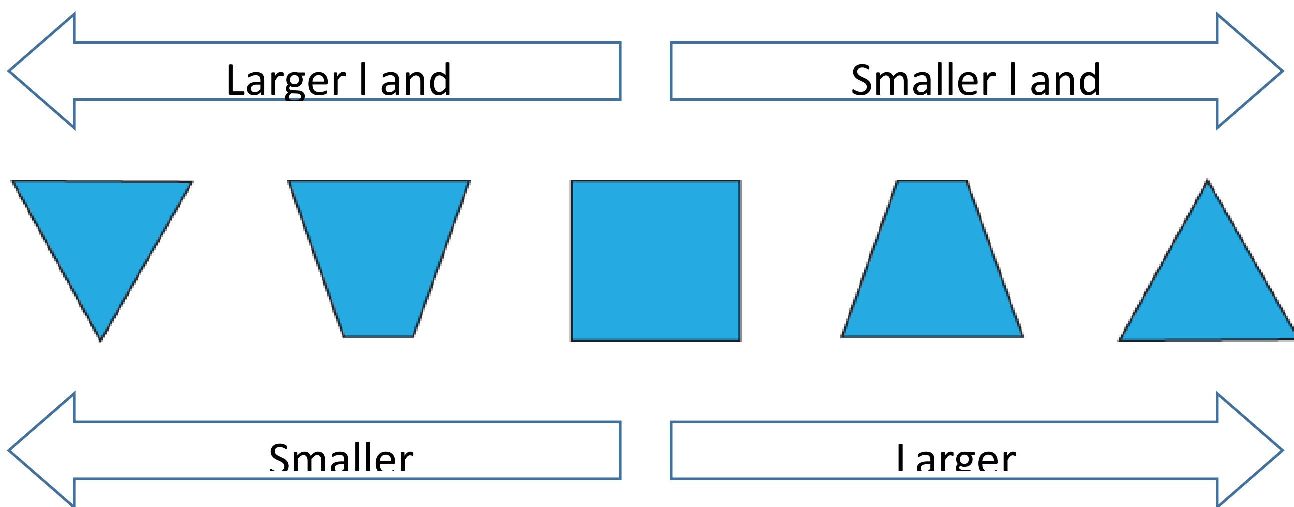


Figure 4 Resulting structures from changes in packing parameter.

Figure 4 depicts the various shapes that can be obtained by changing the various aspects of the packing parameter. Manipulating the various aspects of the packing parameter directly affects the type of packing shape the individual molecules form and thus the structures they assemble into For example the headgroup area of anionic surfactants can be made smaller by increasing the salt concentration or lowering the

pH; the chain length (l) can be decreased by introducing unsaturation.[25] In the case of catanionc vesicles it is proposed that when one surfactant is present in excess to the other it results in a zwiterronionic pairing. This pairing into a zwiterronic molecule decreases the area of the polar head group while simultaneously increasing the area of the hydrophobic region; effectively creating the truncated cone shape characteristic of double-tailed phospholipids opposed to the traditional wedge expected from single tailed surfactants. Thus, at these concentrations it becomes more energetically favorable to spontaneously form vesicles as opposed to infinite bilayers.

Following the initial work of Kaler, other groups built upon this work in order to study the various properties of vesicles and their comparability to liposomes. The main areas of focus were functionalization, encapsulation efficiency, and stability Encapsulation efficiency is of utmost importance in for surfactant vesicles to function as suitable drug carriers. Along with the initial report of vesicle formation, Kaler reported the ability of both anionic and cationic vesicles made from cetyltrimethylammonium tosylate and sodium dodecylbenzyl sulfonate (CTAT/ SDBS) to effectively encapsulate glucose within their aqueous compartments. However, the amount of glucose encapsulated by the vesicles was not reported at the time. Subsequent studies by Calliet using sodium octyl sulfate (SOS) and cetyltrimethylammonium bromide (CTAB) demonstrated the ability of anionic vesicles to encapsulate approximately 1% of the glucose introduced to the system.[17] Further study into the encapsulation efficiency of SDBS/ CTAT was undertaken by Danoff *et. al.*[26] Their study involved measuring the encapsulation of five organic solutes which possessed a charge. It was reported that while both vesicles (anionic

and cationic) were capable of capturing the organic solutes, maximum encapsulation efficiency was obtained when the solute and vesicle possessed opposing charges. This resulted in encapsulation efficiencies ranging from 22-74%.[26] Furthermore, in the case of the anionic dye carboxyflourecein, the vesicles where reported to be stable for 114 days. This development demonstrated the ability of the SDBS/CTAT vesicle system to encapsulate various solutes while maintaining a reasonable stability; important criteria for potential drug carriers.[27]

It has been demonstrated that liposomes can be functionalized in order to improve their association and therefore target specific sites. Groups such as Letourneur have shown that liposomes coated with modified dextrans have an increased affinity to human endothelial and smooth muscular vascular cells. Similar functionalization has also been demonstrated with surfactant vesicle systems. Walker reported the mixing of CTAT, SDBS, and phospholipid linked biotin to form surface functionalized cationic vesicles. Aggregation of the vesicles was observed upon addition of streptavidin.[28] Wang has also shown that the surfaces of ionic surfactant vesicles can be functionalized through electrostatic binding of solutes of opposite charge.[27]

Work within the DeShong group has demonstrated the ability of catanionic vesicles to be functionalized by carbohydrates.[29,30] Lectin-carbohydrate interactions play a part in various biological processes including immune response, pathogenic infections, and reproduction. Though the interactions of proteins and carbohydrates are typically weak, their affinity is increased through multivaleancy. These multivalent interactions have been shown to increase the affinity of these interactions a hundred fold. One of

the most studied interactions has been that of concanavalin A (ConA). ConA binds to glucose, mannose, sucrose, and other carbohydrates. In fact, ConA glucose binding is one the strongest interactions known. It is possible for ConA to exist in two forms; the dimer at pH 5.5 and the tetramer at pH's greater than 7. The tetramer contains four glucose binding sites which allow ConA to bind in a multivalent fashion. Park and co-workers have reported functionalizing catanionic surfactant vesicles using glycoconjugates of varying alkyl chain lengths. The glycoconjugate functionalized vesicles where shown to aggregate upon the addition of ConA or PNA respectively (Figure 6). [29,30]

At this point it has been demonstrated that surfactant vesicles are suitable nanoparticle candidates for drug delivery. Furthermore, they have been shown to have several advantages over the ubiquitously used liposomal technology which is summarized in Table 2. However, there remain several questions about the catanionic system which will be presented and discussed herein.

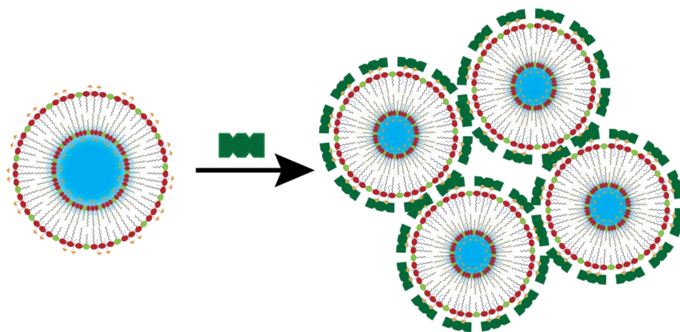


Figure 5 Lectin induced vesicle aggregation. Aggregation of glycoconjugate functionalized vesicles occurs after introduction of lectin (green)

Table 2: Comparison of Surfactant Vesicles to Liposomes

	Surfactant Vesicles	Phospholipid Liposomes
Constituents	Inexpensive surfactants ($1/kg)	Expensive phospholipids ($1000/kg)
Formation	Spontaneous	Sonication , Extrusion
Stability	Years at room temp (in saline)	Days to weeks at room temp (in saline)
Entrap Ionic Solutes	Highly efficient	Inefficient
pH	2-12	6-8
Sterilization	Autoclavable	Not autoclavable
Heating	85 °C max	45 °C max
Lyophilization	Yes	No

Specific Aims

If catanionic vesicle are to be employed effectively for biomedical applications such as targeted drug delivery, the properties and stability of these formulations must be determined. Accordingly, my thesis research was to asses a series of stability parameters of catanionic vesicle preparation methods. The specific aims for this research were to:

1. Evaluate potential differences in the methods used to create functionalized unilamellar vesicles. We proposed to study:
    a. The size and dispersity of vesicles as measured by dynamic light scattering (DLS)
    b. The stability of vesicles to pH
    c. The stability of vesicles to ionic strength
    d. The changes in surface charge (zeta-potential) as a function of increasing glycoconjugate concentration
2. Expand the methods used for preparing unilamellar vesicles for the preparation of multilamellar vesicles

Chapter 2: Catanionc Surfactant Vesicles

Introduction

Previously, the changes to the pharmacological profile of doxorubicin upon encapsulation in a liposomal formulation were discussed. It has been well documented that many cancer focused nanodrugs benefit from the EPR effect (enhanced permeability and retention effect)[31,32]. In order to sustain their rapid growth cancerous tumors stimulate the growth of blood vessels. This increased vascularization along with other factors such as diminished lymphatics around tumor sites make nanoparticles more apt to permeate and be retained in cancerous tissues than in noncancerous tissues. This leads to a higher site specific dosage. However, the ability of nanodrugs to benefit from this effect is inherently tied to the physical properties of the nanodrug. These properties include the size, shape, charge, and stability of the nanoparticles. Due to their similarity to liposomes it is probable that catanionic surfactant vesicles will be able to benefit from the EPR effect as well. However, since there are various preparation methods there is the possibility for differences in the properties of the resulting vesicles. Therefore, it is imperative to discover if the various preparation methods change any of these properties and if so to what degree.

Characterization

Catanioinc Vesicle Preperation

Initially functionalized vesicles were prepared using the three main variations of the Wang procedure: Method 1- mixing solid surfactant and the desired amount of glycoconjugate prior to addition of water; Method 2- preparing bare vesicles followed by addition to the desired amount of solid glycoconjugate; and Method 3- making a solution of the desired amount of glycoconjugate and adding it to the solid surfactants (Figure 6). In order to keep the total percent by weight (wt%) of surfactants and glycoconjugate at 1% the weight of solid surfactants was adjusted in concert with increasing weight of the glycoconjugate Three additional methods were also studied wherein the weights of the solid surfactants were kept constant and only the amount of glycoconjugate was changed. These methods are referred to as Method 4, Method 5, and Method 6 respectively. The resulting colloid solutions for all six methods were purified using size exclusion chromatography

(SEC).

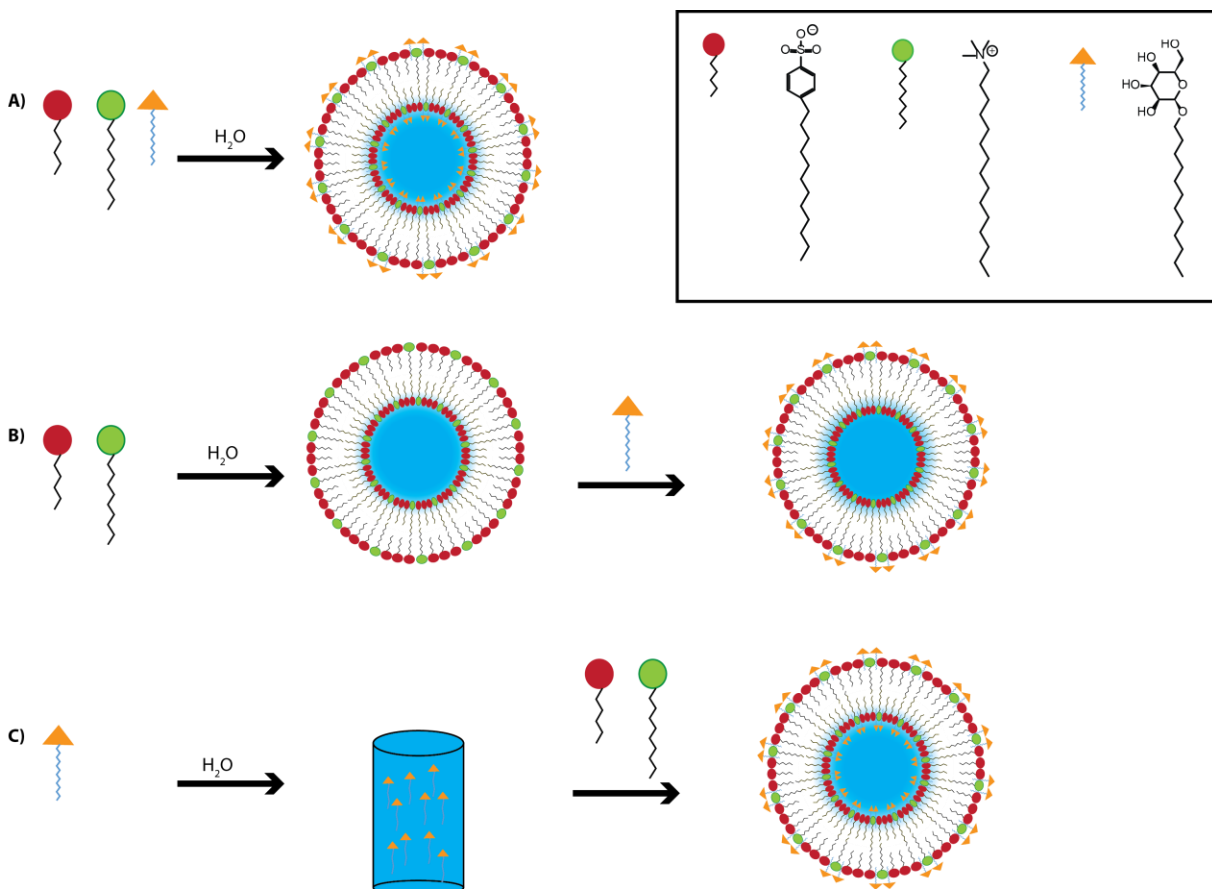


Figure 6 Methods for synthesizing functionalized ULVs. (a) all the solid components were added to vial followed by addition of water to induce vesicle formation (Method 1 and Method 4) (b) water was added to solid surfactants in order to make bare vesicles. Vesicle solution was then added to solid C_{12}-glucose to make functionalized vesicles (Method 2 and Method 5) (c) A solution of solubilized C_{12}-glucose was added to solid surfactants to make functionalized vesicles (Method 3 and Method 6)

Size

Formation of vesicles and their average hydrodynamic radius was confirmed using dynamic light scattering (henceforth referred to radius and DLS respectively). All DLS measurements were taken at 25°C and a scattering angle of 90° using a Photocor Complex Optical unit equipped with a 5mW laser 633nm. The phenol-sulfuric acid assay of vesicle containing fractions further proved incorporation of the glycoconjugate into the vesicles. Previous work in our lab has demonstrated that

vesicles can be functionalized with various glycoconjugates. However, since three methods of preparation were used it was important to ensure that comparable amounts of glycoconjugate were incorporated into the vesicles. During the preparation of vesicles it was found that methods 3 and 6 were unable to dissolve the higher concentrations of the non-ionic C12-glucose. Thus, these methods were not available for further examination. However, results from the phenol-sulfuric acid assay of the other methods indicate that regardless of the method there is a comparable amount of glycoconjugate incorporated into the vesicles.

DLS results showed that there is a noticeable increase in size correlating to the increase of glycoconjugate loading among the four studied methods. Otherwise, all methods produced vesicles that had an average hydrodynamic radius of 72 nm ± 10nm. This suggests that while the order of addition of the solid components does not have a significant effect on the size on the resultant vesicles the amount of glycoconjugate added does.

Stability Studies

Zeta Potential

For colloidal systems, the measurement of the zeta potential will provide a good indication into the stability of the solution. Zeta potential measures the overall surface charge of the particle while in solution. Therefore, collodial solutions that have a large magnitude for the zeta potential will be less apt to aggregate and thus be more stable. This is due to the large increase in repulsion of the particles to each other. Conversely, a lower zeta potential will indicate a system that will more readily aggregate and thus be considered to be less stable. For methods 1 and 2 there was a

slight decrease in the zeta potential as the loading of the glycoconjugate increased. However, this decreasing trend was absent for corresponding methods 4 and 5. For Methods 1 and 2 the amount of surfactant decreases as the amount of glycoconjugate increases. Conversely, with Methods 4 and 5 the amount of the surfactant remains constant as the amount of glycoconjugate increases. Thus, when comparing Methods 1 and 4 the experimental conditions are identical except for the amount of the anionic surfactant SDBS; this is also true in the comparison of methods 2 and 5. Therefore, the overall decrease in the zeta potential for the vesicles prepared by Methods 1 and 2 is due to the overall decrease in the amount of the anionic surfactant SDBS rather than the increase of the nonionic glycoconjugate. The zeta potential of the ULV systems all averaged -60 mV regardless of synthesis method, indicating a good level of stability.

pH

As aforementioned, the structure that is spontaneously formed by amphiphilic molecules is highly dependent on the packing parameter. Thus, any effect that will change the packing parameter will affect the stability of the formed vesicle structures. Since the optimal chain length and volume remain largely unaffected by changes in solution, the easiest factor to manipulate is
the headgroup area. Since the headgroup is negatively charged, its area will relatively easily affected by changes in ionic strength and pH.

It is well documented that cancer cells have a lower pH than the physiological pH of noncancerous cells. This has been accepted as a byproduct of the Warburg Effect. The Warburg Effect is the explanation for the alternative glycolysis route observed in

the cells of most clinical cancers. This route follows the fermentation of lactic acid rather than the oxidation of pyruvate observed in normal cells. Consequently, the rate of glycolysis in cancerous cells occurs at an accelerated rate, often 200 times more, than in normal cells.[33] This increased rate of glycolysis is believed to be the one of the major causes for the acidification of cancerous cells. It is also believed that this acidification plays a role in the rapid proliferation of cancer cells.

Various efforts have been made to exploit the pH difference between host and cancer cells in nanoparticle drug delivery research. The general strategy involves the use of acid labile linkers that bridge a protective group such as PEG to penetrating peptides, targeting agents, hydrophobically modified drugs, or a multifunctional combination of the above.[34–37] One such example is work done by Lee et. al. In summary they developed a multifunctional polymer backbone of N-2-(hydroxypropyl)methacrylamide (HPMA) with hydrazone linked doxorubicin (DOX). The hydrazone linkage was cleaved under the acidic conditions of the tumor environment thus delivering the free DOX directly to the site. This understandably increased the amount of DOX delivered to the site.[36]

The study of effect of pH on the stability of our catanionic system was carried out for a several reasons. First, in order to develop possible pH induced triggers utilizing our catanionic surfactant vesicle system it is first imperative to study the stability of the vesicles under various pH conditions. Due to the acidic nature of tumors, nanodrugs that are basic or not viable in acidic environments will show a diminished benefit and thus result in a lower accumulation in the cancerous cells. Finally, pH is one of the factors that control the headgroup area. As aforementioned, changes to the headgroup

area will influence the packing parameter and thus the shape of the vesicles. Such changes could result in new substructures or different sized vesicles; both of which could have an impact on the circulation and retention times. Figure 7 shows the changes in radius as a function of pH. It can be seen that the radius of C_{12}-glucose functionalized ULVs remains constant throughout a wide range of pH values in agreement with previous results given for bare ULVs.

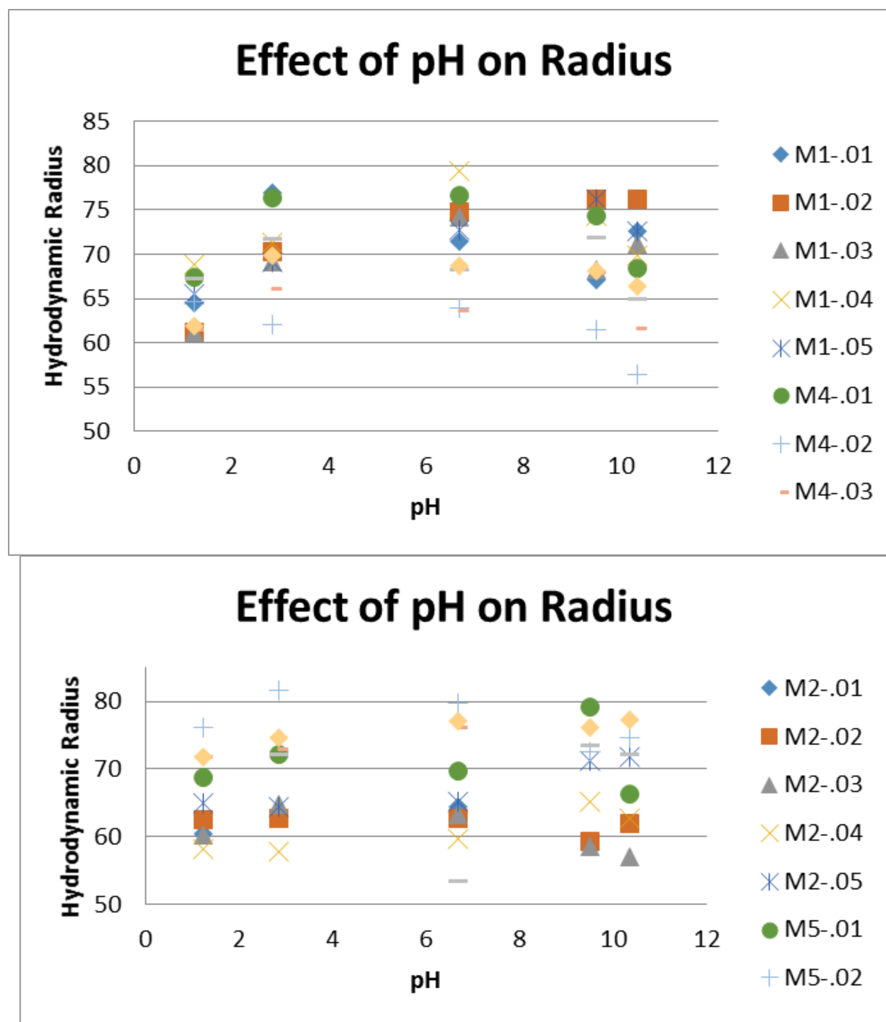


Figure 7 The effect of pH on the radius of ULVs.

Ionic Strength

It is known that increasing the ionic strength of solution containing anionic liposomes will decrease the headgroup area and thus increase the size of the vesicles. Since catanionc vesicles behave differently from their liposomal counterparts, it was desired to determine the effect of ionic strength on the ULV systems. Each procedure introduced the glycoconjugate at different stages; thus, it was hypothesized that there would be variations in the overall distribution. This could in turn affect the ability of the vesicles to adjust to changes in ionic strength. If the differences in bilayer distribution were large enough it should result in varying responses to ionic strength. To test this theory the vesicles were added to solutions of varying salt concentration and the change in size was monitored by DLS (Figure 8). The ULV system demonstrated a rather uniform stability in terms of increasing the salt concentration with significant changes in size occurring when the salt concentration in excess of 0.5M. This suggests that while the distribution of C_{12}-glucose may be different dependent on the method, at this concentration it does not perturb the bilayer composition enough to cause any marked differences in stability to ionic strength.

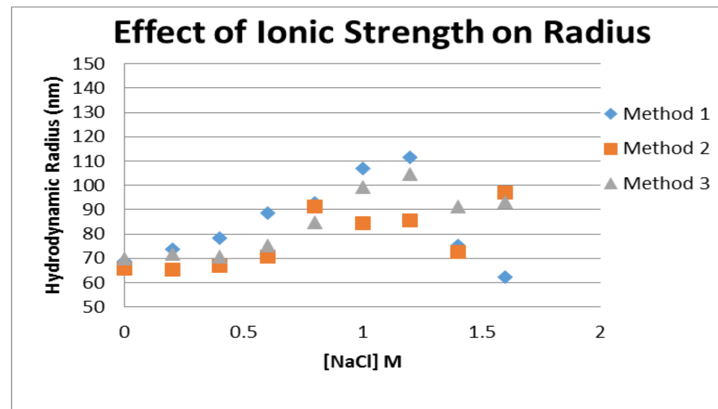


Figure 8 Effect of ionic strength on the radius of ULVs

Cryo-TEM

Cryogenic Transmission Electron Microscopy (Cryo-TEM) was used in order to confirm the sizes and presumed structures of the ULV system. The figure below shows that the vesicles do have a range of sizes as is expected from their spontaneous formation (Figure 9). Furthermore, the size of the vesicle confirms the prior results obtained from the DLS experiments. Most importantly, the vesicles obtained from the methods are shown to in fact be unilamellar systems.

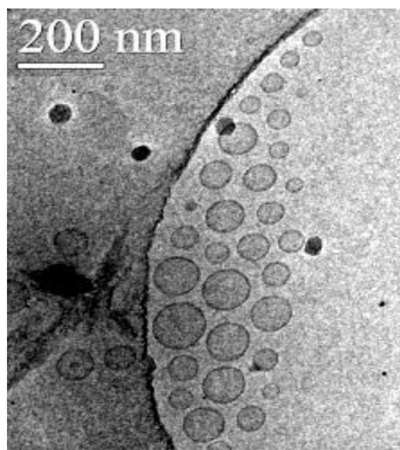


Figure 9 Cryo-TEM of Method 1 ULV

Glycoconjugate Distribution

Based on the methods studied, it was hypothesized that there would be two distinct distributions of C_{12}-glucose; for Methods 1 and 4, where the glycoconjugate is present as the vesicles are formed, there should be glycoconjugate present on the interior and exterior of the vesicles; whereas in Methods 2 and 5, where the vesicles are formed prior to adding the glycoconjugate, there should only be glycoconjugate on the exterior of the vesicles. Previous work has established that the rate of binding is directly correlated to the concentration of C_{12}-glucose (Figure 10); therefore,

differences in the distribution of C_{12}-glucose in the outer portion of the bilayer should affect rates of the binding kinetics.[30] Systems with a higher distribution of C_{12}-glucose in the outer layer should result in a faster increase in the turbidity. Therefore, systematic kinetic studies at each concentration for all methods should be able to elucidate any differences in the distribution amongst these methods.

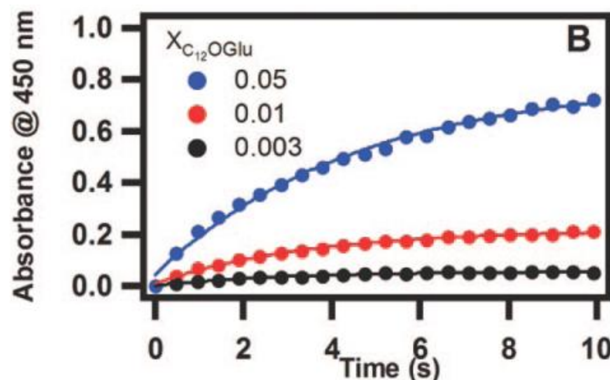


Figure 10 As bilayer glucose concentration increases agglutination rates increase.[30]

With the functionalized vesicles in a cuvette UV-Vis, ConA was added to the vesicles and the change in absorbance continually recorded as a function of time for 60 seconds. Immediate aggregation was observed within 1s of ConA addition for all concentrations of C_{12}-glucose. Figure 11 shows the normalized data from these experiments. This data shows that for the corresponding glycoconjugate concentrations the rates is agglutination between Methods 1 and Method 2 were comparable to each other. Likewise the agglutination rate between Method 4 and Method 5 were also comparable. This indicates that despite the preparation method, the distribution of the glycoconjugate in resultant vesicles are the same. However, the agglutination rates of Methods 1 and 2 were discernibly faster than that of Methods 4 and 5.

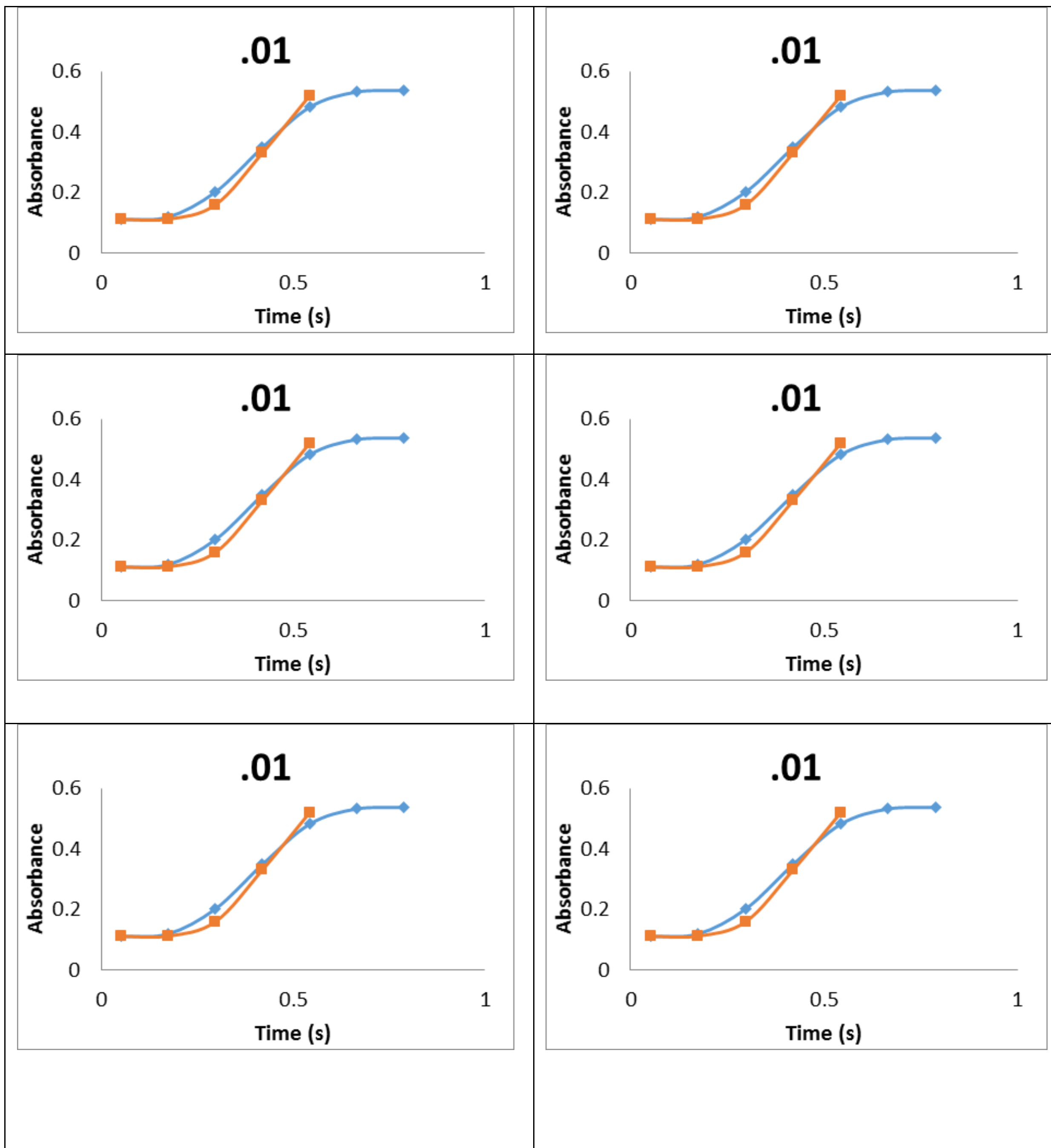


Figure 11 Kinetic Data

<u>*Current Work*</u>

It is well documented that the addition of cholesterol to liposomes can increase their stability presumably in the same manner in which cholesterol stabilizes cellular

structures. We sought to determine if the same would hold true for our catanionc system. We have been able to synthesize cholesterol-C_{12}-glucose vesicles utilizing Method 1 with cholesterol loadings up to .02 mole fraction. These vesicles have demonstrated a comparable size to their non-cholesterol counterparts and are capable of aggregation upon addition of ConA. Preliminary turbidity and DLS studies suggest that C_{12}-glucose vesicles with added cholesterol show increased stability in regards to changes in ionic strength. A more in depth study covering the full range of stability studies of these vesicles will be explored.

Conclusions

Presented here is a description of **six** different methods for the preparation of C_{12}-glucose functionalized ULVs. The inability to create solutions of the glycoconjugate at higher concentrations invalidated two of the proposed preparation methods, thus only four methods were studied further.

The results from the phenol-sulfuric acid assay demonstrated that regardless of preparation method the amount of glucose associated with the vesicles remained the same. This suggests that while the order in which C_{12}-glucose was added to the vesicles was different, that the majority of the glucose associated with the vesicles after SEC remained the same. Additional characterization of the various preparation methods further confirms that the vesicles formed shared analogous stability and characteristics. The size of the resultant vesicles remained the same for the corresponding C_{12}-glucose concentration. Furthermore, all four methods showed remarkable stability to a wide range of pH values as well as ionic strength.

The kinetic studies indicated that even at higher concentrations of the glycoconjugate there is no discernible difference in the distribution between the interior and exterior leaflet. This suggests that there is a preference for the glycoconjugate to be in the exterior leaflet of the vesicles. This is most likely due to the packing parameter and the shape of the glycoconjugate.

Since has been shown that these four methods produce analogous vesicles any of them could be used for the preparation of vesicles for future drug delivery purposes. Furthermore, since the surfactant concentration can be kept constant without any adverse effects methods 4 and 5 can be used for the added ease of preparation.

Future Work

Loading Capacity

Prior work within our group has demonstrated that varying amounts of C_{12}-glucose may be loaded into the catanionic surfactant vesicles loading up to 0.3 mol fraction.[29,30] However, this work was performed on vesicles made by a procedure analogous to method 1 reported here. Currently it is assumed that the three methods differ in the final location of C_{12}-glucose in the bilayer based on the order of addition of the glucose (Figure 12). However, it is unknown whether the order of addition affects the overall loading capacity. At higher concentrations of C_{12}-glucose differences distribution may have an effect on the maximum loading capacity. Thus, this research shall be expanded in order to elucidate any differences in the loading capacities of the three methods. The amount of glucose associated with the vesicles prepared by each method will be compared using the phenol-sulfuric acid assay. Previous results indicate a direct correlation between the results obtained from this

assay to the amount of glycoconjugate added to the vesicles (Figure 10).[29] These studies will be expanded to other moieties specifically hydrophobicly modified folate.

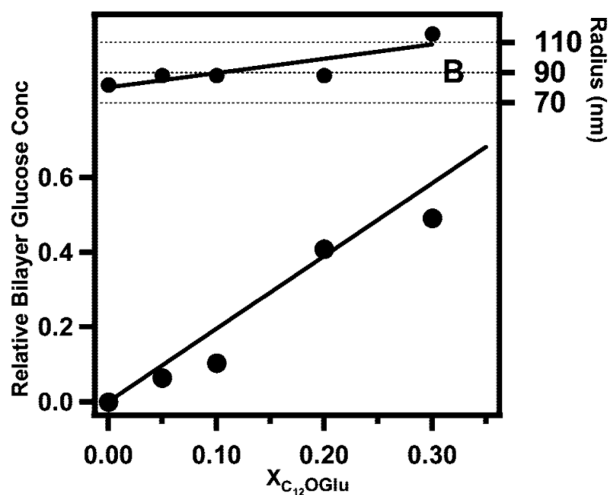


Figure 12 Plot of detected glucose (proportional to UV - vis signal of colorimetric assay) versus initial mole fraction of $C_{12}OGlu$[30]

Doxurubicin

Other members within the group have performed preliminary studies involving the loading of doxorubicin into our catanionic vesicles. This works has provided a broad range of loadings which have been used for successful mice studies. The maximum loading of doxorubicin has been estimated to be ~.02 mole fraction as evident by the precipitation of excess doxorubicin from the vesicles. However, a systematic study into the encapsulation efficiency, loading capacity, and characterization of these vesicles has not been performed. Thus, I will focus on exploring these aspects of doxorubicin loaded vesicles between the ranges of .01-.02 mole fraction.

Utilizing the methods reported by others in the field, the encapsulation efficiency of vesicles will be measured. This may be done by directly measuring the amount of doxorubicin associated with the vesicle fractions as a ration to the amount of

doxorubicin initially loaded into the vesicles. The levels of doxorubicin can be monitored by the absorbance peak at 490 nm by UV-Vis. This systematic approach will permit a quantitative determination of the maximum load of doxorubicin that the catanionic system is capable of delivering. The resulting system(s) will be fully characterized in the same manner previously described. Finally, the creation of a multifunctional catanionic system will be studied by creating vesicles containing doxorubicin along with targeting molecules such as folate. Analogous work to that reported will be done to determine the optimal loading of each component, i.e. doxorubicin and folate, the effect of folate and doxorubicin on the stability of vesicles.

Chapter 3: Multilamellar Catanionic Surfactant Vesicles

Introduction

Prior to the advent of nanoparticles methods to increase solubility were limited to structural changes or changes in the formulation. Such methods often have adverse effects on the selectivity and toxicity of the desired therapeutic. Aforementioned were the various methods employed by nanocarriers to overcome these challenges. Liposomes in particular have been studied as drug carriers since they allow for the transport of both hydrophobic and hydrophilic molecules. However, since the therapeutic effect is highly dependent on the amount of drug delivered the encapsulation efficiency of drug molecules by liposomes is very important. Introduction of the desired pharmaceutical may change the properties of the bilayer, size, and charge of the carrier particle; all of which affects the inherent stability of the molecule. Thus a delicate balance must be made between stability of the nanocarrier and the payload delivery. As a result encapsulation efficiency remains one of the many challenges faced in the development of novel drug carriers.

One of the methods used in increase the encapsulation efficiency of hydrophobic drugs within liposomal drug carriers is multilamellar liposomes. By increasing the amount of lamellae present the overall area of hydrophobic areas within the liposome is increased. This allows for a greater entrapment of the hydrophobic molecule within the bilayers. Theoretical experiments have calculated that the structures of multilamellar liposomes could provide some benefits such as greater stability with increasing lamellae and reduced stress due to osmotic shock.[38] However, outside of the theoretical calculations few reports of actual multilamellar

liposome used for drug delivery have been reported. This is due mainly to non-uniformity in size, non uniform lamellarity, poor long term stability, and lack of a standard preparation.

A more recent development within this field is the advent of DepoFoam (Figure 13). DepoFoam is described as being a unique liposomal drug release formulation that may be described as being a multivesicular system (MVL).[39,40]Each DepoFoam particle encloses multiple nonconcentric chambers each enclosed by a single lipid bilayer. This results in multiple aqueous and lipid areas in which molecules of interest may be encapsulated. Thus, as a result of the increased encapsulation there is a higher drug delivery to the targeted site. However, the enclosed multiple bilayers create slower and sustained release lowering the toxicity of the encapsulated drug while increasing the drug load.[39–43] The development of DepoFoam technology has been used in of several FDA approved formulations such as DepoCyt and DepoDur which are used for the sustained release for cytabrine and morphine respectively.

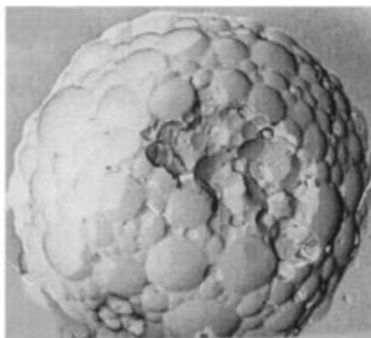


Figure 13 Transmission electron microscope (TEM) image of DepoFoam Particle. Multivesicular system consisting of a large spherical liposome containing many smaller spherical liposomes.

Previously, it was mentioned that one of the advantages of the catanionic surfactant vesicle system over liposomes was the ease of reproducibly creating ULVs.

Liposomal techniques all require some form of mechanical energy during their preparation, i.e. sonication or extrusion, in order to homogenize the system and create the metastable unilamellar system. The apparent absence of spontaneous multilamellar vesicle formation utilizing a process presents a unique challenge in creating additional hydrophobic sites for increased drug loading.

During inquiry into the multivalent capabilities of surfactant vesicles, Dr. English serendipitously discovered that upon disaggregation, several of the vesicles were multilamellar in nature (Figure 14).[44] We sought to explore this feature and develop a reproducible method of creating mulitlamellar vesicles hereto referred to as MLVs. Since the lamellae of these MLVs would be linked through a lectin-ConA-lectin linkage it was believed that these vesicles should impart similar if not greater stability when compared to the ULV counterparts. Theoretically, initial hydrophobic loading could occur during the initial vesicle formation followed by an additional loading step after MLV formation. This would allow for increased loading of one drug or a possible tandem drug delivery system.

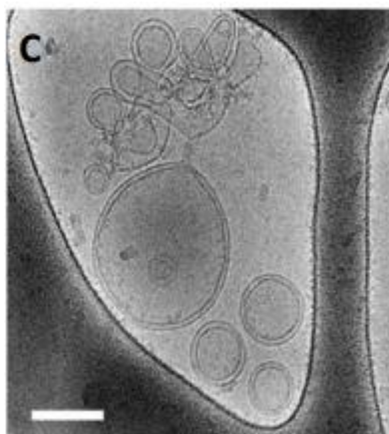


Figure 14 Cryo-TEM of C_{12}-glucose vesicles after aggregation with ConA. [44]

Preparation of Multilamellar Vesicles

In order to create MLVs, the C_{12} functionalized ULVs that were previously synthesized were treated with concanavalin A (ConA). In accordance with previous research, this resulted in immediate aggregation and a visible increase in turbidity. This aggregated colloid system was subsequently treated with a concentrated glucose solution effectively disaggregating the system (Figure 15). Finally, the presumed MLVs were purified by SEC using Sephadex 100-G saturated in glucose. Fractions 3 and 4 were collected and vesicle formation was confirmed by DLS.

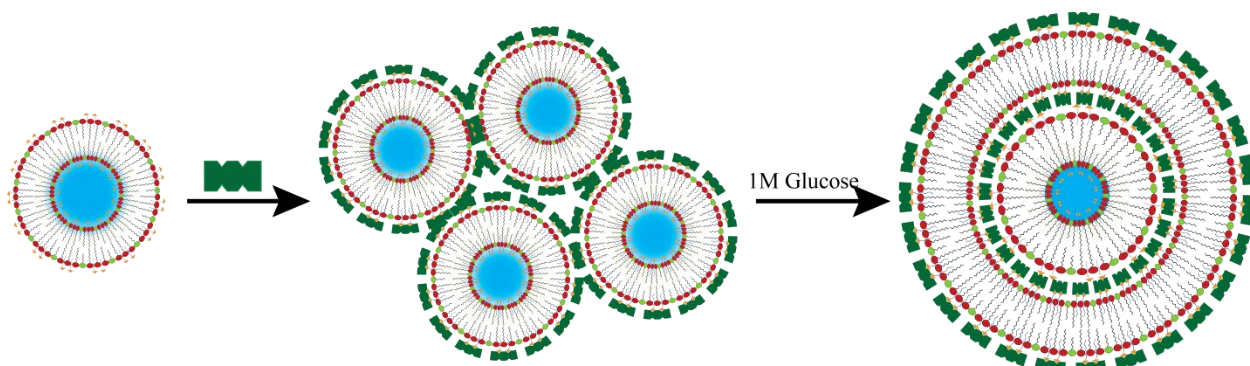


Figure 15 Proposed synthesis of MLVs. ULV previously created were treated with ConA (green) followed by addition of glucose.

Stability Studies

Zeta Potential

To compare the potential stability of the MLV system to the ULV system the zeta potential was once again obtained. Results showed that the zeta potential of the MLV system was significantly lower than that of the ULV system, averaging -30mV. Once again, there was only one population responsible for the potential correlating to the data obtained from DLS. The lower potential indicates that the MLV systems are less

resistant to aggregation than the ULV system and thus there may be a higher propensity for the system to aggregate overtime.

Ionic Strength

Like ULVs the packing parameter of the MLVs can be manipulated by changes in the ionic strength of the solution. When the ionic strength containing MLVs was increased there a dramatic increase in size a lot earlier than that seen in the ULV system. Furthermore, complete dissolution of the MLV system occurred at a lower ionic strength as well. Once again this may be explained by the packing parameter. Since the size of the MLVs are larger than that of the ULVs. This means that the ability of the MLVs to rearrange in order to accommodate such changes is greatly diminished. Interestingly, while all of the ULV preparations demonstrated a rather uniform response to changes in ionic strength in the MLV system the vesicles prepared by the VAM method demonstrated a marked difference.

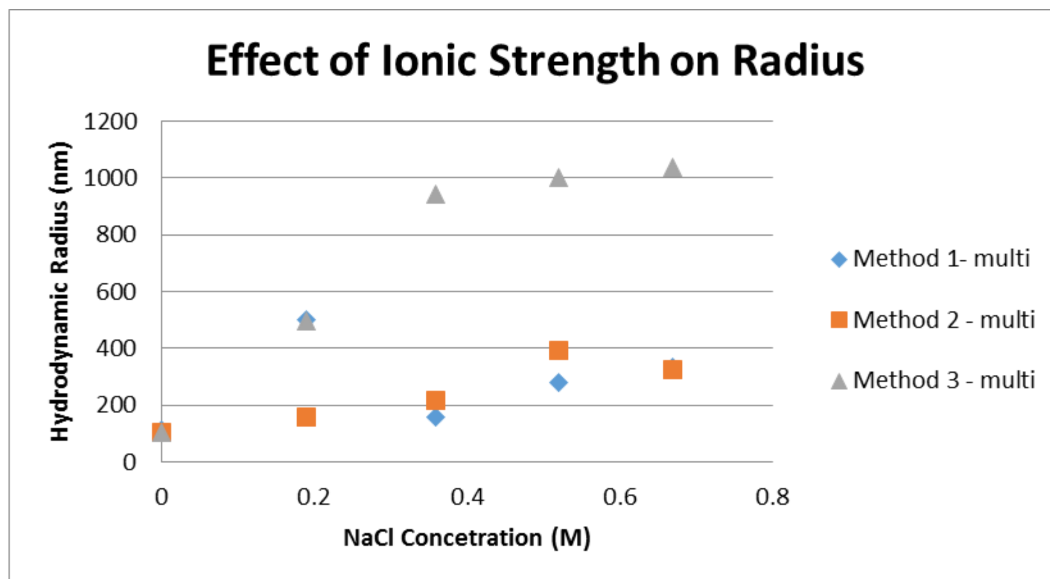


Figure 16 Effect of ionic strength on the radius of MLVs

Cryo-TEM

The prior results all indicate that the resulting solution formed after disaggregation behaves significantly different from the originally developed ULV system. This suggested that the MLV system must have an inherent structural difference in order to account for these differences. As aforementioned, the results reported by English *et. al.* demonstrated MLV vesicle formation after disaggregation of the ConA vesicles. Thus, it was expected that cryo-TEM images of our MLV system would show more of the same. However, Figure 17 shows that while larger vesicles are indeed formed, they are not multilamellar in nature. The TEM images below confirm that the vesicles in the MLV systems are larger. However, they fail to elucidate any other structural difference between the two systems. Thus, it would seem that the difference in behavior is due solely to the larger size of the vesicle system.

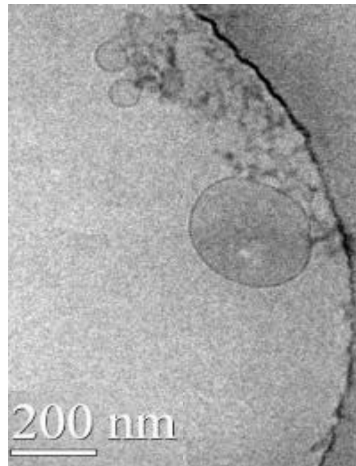


Figure 17 Cryo-TEM of the MLV (Method 1 - multi shown)

Dialysis

The ability of the MLVs to be disaggregated by the addition of glucose piqued interest in the system being completely reversible. Therefore, it was believed that removal of the excess glucose concentration should result in the aggregation of the

system which could then again be disaggregated. Dialysis was determined to be the most suitable method to carry out this goal. Thus, when the disaggregated vesicles were subjected to dialysis the excess glucose was removed which resulted in the system aggregating. However, when these aggregated vesicles were treated with an equal amount of glucose the system failed to disaggregate again.

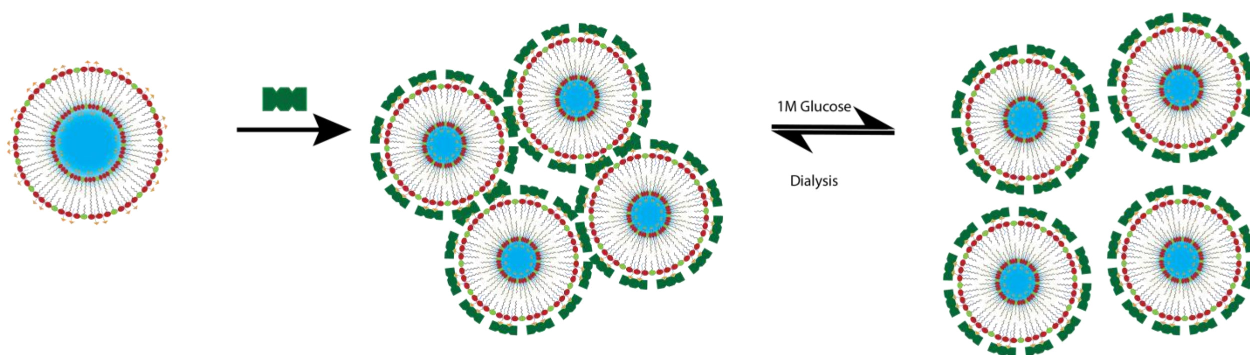


Figure 18 Proposed reversible aggregation process.

Conclusions

Attempts to create a reproducible and systematic method for the preparation of MLVs were successful in producing larger vesicles. These larger vesicles were more sensitive to environmental changes and demonstrated a lower zeta-potential. This indicated that they are most likely less stable than their ULV counterparts. This marked decrease in stability indicates that these larger vesicles are close to the critical size for the given surfactant system. Thus, they are unable to accommodate environmental changes that would affect the packing parameter by increasing their size. These MLVs are not fully reversible, capable of undergoing only one aggregation-disaggregation-aggregation cycle. Furthermore, the cryo-TEM imaging showed that these larger vesicles were not in fact multilamellar; but rather larger ULVs.

Future Work

Further studies on the MLV system are planned in order to elucidate the exact mechanism behind the formation of the multilamellar system exhibited in the work of English *et. al*. TEM experiments taken at various stages namely; after aggregation, immediately following disaggregation, and of the dialyzed material, will help to determine if there is indeed a return to the unilamellar phase after multilamellar formation. Furthermore, studies of the vesicles after dialysis will be obtained; specifically, determination of size, response to environmental changes and cryo-TEM. Hopefully this will elucidate mechanism behind the "reversible" process. Finally, it is possible that the increased size of vesicles with increasing ionic strength could result in a change in the assembled structure. If there is a new structure and it is in fact multilamellar this could give rise to a new technique for MLV creation.

Experimental

Materials

All chemicals and solvents were purchased from commercial suppliers unless otherwise noted.

Equipment

Absorbance spectra was collected using CHEM2000-UV-vis-spectrometer, Ocean Optics, Inc. Average hydrodynamic radius was determined using dynamic light scattering (DLS) using a Photocor Complex Optical unit equipped with a 5mW laser 633nm. All DLS measurements were taken at a 90° scattering angle and at 25°C. Size distribution, polydispersity index, and hydrodynamic radius of vesicle samples were determined using an autocorrelation function utilizing the instruments software (Photocor Software). Cryogenic transition electron microscope (TEM) images were taken using (JEOL JEM 2100).

Zeta potentials were collected using Malvern Zetasizer at 25°C.

Size exclusion chromatography (SEC) was performed using A column (5.5cm length, 1.5cm diameter) packed with Sephadex G-100 (Sigma) suspended in 18 MΩ Millipore H_2O for initial vesicle preparation and 1M glucose for preparation of multilamellar vesicles.

General Procedures

Carbohydrate Assay

To determine the amount of carbohydrate present in each fraction a standard phenol-sulfuric acid assay was used. A 0.250mL aliquot of the vesicle containing SEC fraction was transferred to a separate test tube where 0.125mL of 0.530M aqueous

phenol (13.3 mmol) was added, followed by the addition of 0.625mL of concentrated H_2SO_4 (18M) directly to the liquid surface. Samples where then vortexed and allowed to sit at room temperature for 1h to permit color formation. Presence of carbohydrate was indicated by the formation of a yellow/ orange color. 0.250mL of ethanol was then added to the samples and vortexed again. After 10 min at room temperature the absorbance was measured at 490 nm.

Dialysis

1 mL of vesicle solution was added to dialysis tubing (Spectra/Por MW, 1,000) and stirred in 18 MΩ Millipore water. Water was exchanged twice (every 2hrs) and then allowed to stir overnight. Vesicle solution was removed the following day.

NaCl/ Size Tests

A 4M stock solution of NaCl was prepared and then diluted to the desired concentrations. 100 µL of vesicle solution was added to .5mL of corresponding salt solution. The solution was allowed to sit at room temperature for 5 min. before taking DLS measurements.

pH Studies

Solutions of the desired pH were prepared using concentrated HCl (12 M) and concentrated NaOH (19 M). 100 µL of vesicle solution was added to .5mL of corresponding solution and allowed to sit at room temperature for 5 min. before taking DLS measurements.

Kinetics

To evaluate the potential differences in the glucose distribution a kinetic assay was used. The absorbance at 450 nm was monitored over time after the addition of

buffered Con A to vesicles conjugated with different mole fractions of C_{12}-glucose. A blank containing 400 μL of vesicle sample and 100 μL buffer with no ConA was used in each experiment. Each run was performed by first adding 400 μL of vesicle sample to the cuvette, then placing the cuvette in the UV-vis spectrometer, adding 100 μL of buffered Con A, then immediately starting acquisition of the kinetics data. The concentration of Con A used was 5 μM. For each kinetics run, the initial rate was found from the slope of the initial linear region of the absorbance plot. The initial rate of aggregation was monitored to avoid complications associated with a nonlinear response from the formation of large aggregates.

Vesicle Preparation

All vesicles were prepared with an excess of the anionic surfactant SDBS and are thus referred to as anionic vesicles.

Method 1

9.90 mL of 18 mΩ Millipore H2O was added to a vial containing of SDBS of CTAT, and C12-glucose adjusted for the desired w/w ratio of surfactants to C12-glucose. Solution was stirred for 1hr resulting in a milky colloid solution which was then further purified using size exclusion chromatography (SEC)

Method 2

9.90 mL of 18 Ω Millipore H2O was added to a vial containing of SDBS and of CTAT. Solution was stirred for 1hr resulting in colloidal suspension of bare anionic vesicles. This solution was then added to a vial containing C12-glucose and stirred for an additional hour which was then further purified using SEC

Method3

9.90 mL of 18 Ω Millipore H2O was added to a vial containing C12-glucose. Solution was stirred until glucose was dissolved. The resulting solution was then added to a vial containing SDBS and CTAT adjusted for the desired w/w ratio. Solution was stirred for 1hr resulting in a colloidal suspension milky in appearance which was then further purified using SEC

Method 4

9.90 mL of 18 mΩ Millipore H2O was added to a vial containing of 70.0 mg of SDBS, 30.0mg of CTAT, and the desired amount of C12-glucose adjusted for the desired w/w ratio of surfactants to glycoconjugate. Solution was stirred for 1hr resulting in a milky colloid solution which was then further purified using size exclusion chromatography (SEC)

Method 5

9.90 mL of 18 Ω Millipore H2O was added to a vial containing 70.0 mg of SDBS and 30.0 mg of CTAT. Solution was stirred for 1hr resulting in colloidal suspension of bare anionic vesicles. This solution was then added to a vial containing the desired amount of C12-glucose and stirred for an additional hour which was then further purified using SEC

Method 6

9.90 mL of 18 Ω Millipore H2O was added to a vial containing the desired amount of n C12-glucose. Solution was stirred until glucose was dissolved. The resulting solution was then added to a vial containing 70.0 mg of SDBS and 30.0 mg of CTAT.

Solution was stirred for 1hr resulting in a colloidal suspension milky in appearance which was then further purified using SEC

Multilamellar Vesicles

To each of the previously formed vesicle solutions 0.200 mL of 5µM ConA was added, resulting in visible aggregation. Solutions were allowed to sit at room temperature for 45 min, after which 0.100 mL of 1M glucose was added. Solution was then stirred for 1h at room temperature resulting in a milky colloidal solution. The resulting vesicles were then purified by SEC prior to analysis.

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