Properties of vesicles containing natural and synthetic lipids formed by microfluidic mixing

by

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BSc, University of Waterloo, 2013

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

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University of Victoria

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Supervisory Committee

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Abstract

A series of sulfonate anionic lipids esters derived from 4-sulfobenzoic acid (single chain) or 5-sulfoisophthalic acid (double chain) with chain length from C_{14} to C_{18} were synthesized and characterized. The sodium salts were uniformly insoluble in ethanol; the tetramethylammonium salts of the single chain derivative from oleyl alcohol and the double chain derivative from 2-octyldodecan-1-ol were sufficiently soluble for subsequent experiments.

Lipids in ethanol and aqueous buffers were mixed in a microfluidic system (NanoAssmblr® microfluidic mixer) to prepare a lipid dispersion containing vesicles and/or nanoparticles. Initial studies on prediction and controlling vesicle size based on lipid geometric parameters showed that particle size could be successfully affected and controlled by altering lipid compositions consistent with the formation of vesicles. A survey using high resolution cryo-Scanning Transmission Electron microscopy of the sample made by the microfluidic mixer demonstrated that vesicles were formed but a majority of the sample reformed to other aggregates, which complicated the interpretation of the initial product distribution. Further investigation on the efficiency of incorporation of phospholipids into vesicles indicated that 55% of the initial phospholipid appeared in the vesicle fractions. Sulfonate anionic lipids are incorporated into vesicles with lower efficiency and reach a threshold beyond which the sulfonate lipid is not incorporated. Entrapment efficiency was studied with three dyes. Different concentrations of the hydrophobic neutral dye Nile red, the hydrophilic cationic dye neutral red and the hydrophilic anionic dye hydroxypyrene trisulfonate (HPTS) were prepared. The entrapment efficiency was quantitatively analyzed by HPLC, and electrospray mass spectrometry; up to 15% of the initial dye present could be entrapped. Vesicles permeability assays using the ion channel
gramicidin and the ion carrier valinomycin with HPTS-loaded vesicle samples showed that vesicle samples made by the microfluidic mixer and made by a conventional extrusion method appeared to behave in the same manner. Addition of a sulfonate anionic lipid to the lipid mixture resulted in vesicle leakage. The unilamellar proportion of HPTS loaded vesicle samples was assessed using a mellitin assay. A vesicle sample made by the microfluidic mixer was 80% unilamellar; a vesicle sample made by the extrusion method on the same lipid mixture was 60% unilamellar.
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# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>Brain PS</td>
<td>1-octadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phospho-L-serine (sodium salt)</td>
</tr>
<tr>
<td>Chol</td>
<td>Cholesterol</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DSPE-mPeg</td>
<td>1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt)</td>
</tr>
<tr>
<td>E coli PE</td>
<td>L-α-phosphatidylethanolamine (E. coli)</td>
</tr>
<tr>
<td>Egg PG</td>
<td>L-α-phosphatidylglycerol (Egg, Chicken) (sodium salt)</td>
</tr>
<tr>
<td>Equiv</td>
<td>Equivalent</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>Gram</td>
<td>Gramicidin</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>POPC</td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>SAdiC14:0</td>
<td>3,5-bis((tetradecyloxy)carbonyl)benzenesulfonic acid (Tetramethylammonium salt)</td>
</tr>
<tr>
<td>SAdiCbr20</td>
<td>3,5-bis(((2-octyldodecyl)oxy)carbonyl)benzenesulfonic acid (Tetramethylammonium salt)</td>
</tr>
<tr>
<td>SAmmonoC18:1</td>
<td>(Z)-4-((octadec-9-en-1-yloxy)carbonyl)benzenesulfonic acid (Tetramethylammonium salt)</td>
</tr>
<tr>
<td>SAmmonoCbr20</td>
<td>4-(((3-octyltridecyl)oxy)carbonyl)benzenesulfonic acid (Tetramethylammonium salt)</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>Vln</td>
<td>Valinomycin</td>
</tr>
<tr>
<td>1H-NMR</td>
<td>Proton nuclear magnetic resonance</td>
</tr>
<tr>
<td>13C-NMR</td>
<td>Carbon-13 nuclear magnetic resonance</td>
</tr>
<tr>
<td>18:0 Lyso PC</td>
<td>1-octadecanoyl-sn-glycero-3-phosphocholine</td>
</tr>
</tbody>
</table>
List of numbered compounds

1-3c

1-3d

1-3e

1-4a

1-4b

1-4c
1-8b (SAmonoC16:0)

1-8c (SAmonoC18:0)

1-8d (SAmonoCbr20:0)

1-8e (SAmonoC18:1)
Acknowledgments

I would like to express my deep sense of appreciations to my supervisor Dr. Tom Flyes who guides me with this project. He is the most acknowledgeable, intelligent and nicest person I have ever met. I feel so lucky to be his student since he is always there for you when you need help. During my entire study, he does not only have faith on me, but also encourages me to achieve more, and inspires me with his wisdom.

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To my friends, thanks for listening and providing me advice, and thanks for all the happy time we spent together, and all the beautiful moments we shared. This would not be possible without all of you.
Chapter 1 Introduction

1.1 Lipids and lipid geometry

Biological membranes, which consist of different lipids and proteins, function as barriers to separate cellular environments, thus they play important roles in regulating cell life by controlling exchange of materials and information with outside environment of the cell\(^1\). Lipids are amphiphilic molecules with polar phosphate headgroups and nonpolar fatty acid tails. Polar and charged headgroups have favorable interaction with water, while hydrocarbon tails have unfavorable interaction with water, thus lipids tend to shield long hydrocarbon tails from water and expose headgroups in water to self-assemble into different morphologies (bilayers and micelles)\(^2\). The general structure of natural lipids has a glycerol backbone, which esterify with different fatty acids to form glycerolipids with mono-, di, and tri-substitution. Triglyceride, which is tri-substituted glycerol, plays an important role in metabolism as an energy source. Di-substituted glycerol esterify with two fatty acids leaves another hydroxyl group which is further esterified with different polar headgroups in the \(sn-2\) position to form different classes of phospholipids. Figure 1-1 illustrates some examples of natural phospholipids involved in biological membranes: phosphatidylcholine, phosphatidylethanolamine, phosphatidylerine, phosphatidylglycerol, and phosphatidylinositol\(^3\). Among these, phosphatidylcholine are the most abundant lipids in plant and in animals.

Lipids are separated into different categories based on the headgroups in the molecules; however, the fatty acids chains in individual lipids are the key factor to determine the behavior of lipid. The characteristics of phospholipids are changed by a difference in the number of carbon atoms and the degree of unsaturation in the fatty acid\(^3\). Lysolipids are derived from the hydrolysis of one ester chain to give a free alcohol on the glycerol. They usually are found in a cell membrane in small amounts, but in spite of the small amount present, they play an important role in regulating membrane-membrane interactions\(^4\). Other natural lipids include sphingolipids and sterol lipids. Cholesterol is a well-known sterol lipid, and previous research has exhibited that cholesterol plays an important role in helping maintain the rigidity of cell membranes due to a lipid mixture with a certain amount of cholesterol appears to improve the membrane fluidity and reduce the permeability of water-soluble molecules through membranes\(^4,5\).
Triglyceride

POPC (16:0-18:1 PC)

E coli PE (16:0-18:1 PE)

Brain PS (18:0-18:1 PS)

Egg PG (16:0-18:1 PG)

Cholesterol
Molecular geometry is an important factor to affect and control the shape and size of aggregates formed from lipids in water. Attempts to understand lipid packing led to the study of interaction free energies between lipids as controlled by the lipid shape. From that, a dimensionless shape parameter was developed\(^5\). Lipids self-assemble into different morphologies which are governed by a shape parameter given by \(S=V/a_0l_c\), where \(V\) is the volume of the hydrocarbon chains, \(a_0\) is the area of the lipid head group at equilibrium in the aggregate, and \(l_c\) is the critical hydrocarbon chain length. The values of \(V\), \(a_0\), and \(l_c\) have traditionally been determined by experiments; however, for a prediction purpose, \(S\) can be computed based on standard bond lengths, angles and the partial atomic volumes of the atoms in a given lipid\(^5,6\). In general, a lipid which possesses of a sufficiently large headgroup and a sufficiently small hydrocarbon volume tends to form micelles, and the lipid has wedge shape with very small \(S\) value (\(S<0.5\)). Lipids that self-assemble into inverted micelles or other inverted phases (hexagonal, cubic) have relatively large hydrocarbon chain volume and small head group area; these lipids usually have truncated cone shape with an \(S\) value greater than 1. Cylindrical shape lipids with \(S\) value approximately 1 appear to form flat bilayers. Biological lipids usually have \(S\) value about 0.7~1, which leads to the formation of bilayers with curvature that results in vesicles (liposomes)(In this thesis, the terms of “liposome” and “vesicles” are interchangeable, and both of them refer to a closed bilayer structure composed by natural and synthetic lipids, in biology, “vesicle” can be have other meanings\(^4,5\). Phospholipids such as POPC, which have shape parameter values about 0.85, are approximately cylindrical, and enable self-assembly into bilayers. However, cone-shaped cholesterol having a shape parameter value greater than 1(1.14-1.24 depending on the system\(^5\) is not able to form a bilayer by itself. Instead, self-assembly into an inverted micelle occurs under
some conditions\textsuperscript{5}. Inverted wedge shaped lipids such as lysoPC tend to form micelles in water.

![Diagram of lipids and micelles]

Figure 1-2 Morphologies of lipids with different shape parameters

Natural membranes contain mixture of lipids. In simple binary mixture of lipids such as a mixture of cholesterol and phospholipids, cholesterol tends to closely interact with the fatty acid chains on phospholipids\textsuperscript{4}. If cholesterol concentration is low enough, the mixture forms a bilayer; however, increasing cholesterol concentration leads to the formation of inverted micelles. The fact that different shapes of aggregates are formed from the same lipid mixtures suggests the shape and size of aggregates depend on both the molecular geometry of the lipids and the molar weighted average $S$ of the two lipids\textsuperscript{5}. Apart from cholesterol and phospholipid binary mixtures, early reports of membrane
structure also exhibited that bilayer structure was observed for combination of phospholipids and lysophosphatidylcholine. The result is general- in mixed lipids systems with complementary shapes between the hydrocarbons, the lipids self-assemble, and the value of $S$ for the mixture is molar weighted average, and the resulting aggregate has the predicted geometry as if it were a single lipid having the average $S^{4,5}$.

1.2 Prediction of vesicle' diameter

Single lipids having shape parameter values about 0.85 are able to self-assembly into enclosed lipid bilayer membranes (vesicles), and two lipids having complementary structures enable formation of closed bilayers as well. For a two component system, how are the two lipids distributed between the inner and outer layers of the bilayer? General speaking, a lower aggregation number (fewer lipids per vesicle) leads to lower the total system free energy. But smaller vesicles have tighter curvature on both the inside and outside leaflets of the bilayer, and the difference becomes more pronounced as the vesicle radius decreases. On the other hand, the entropy favored state is the situation where two lipids have equal distribution between inner and outer layers. Assume that two lipids (A, B) have the same critical chain length, and that the headgroup of A is larger than B. In this case $S$ for A is less than $S$ for B (Figure 1-3).

Figure 1-3: Lipid distribution in bilayers
Based on a pictorial analysis in Figure 1-3, the bilayer symmetric system C, and asymmetric system D have the same total number of the two lipids A and B. In the system C, two lipids are equally distributed in both leaflets; however, in the asymmetric system D, more lipids B are placed in the inner leaflet\(^6\). The asymmetric distribution also leads to a smaller diameter as placement of the small headgroup B is more favourable on the inside. In three dimensions, it turns out that the system D is more reasonable and physically correct for the fact that the area in inner leaflet is smaller than the area in the outer leaflet. The negative curvature on the inner leaflet is better accommodated by lipid B with the higher value of S while lipid A is better located in the outer leaflet where the lower S fits with the positive bilayer curvature\(^4,5,6\). The general conclusion is that lipids distribute asymmetrically in the inner and outer leaflets in binary lipid systems in order to minimize the Gibbs free energy of the system. In addition, there are a large number of experimental results which have shown that vesicles from mixed lipids have an asymmetric distribution of lipids\(^6,7\).

The discussion above presents that the diameters of vesicles are related to the headgroups and the hydrocarbon chains of lipids. Previous publications have shown that the vesicle diameter in a pure lipid system can be predicted using geometric variables \((a_0, V, l_c)\). The same theory can be applied for binary systems, by a numerical method that minimizes the total Gibbs free energy of the system as a function of the asymmetric distribution of the two components. The results are comparable to the assumption that the binary mixture has the molar weighted average value of the geometric variables \(V, a_0, l_c\)\(^4,6\).

### 1.3 Drug delivery and gene therapy

Macromolecular drug delivery systems refer to the complexes in which drugs are attached to carriers such as liposomes, synthetic polymers\(^8\), and dendrimers, where dendrimers are synthetic, highly branched globular molecules\(^9\). Targeting drugs by carriers has been an important theme of research in therapeutics since most drugs are usually unable to directly achieve therapeutic concentrations in targeting sites without harmful toxic effects in normal tissues\(^7,8,10\). Most drugs lead fail to go through clinic trails as a result of non-target toxic effects, which indicates there is a need for developing reliable and efficient delivery systems. Ideal drug carriers should be biodegradable, and non-toxic, able to protect the drugs from degradation until they reach the desired site of
action, and then be capable of releasing the drugs\textsuperscript{8,9}. In recent years, lots of effort has been devoted to the design and preparation of reliable drug delivery systems with the aim of minimizing the side effects and improving site specificity.

Biodegradable polymers have been widely synthetized for drug delivery\textsuperscript{11}. Polymer-drug conjugated systems are well-studied systems for delivering drugs, where one or more drugs are attached to the functional groups on polymers by covalent bonding or through a spacer\textsuperscript{12}. In addition, block copolymers with amphiphilic character assemble to core-shell architecture micelles in aqueous solution; thus, drugs are encapsulated in the inner hydrophobic core\textsuperscript{11,13}. On the other hand, interest has been raised in the application of dendrimers as drug delivery systems. Started in the mid-1980s, lots of research has focused on investigating of synthetic methods, physical and chemical properties of dendrimers\textsuperscript{14}. Dendrimers can be used as drugs delivery systems in two ways: drugs can be entrapped on the internal cavity of dendrimers, or they can be covalently linked between the functional groups on the surface of dendrimers and drug molecules. Furthermore, graft dendrimers with polyethylene glycol (PEG) improves drugs encapsulation efficiency\textsuperscript{9,15}.

Although many drug delivery systems have been established, a majority of them suffer from low entrapment efficiency and high toxicity, which implies there is a requirement for development of more efficient systems.

Solid lipid nanoparticles and liposomes consisting of natural or synthetic lipids are the most investigated systems and have attracted interest due to the advantage of biologically inert and nontoxic components; hence, their great potential application for gene and small-molecule drugs delivery. Solid lipid nanoparticles (SLNs) introduced in 1991 represent an alternative colloidal carrier, which are composed by a solid core coated with surfactants, where the solid lipid matrix core consists of high melting point lipid and drugs (Figure 1-4)\textsuperscript{16}. The use of crystalline lipids instead of liquid state lipids aims to decrease the leakage of incorporated drugs and increase the stability of the nanoparticles. SLNs represent a promising delivery system for a wide range of applications for drugs, gene therapy and in the food industries\textsuperscript{17}. On the other hand, liposomes, which are enclosed lipid layers, act as effective delivery systems for small molecule drugs with a wide range of lipophilicity. Specifically, drugs
with intermediate lipophilic character are partitioned between lipid bilayers, while strongly hydrophilic drugs are entrapped in the aqueous volume (Figure 1-4)\textsuperscript{18}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{liposome_slp.png}
\caption{Figure 1-4: Sketch of a liposome and a solid lipid nanoparticle as drug carriers\textsuperscript{11,12}}
\end{figure}

To date, non-viral gene delivery vectors using liposomes or SLPs are a common strategy for addressing many untreatable diseases. To address the problem of difficulty in purification and easy decomposition at room temperature of commercially available lipids, exploration of drugs and gene carriers in terms of developing new lipids is a key factor for drug delivery and gene therapy. Many synthetic lipids have shown good incorporation ability to liposomes and solid lipid nanoparticles\textsuperscript{19}, which leads to the thought that using the shape of synthetic lipids as a controlling factor to affect the shape and properties of lipoplexes and SLPs. In Dr. Fyles’ group at the University of Victoria, a large library of pyridinium lipids was established\textsuperscript{20}. Pyridinium cationic lipids with high shape parameters were synthetized by utilizing pyridine 3,5-diesteres esterified with different alcohols, and then methylated with tetramethyloxonium tetrafluoroborate\textsuperscript{20}. Pyridinium lipids with branched alkyl chains, linear alkyl chains, and chains having various degree of unsaturation have been synthetized, and the relationship between lipid geometry and the toxicity and gene transfection of the lipoplex has been investigated\textsuperscript{20}.

\textbf{1.4 Liposome classes and preparations}

Liposomes are potential drug carriers for a broad range of lipophilic or hydrophilic drugs, which consist of an aqueous core entrapped by one or more lipid bilayers. Due to different size and lamellarity, liposomes can be classified into the following categories:
multi-lamellar vesicles (MLV), large unilamellar vesicles (LUV), and small unilamellar vesicles (SUV)\textsuperscript{18, 21}. Multi-lamellar vesicles have more than one layer, and generally they have a broad range of size depending on lamellarity. The high lipid content of these MLVs owns a great ability to encapsulate lipophilic drugs. LUVs comprise a single lipid layer with diameter greater than 1µm, and they have large aqueous core, which allows them to capture hydrophilic drugs. SUVs, which are prepared by reducing the size of LUVs by sonication, have uniform diameter, and small aqueous core. The size of liposomes is an important factor, which determines the rate of liposomes uptake by the cells of the reticuloendothelial system (RES)\textsuperscript{22}. The rate of liposome uptake by the RES increases with the size of the vesicles\textsuperscript{22}. For example, small liposomes can passively transform through tumor capillaries more easily than large liposomes; hence, liposomes in the diameter about 100nm show high potential in drug delivery\textsuperscript{22}.

![Diagram of liposome types](image)

**Figure 1-5: Different classes of liposomes\textsuperscript{21}**

### 1.4.1 Liposome preparation

Liposomes with different sizes and characteristics require different preparation methods. Drug molecules can be entrapped in vesicles by the thin-film hydration technique\textsuperscript{18} and the freeze-thaw method\textsuperscript{23}. During a thin-film hydration process, lipids are dissolved in an organic solvent, and then, evaporation of solvent under vacuum yields a lipid film with a trace amount of residual solvent. Hydration of the lipid thin film is accomplished by adding desired buffer solution. The drugs to be encapsulated are either in the aqueous core (hydrophilic drugs dissolved in the buffer), or in the lipid film (lipophilic drugs).
MLVs produced by thin-firm hydration can be further disrupted by sonication or extrusion to produce SUVs. Alternatively, the freeze-thaw method involves hydration of dry lipid film in a buffer containing hydrophilic drugs, the mixture is vigorously shaken by vortexing; the resulting MLV suspension is frozen in liquid nitrogen and then thawed in warm water to melt lipid mixture. The freeze-thaw is repeated for five cycles to obtain the MLV with a maximum level of drugs entrapped\textsuperscript{23}, and then, the resulting suspension was forced to extrude through a polycarbonate membrane with specific pore size to generate uniform size distribution; the non-entrapped drug was removed by size exclusion chromatography. Previous publications presented that the entrapment efficiency could be as high as 31\% when lipid mixture undergoes 5-cycles of freeze-thaw; furthermore, increasing the freeze-thaw cycles reduced the entrapment efficiency, and higher drug encapsulation efficiency might be achieved by increasing the initial lipids concentration\textsuperscript{23}.

\textbf{1.4.2 Literature review for microfluidic mixing technique in drug delivery}

Numerous methods have been developed for preparing carriers for drugs and gene delivery; for example, liposome could be generated by freeze-thaw methods, and polymeric particles could be obtained from nanoprecipitation, and solvent diffusion salting out; however, these methods generate nanoparticles by bulking mixing, which suffers from a lack of reproducibility for the large-scale requirements of drug manufacturing and precise tenability, which has limited the widespread application of these techniques\textsuperscript{24}. There is a need to develop a convenient and simple technique to prepare liposomes in large scale with good reproducibility. The microfluidic mixing technique is a promising technique that currently used for generating liposomes and lipid nanoparticles. The technique allows multiple samples to be mixed at a millisecond time scale, and has demonstrated good batch-to-batch reproducibility\textsuperscript{25}.

What is a microfluidic system? It is defined as a system that has at least one dimension in the micrometer range\textsuperscript{26}. Microfluidic mixing is a new and promising technique, and has impacted various fields including chemistry, biomedicine, and pharmaceutical science. Due to the advantages of microfluidic systems, such as short mixing time, small volume consumption and good reproducibility, it is widely applied in formulating particles for gene and drug delivery. There are various microfluidic platforms for fabrication of drug
carriers, such as co-flow devices, flow focusing devices, cross-flow devices\textsuperscript{27}, and staggered herringbone structure micromixer\textsuperscript{28}. These differ in how the fluid flow induces shear forces in the fluid and in the sequence the components are combined. A flow focusing microchannel device is based on the principle of hydrodynamic focusing. The dispersed phase flows along the central channel, and continuous phases are delivered on two sides via channels\textsuperscript{28}. The central flow is squeezed by two adjacent flows, thus, the rapid mixing was achieved through diffusion. Drug-encapsulated polymeric nanoparticles could be obtained using flow focusing microchannel microfluidic mixing. The example demonstrated that polymeric poly (lactic-co-glycolic acid) (PLGA)-PEG nanoparticles were produced by self-assembly in a microfluidic channel by rapid mixing of an acetonitrile-polymer solution and water to produce nanoparticle diameter of 20-25 nm\textsuperscript{27}. Furthermore, the docetaxel drug encapsulation efficiency was 51% by microfluidic mixing, while the encapsulation efficiency was 45% for a bulk mixing method. Another example demonstrated that monodisperse and biodegradable drug-loaded microparticles could be form via a flow-focusing microfluidic mixing\textsuperscript{11}. PLGA dissolved in organic solvent such as dichloromethane was introduced in central channel, and the two side channels were occupied by aqueous solution as continuous phase. Droplets of dichloromethane/PLGA/drug were formed at the junction of the three inlets. The nanoparticles have uniform size of 40nm with drug-loading efficiency of 20 w/w\%\textsuperscript{11}.

Co-flow microchannel device, the dispersed and continuous phases flow parallel to each other, and the inner capillary tube contains a tapered tip made by microforging, and droplets are produced from the tip of the inner tube. Cross flow device the dispersed phase flows is perpendicular to the continuous phase\textsuperscript{24}. The aim of microfluidic mixing is to increase the contact area between the species to be mixed in a shooter time\textsuperscript{29}. The microfluidic mixer with staggered herringbone structure provides very fast mixing of two input steams. Two solutions from different inlet ports are combined and pass through a series of herringbone structures that induce rotational flow, which wrap the flow onto each other to achieve further mixing. This microfluidic based formulation process provides a quick and straightforward method for procuring vesicles and nanoparticles with size in the range of 20-200nm\textsuperscript{30}.
1.4.3: Microfluidic mixing with staggered herringbone structure platform in producing vesicles and lipid nanoparticles

Many microfluidic mixing devices have been developed for generation of drug encapsulated biodegradable polymeric nanoparticles, and the drug encapsulation efficiency was improved compared the bulk mixing method. Cullis et al demonstrated that lipid nanoparticles could be obtained by microfluidic mixing with staggered herringbone structure platform with siRNA entrapment efficiency of 95%\textsuperscript{30-31}. The microfluidic mixing device with staggered herringbone structure platform produced by Precision NanoSystems consists of a dual syringe pump controller that feeds solution to a microfluidic mixing device that contains a staggered herringbone micromixer section (Figure 1-6)\textsuperscript{30}. The two inputs, where lipids dissolved in ethanol are injected via the right inlet port, and buffer solution is injected in left inlet port are combined and mixed in the micromixer section. The microfluidic chip provides very fast mixing of two input streams by microstructure-induced chaotic advection. That flow orientation changes between half cycle leads to further mixing two solutions. Fast mixing of the solutions leads to a rapid rise in solvent polarity; as a result, the hydrophobic components are assembled into different morphologies depending on the lipid mixture\textsuperscript{30}.

The work has been done related to the production of limit size nanoparticle as gene and drug carriers by microfluidic mixer with staggered herringbone structure, and there has been much less published work on the use of microfluidic mixing to produce vesicles.

Figure 1-6: The chip for the NanoAssembler\textregistered microfluidic mixing device
1.5 Project goals and thesis overview

The limitation of traditional liposome preparation methods encourages us to develop new techniques for the large-scale requirement of drug formulation with a good reproducibility. The microfluidic mixing technique that provides a fast mixing process and good reproducibility has been applied for making nanoparticles or vesicles not only on bench scale but also scaled up to the 100-liter scale\textsuperscript{30}. Although the development of microfluidic mixing technique has a huge potential impact on drug delivery; there are some questions that need to be solved, such as:

- What type of aggregates the microfluidic mixer device makes; are the so-called “vesicles” produced real vesicles or something else?
- If the device can make vesicles, can the size of vesicles be affected by altering lipid composition in accord with the underlying theory? For example, do lipids with high shape parameters stabilize the interior curvature of small size vesicles?
- How much lipid ends up in the vesicles compared to losses during preparation and purification?
- What is the entrapment efficiency? Can it be improved via manipulation of the lipids and dyes?
- Does the vesicle membrane present the same sort of barrier as in vesicles made by other methods?

The overall goal of this thesis is to explore these unknowns experimentally. This includes synthetizing some anionic lipids, and using the microfluidic mixer to produce vesicles from lipid mixtures to uncover the vesicle’s properties, which in turns of investigating the size of aggregates, studying entrapment efficiencies and permeability and comparison with conversional methods of making vesicles.

Since a majority of drugs bear positive charges at physiological pH as they are weak bases and are protonated in near neutral pH, it would be interest to synthetize some anionic lipids to potentially assist in improved drug entrapment efficiency and liposome stability by charge-neutralization entrapment. From an awareness of a shape parameter and its potential effect on the morphology of lipid aggregates, pyridinium cationic lipids with very high shape parameters have been successful synthetized and applied in the study of transfection efficiency\textsuperscript{20}. On the other hand, lipids with high shape parameters
are required to stabilize the high curvature of inner leaflet in small size vesicles\textsuperscript{5,6}. In this thesis, the syntheses of anionic lipids with very high shape parameters were explored, and the properties of small size vesicles were investigated. Previous work has demonstrated that a branched alkyl hydrocarbon chain has a significant effect on lipid shape parameter since it increases the hydrocarbon chain volume without increasing the chain length\textsuperscript{6,20}. In this thesis, synthetic anionic lipids are prepared by esterification of 5-sulfoisophthalic acid with alkyl bromides having different chain length; thus, the possible structures for anionic lipids would be the lipids that bear sulfonate headgroups with branched and/or linear alkyl chains. One proposed structure with very high shape parameter (S=2.01) was presented in Figure 1-7. Due to the chiral center in the branched alkyl chain, the compound proposed is a mixture of diasteromers with two enantiomers and one meso compound. It is expected that these isomers will not be separable and will act similarly within the mixture.

![Figure 1-7: Prototype structure of anionic lipids](image_url)

In the second chapter of this thesis, a brief discussion about the synthesis of anionic lipids is presented. The third chapter describes the investigation of the properties of anionic vesicles which include some experimental work and discussions from different aspects: predict and control vesicle size, the efficiency of incorporation of lipids into vesicles, dye entrapment with three different dyes (anionic dye HPTS, cationic hydrophilic dye neutral red, and lipophilic neutral dye Nile red), investigation of vesicle stability via permeability
assays with ion channel gramicidin and ion carrier valinomycin, and the lamellarity of vesicles. The appendix A discusses the synthesis of sulfonate lipids in detail. Appendix B includes $^1$H-NMR spectra and $^{13}$C-NMR spectra for compound characterization, and Appendix C describes the extrusion method and the microfluidics mixing techniques to generate vesicles, outlines the vesicle formulations discussed, and gives the spectra from HPTS florescence experiments.
Chapter 2 Synthesis of sulfonate lipids

2. 1: Synthesis
In order to establish that the sizes of liposomes could be controlled via the shape parameters of lipids in a mixture, and to investigate charge-neutralization entrapment of cationic drugs, the synthesis of anionic sulfonate lipids with various shape parameters is required. The synthetic route is summarized in Scheme 2-1, and the general features of the synthesis are outlined. Full details of synthesis and compound characterizations including $^1$H-NMR and $^{13}$C-NMR are given in Appendix A and Appendix B respectively.

Scheme 2-1: Synthetic route for sulfonate lipids
2.1.1 Nucleophilic substitution of alcohol to bromide by Appel Reaction

The desired alkyl bromides presented on the scheme were synthetized by Appel reaction via the corresponding alcohols in the presence of CBr₄ and PPh₃. The products showed the expected ¹H-NMR spectra in that the methylene adjacent to bromide showed an upfield shift compared to the starting alcohols, which proved the successful replacement of hydroxyl groups by bromide. The pure alkyl bromides were obtained by silica gel chromatography in hexane and dichloromethane solvent mixtures with 1-3c in 92% yield, 1-3d in 97% yield and 1-3e in 90% yield.
2.1.2: Ester coupling

The sulfonate diester compounds for the scheme were synthesized by reacting 5-sulfoisophthalic acid (sodium salt) with relative alkyl bromides in DMSO in the presence of Cs$_2$CO$_3$, and NaI at 100 °C. After 12 hours, chloroform was added to the reaction mixture. The sulfonate diester compounds 1-4(a-e) were precipitated out of the solution due to their low solubility in chloroform. The precipitates were filtered and washed by chloroform to remove residual alkyl bromide to afford the pure products in yields varying from 50-75%. The resulting compounds 1-4(a-e) were identified by $^1$H-NMR: the methylene ($\delta=4.3$) adjacent to the ester oxygen was downfield shifted relative to starting alkyl bromide ($\delta=3.4$); furthermore, the correct integration between the aromatic ring and the alkyl chain was as expected for the products, the integration between the aromatic protons ($\delta=8.4, \delta=8.3$), and the methylene next to ester oxygen ($\delta=4.3$) was 1:2:4.

Obtaining the products of 1-4d, and 1-4e by applying the same method presented was unsuccessful since there was no precipitate produced after adding chloroform to the reaction mixture. Instead, addition of water and stirring the reaction mixture for 30 minutes and followed with evaporation of water under reduced pressure afforded while
particles. The while particles were washed by hexane dropwise to remove residual alkyl bromides present in the products. The desired compounds were confirmed by $^1$H-NMR, where the downfield shift of the methylene next to the ester oxygen in the products was observed by comparing with the methylene adjacent to bromide in the starting bromide. The integration ratio between the aromatic ring protons and the methylene protons adjacent to ester oxygen was correct (ratio is 1:2:4).

![Scheme 2-4: Ester coupling from alkyl bromides with 4-sulfobenzoic acid](image)

The sulfonate esters with single chains were synthesized by reacting 4-sulfobenzoic acid with relative alkyl bromides in the present of Cs$_2$CO$_3$, and NaI in DMSO as solvent at 100 °C for 12 hours. Chloroform was added to quench the reaction, and the sulfonate monoesters (potassium salt) 1-7(a-c) were precipitated due to their low solubility in chloroform. The precipitate was filtered and washed with chloroform to remove residual alkyl bromide to afford the pure compound. The products of 1-7(a-c) were obtained in yield varying from 50-65%. The resulting compounds were confirmed by $^1$H-NMR. The methylene ($\delta=4.3$) next to the ester oxygen showed a larger chemical shift compared to
the starting alkyl bromide ($\delta=3.4$) due to the deshielding effect of the ester group. Furthermore, the correct integration between the aromatic ring and the alkyl chain was expected for the products, in which the integration between the aromatic ring protons ($\delta=7.9, \delta=7.7$), and the methylene protons next to ester oxygen ($\delta=4.3$) was 1:1:1.

Due to the liquid-like nature of compounds of 1-8d and 1-8e, the purification steps listed for 1-8(a-c) were not applicable. Instead, the products of 1-8d, and 1-8e were purified by anion exchange chromatography using MeOH in the presence of NaI. The reaction mixture was passed onto the anion exchange resin to immobilize the desired compound and to allow non-ionic species to be washed out. Then the column was eluted with NaI in MeOH to release the product in excess NaI. The NaI mixed in the products was removed by dissolving them in chloroform; undissolved NaI was filtered out to afford the pure products. Successfully purification of products 1-8d, and 1-8e were proved by $^1$H-NMR, which showed the correct 1:1:1 integration ratio between the aromatic ring protons and the methylene protons next to the ester oxygen. In addition, the methylene protons next to the ester oxygen presented the chemical shift of 4.3, which was downfield shift compared to the methylene protons in starting bromide compounds.

2.1.3: Converting sodium and potassium salts to tetramethylammonium salts

\[
\begin{align*}
\text{SO}_3\text{Na}^+ & \quad \text{Acetone, 24h, NMe}_4\text{Cl} & \quad \text{SO}_3\text{NMe}_4^+ \\
1-4(a-e) & & 1-5(a-e)
\end{align*}
\]
Aiming to improve the solubility of the sulfonate diester (sodium salt), and sulfonate monoester (potassium salt), the salts were converted to tetramethylammonium salts in the presence of NMe₄Cl using acetone as solvent. After stirring for 12 hours at room temperature, unreacted solid was filtered out, and the filtrate was evaporated under pressure to afford the final products with the yield of 20%-40%. The final compounds were confirmed by ¹H-NMR and mass spectrometry. The integration in the ¹H-NMR spectra showing the 12 protons for four methyl groups at 3.1 ppm, the 2 protons in the aromatic range at δ= 7.9 ppm, and another 2 protons in the aromatic ring at δ= 7.2 ppm confirmed the products were obtained; furthermore, the molecular ion mass values that were given by negative mode of ESI mass spectrometry, and given by calculation were presented on Table 1.
Table 1: Observed molecular ion masses for different compounds obtained by ESI Mass spectrometry and by calculation

<table>
<thead>
<tr>
<th>Compounds name</th>
<th>M/Z given by ESI mass spec</th>
<th>M/Z given by calculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-5a</td>
<td>637.80 amu</td>
<td>637.41 amu</td>
</tr>
<tr>
<td>1-5b</td>
<td>693.48 amu</td>
<td>693.93 amu</td>
</tr>
<tr>
<td>1-5c</td>
<td>947.93 amu</td>
<td>749.53 amu</td>
</tr>
<tr>
<td>1-5d</td>
<td>806.07 amu</td>
<td>805.60 amu</td>
</tr>
<tr>
<td>1-5e</td>
<td>745.93 amu</td>
<td>745.51 amu</td>
</tr>
<tr>
<td>1-8a</td>
<td>397.60 amu</td>
<td>397.21 amu</td>
</tr>
<tr>
<td>1-8b</td>
<td>425.67 amu</td>
<td>425.67 amu</td>
</tr>
<tr>
<td>1-8c</td>
<td>453.73 amu</td>
<td>453.27 amu</td>
</tr>
<tr>
<td>1-8d</td>
<td>481.73 amu</td>
<td>481.30 amu</td>
</tr>
<tr>
<td>1-8e</td>
<td>451.73 amu</td>
<td>451.25 amu</td>
</tr>
</tbody>
</table>

2.2 Solubility of sulfonate anionic lipids
A key factor in determining the efficiency of the microfluidic mixer is the mixing rate between components from the two inlet ports. The solvent in which lipid mixture is dissolved needs to be miscible with water and the lipids must be at a high enough concentration so that the aggregates produced are the most stable form available to the mixtures. The previous publications have demonstrated using ethanol as the preferred solvent for lipid mixture; thus, the solubility of synthetic anionic lipids and some natural lipids in ethanol was examined and the results are presented in Table 2. The fact that the solubility values for several of the synthetic sulfonate lipids are below 10 mg/ml, limits the extent to which these compounds can be explored in vesicles.
Table 2: solubility of some synthetic and natural lipids

<table>
<thead>
<tr>
<th>Lipids</th>
<th>Solubility (mg/ml) in Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>POPC(16:0-18:1 PC)</td>
<td>40</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>10</td>
</tr>
<tr>
<td>DSPE-mPEG</td>
<td>25</td>
</tr>
<tr>
<td>LysoPC18:0</td>
<td>&lt;10</td>
</tr>
<tr>
<td>SAdiC14:0&lt;sup&gt;b&lt;/sup&gt; (1-5a)</td>
<td>&lt;10</td>
</tr>
<tr>
<td>SAdiCbr20:0&lt;sup&gt;b&lt;/sup&gt; (1-5d)</td>
<td>10</td>
</tr>
<tr>
<td>SAdiC18:1&lt;sup&gt;b&lt;/sup&gt; (1-5e)</td>
<td>&lt;10</td>
</tr>
<tr>
<td>SAdiC14:0&lt;sup&gt;b&lt;/sup&gt; (1-8a)</td>
<td>&lt;10</td>
</tr>
<tr>
<td>SAdiCbr20:0&lt;sup&gt;b&lt;/sup&gt; (1-8c)</td>
<td>&lt;10</td>
</tr>
<tr>
<td>SAdiCbr20:0&lt;sup&gt;b&lt;/sup&gt; (1-8d)</td>
<td>10</td>
</tr>
<tr>
<td>SAdiC18:1&lt;sup&gt;b&lt;/sup&gt; (1-8e)</td>
<td>10</td>
</tr>
</tbody>
</table>

<sup>b</sup>This table listed two naming systems, and the naming system indicated as “b” will be used in the rest of the thesis. All the synthetic lipids are tetramethylammonium salts.
Chapter 3 Properties of vesicles formed by microfluidic mixing

The overall goal of this chapter is to characterize the products of the microfluidic mixer using natural and synthetic lipid mixtures. Subsections of 3.1 and 3.2 explore if the products are vesicles. The focus then moves to investigate the efficiency that lipids are incorporated into vesicles (section 3.3), and further studies entrapment efficiency using dyes as surrogates of drugs (section 3.4). The last section (section 3.5) includes the comparisons between the vesicles made by the conventional extrusion method and the products from the microfluidic mixer with respect to the permeability of the bilayers and the properties of the distribution of the products.

3.1 Are vesicles formed by microfluidic mixing? Size control via molecular parameters

The purpose of this section is to evaluate the products of the microfluidic mixer by the underlying theory of the molecular parameters in the control of vesicle size. The logic is if the products coming out from the microfluidic mixer are vesicles, and if the size of these vesicles can be predicted, then if changing the lipid mixture can lead to variations in vesicle size.

Lipids self-assemble into different morphologies driven by the hydrophobic effect between hydrocarbon chains and water, and by the hydrophilic and electrostatic properties of headgroups. The morphologies of lipid aggregates and the molecular parameters that control the aggregates formed were discussed in the introductory section. Furthermore, it has also been illustrated that lipid mixtures with complementary shapes could form bilayers, although a single lipid within the mixture might not form a bilayer by itself. The morphologies and size distribution of lipid aggregates are restrained by molecular parameters\textsuperscript{11}. The overall size of aggregates is controlled by hydrocarbon chain volume, cross sectional area and critical chain length of the lipids, which combine with the electrostatic repulsions between headgroups to produce a free energy-minimized aggregate\textsuperscript{5}. As discussed in the introduction, the shape parameter is defined as

\[ S = \frac{V_c}{a_o l_c} \]

To predict the properties of a single lipid and lipid mixtures, the terms of \( V_c \), \( l_c \) and \( a_o \) were obtained from partial atomic volume increments\textsuperscript{5}. The volume of the tail or the
headgroup was obtained by adding the partial molar volumes of the atoms present. The headgroup was assumed to be a sphere; hence, the cross-sectional area of the headgroup could be estimated. The fully extended chain length (in Å) was calculated using the equation \( l_c = 1.256 (n-1) + \text{(correction for cis alkene; -0.875)} + \text{(term for the terminal – CH}_3; +2.72) \), where \( n \) is the number of carbons in the chain. This equation assumed that the angle between carbon-carbon single bond is 109°. The critical chain length should account for the presence of gauche segments; thus, the value of \( l_c \) is 80% of the fully extended chain length\(^5\), \(^{20}\). The packing parameters for known single-chain lipids and double-chain lipids have been successfully predicted by this method, which agreed well with experimental values\(^5\). The structures for some natural lipids and synthetic lipids have been illustrated in the introductory section; the structure of the Peg lipid used in some experiments is given in Figure 3-1. A Peg lipid, which refers to a lipid that is conjugated with poly(ethyleneglycol) to anchor the surface of a liposome, is useful to stabilize the small size vesicles from self-aggregation\(^{14}\). The molecular parameters of the lipids explored experimentally were calculated and are presented in Table 3. The synthetized lipids have various shape parameters over a range from 0.7-2.1, which could be potentially applied to make vesicles with different sizes.

![DSPE-mPeg(Peg 2000)](image)

**Figure 3-1: The structure of DSPE-mPeg (Peg 2000) lipid**
Table 3: The calculated molecular parameters for different lipids

<table>
<thead>
<tr>
<th>Lipids</th>
<th>( l_c (\text{Å}) )</th>
<th>( a_o (\text{Å}^2) )</th>
<th>( V_c (\text{Å}^3) )</th>
<th>Shape parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>POPC</td>
<td>18.0</td>
<td>54.9</td>
<td>922</td>
<td>0.93</td>
</tr>
<tr>
<td>DSPE-mPEG</td>
<td>18.7</td>
<td>257.7(^a)</td>
<td>955</td>
<td>0.2</td>
</tr>
<tr>
<td>Chol</td>
<td>17.5</td>
<td>19.0</td>
<td>400</td>
<td>1.20</td>
</tr>
<tr>
<td>SAdiC14:0</td>
<td>15.3</td>
<td>41.2</td>
<td>775</td>
<td>1.23</td>
</tr>
<tr>
<td>SAdiC18:1</td>
<td>18.7</td>
<td>41.1</td>
<td>970</td>
<td>1.26</td>
</tr>
<tr>
<td>SAdiC(br20:0)</td>
<td>13.3</td>
<td>41</td>
<td>1097</td>
<td>2.01</td>
</tr>
<tr>
<td>SAmonoC(br20:0)</td>
<td>12.3</td>
<td>37.1</td>
<td>566</td>
<td>1.24</td>
</tr>
<tr>
<td>SAmonoC18:1</td>
<td>18.7</td>
<td>37</td>
<td>491</td>
<td>0.71</td>
</tr>
</tbody>
</table>

\(^a\) Calculated assuming the Peg headgroup adopts a hard sphere. This is unreasonable and the value is probably much smaller. This would act to increase \( S \) above the low value given.

In lipid mixtures, the \( S \) value for the mixture was computed as the molar weighted average of the \( S \) values of the component pure phospholipids\(^5\). In a binary mixture of phosphatidylcholines (\( S = 0.85 \) from experiment) and cholesterol (\( S = 1.2 \) estimated)\(^5\), experimental work shows that the vesicles increase in diameter as more cholesterol is added\(^4\). This is physically correct according to the shape parameter logic since increasing the cholesterol proportion leads to an increase in the weighted average shape parameter of the mixture. As \( S \) approaches 1, the curvature decreases and it leads to larger and larger vesicle size. Conversely in the experimental lysophosphatidylcholine (\( S = 0.42 \) from experiment\(^5\)) and phosphatidylcholine system, the diameter of the vesicles decreases; as the fraction of lysophosphatidylcholine in the vesicle is increased\(^4\). This is simply a result of the packing property between different lipids. In the pure lysophosphatidylcholine system, only small micelles are formed; however, in the pure phosphatidylcholine system, the vesicular structure is formed with a particular curvature\(^4\). By combining these two lipids, the vesicle aggregate is formed with an intermediate curvature hence an intermediate size\(^4\).

Quantitatively aggregate size is affected by molecular parameters, and the discussion in the introduction also shows that two lipids are distributed in an asymmetric way between the inner and outer leaflets of a bilayer\(^4,5,7\). Previous research has exhibited that the
vesicle size can be predicted for binary lipid mixtures based on a numerical minimization of the energetic and geometric parameters involved\textsuperscript{4}. The prediction of vesicle size was achieved by generating an Excel spreadsheet incorporating the method that Israelachvili developed\textsuperscript{4}. Additive partial molar volume values of $V$, $a_o$ and $l_c$ were used as inputs. The solver function in Excel was used to increase the vesicle size until the constraints of geometry, lipid asymmetric distribution, and curvature energy were satisfied. The method correctly reproduces the published data on the size of vesicles containing phosphatidylcholine/cholesterol and vesicles containing phosphatidylcholine/lysophosphatidylcholine\textsuperscript{4}, but is a brute force method and does not guarantee to find the global free energy minimum of the system.

Traditional methods for making vesicles suffer from a slow mixing speed which leads to a heterogeneous local lipid component concentration, which results in high sample heterogeneity, and batch to batch variability\textsuperscript{24, 30}. On the other hand, the PNI microfluidic mixing technique (NanoAssmblr ®) provides millisecond mixing at the nanoliter scale, with a short mixing time, and is claimed to enable the production of the equilibrium vesicle size with high uniformity and reproducibility\textsuperscript{27, 30-31}.

The reproducibility of the PNI microfluidic mixer was initially examined by making (assumed) vesicle samples with a constant lipid composition of molar percentage of 59\% of POPC, 40\% of cholesterol and 1\% of DSPE-mPeg in 10 mg/ml of initial lipid concentration in ethanol. The total flow rate of the microfluidic mixer was set to 12 ml/min with the lipid to buffer mixing flow rate ratio of 1:3. Five samples were prepared; the diameters and polydispersity index (PDI) were measured by dynamic light scattering (Figure 3-2). POPC was chosen owing to the low transition temperature (-2°C)\textsuperscript{33}; it is already in disordered liquid crystalline phase at room temperature so the vesicles would have a bilayer in a fluid disordered phase and there would be no interference from a gel (crystalline) lipid phase. Cholesterol was applied for vesicle formulation since previous research has demonstrated that cholesterol can improve the rigidity of bilayers\textsuperscript{1,4}. Figure 3-2 gives two experimental parameters—the apparent diameters and the error bars, which represent the experimental errors due to small uncertainty related to the statistical fitting. The error bar shows the standard deviation for triple measurements for a single sample. The figure demonstrates the good reproducibility between replicates due to the small
standard deviation value and close diameter value from each sample. The average
diameter for these five samples is (46.9±1.8) nm, and the average PDI for the samples is
0.24±0.08.

Figure 3-2: Replicate formation of vesicles from the NanoAssembler® microfluidic mixer.
Lipid composition: 59% POPC, 40% cholesterol, and 1% DSPE-mPeg; lipid concentration
10 mg/ml

Knowing the good reproducibility of the microfluidic mixer, the further experimental
work including investigating whether the microfluidic mixer makes nanoparticles or
vesicles was carried out. Geometric and packing restrictions could be applied to establish
the possible structures. Theoretically, the diameters of vesicles strongly depend on the
shape parameters, thus a change of lipid mixture should lead to a significant change in
diameters of vesicles. On the other hand, lipid composition and concentration have only a
limited effect on a solid nanoparticle size due to the volume increase for adding another
molecule to a particle that translates to the cube root of the volume increment as an
increment in the particle diameter\(^{16}\). From the theories related to the prediction of vesicles
size that Israelachvili developed\(^4,6\), the diameters of vesicles can be calculated based on
binary lipid mixture compositions. The experimental measurements were performed in
tertiary mixture of POPC and cholesterol, and DSPE-mPeg lipid, where the Peg lipid was
held in constant 3% molar percentage. Assuming that contribution of the Peg-lipid is
small due to the small amount and low predicted shape parameter value, the predicted
diameter value for the vesicles can be calculated based only on the POPC and cholesterol
ratio.
The experimental results are presented in Figure 3-3. The column presents the average diameter of vesicle from triple measurements, and the error bar was obtained based on the standard deviation associated with three measurements for a sample from dynamic light scattering. As the amount of cholesterol increased from 10 mol% to 30 mol%, the diameters measured increased, which was in agreement with a simple analysis. In the presence of 10% cholesterol, 87% POPC and 3% DSPE-mPeg, the vesicle had the size of 43 nm, and the vesicle size increased to 56.9 nm as cholesterol amount increased to 25%, and the PDI increased over the same range. In the lipid mixture of 10% cholesterol, 87%POPC, and 3% DSPE-mPeg, the shape parameter of the system is 0.93. Figure 3-3 showed that when shape parameter of a system is less than 0.93, the predicted value is always larger than the experimental value, which suggests the theory is headed to larger products than the microfluidic mixer produced, and if the products from the microfluidic mixer are vesicles, they should be more curved than prediction. In the mixture of 25% cholesterol and 72% POPC, and 3% DSPE-mPeg, the calculated shape parameter of the system is 0.98; hence, as shape parameter of the system is close to 1, the system is getting up to the point where the curvature of vesicles is decreased so the size is getting larger. At this point, the solver could not find a suitable value for the system. However, the microfluidic mixer still produced some particles for the lipid mixture system having shape parameter larger than 1.

To conclude, the trend in the size is consistent with the device making vesicles as the vesicle size does increase with an increase in S, but the vesicles made by the NanoAssemblr® microfluidic mixer may not be the same as would be expected by conventional methods. One possibility is that these smaller vesicles are multi-lamellar. Another possibility is that the vesicles are accompanied by small nanoparticles, which was confirmed by the MSD (Mean Square Displacement) summary table in dynamic light scattering, which showed a minor portion of aggregates having diameter about 10-20 nm.
A second set of experiments was carried out to explore the effect of a combination of synthetic lipids with commercially available lipids. Single chain SAmonoC18:1 was co-formulated with commercially available lipids cholesterol, DSPE-mPeg and POPC. In different mixtures, POPC and DSPE-mPeg were held constant at 72 mol%, and 3 mol% respectively, and the sum of cholesterol and SAmonoC18:1 was 25 mol% with a variable amount of SAmonoC18:1 plotted in Figure 3-4. The range of PDI in these measurements is small from 0.218-0.272, and the standard deviations which were presented as error bars on the columns are also small. The experimental results exhibited that replacing a high S cholesterol component with a low S SAmonoC18:1 component led to shrinkage in the aggregate size, which was expected since reducing the shape parameter in the lipid mixture system led to a decrease in diameter.
Previous results showed that vesicle size, which strongly depends on molar averaged shape parameters of the system, increases with increasing the shape parameter of pure lipids. The substitution of the low shape parameter SAmonoC18:1 with high shape parameter SAdiCbr20 was then explored in another set of vesicles. The high shape parameter of SAdiCbr20 (S=2.01) was assumed to lead to its preferential packing in the inner leaflet. Holding the amount of cholesterol and DSPE-mPeg constant at 25% and 3% respectively, and the sum of POPC and SAdiCbr20 was 72% with a variable amount of SAdiCbr20 plotted in Figure 3-5. The Figure 3-5 presented the vesicle diameters for lipid mixtures with increasing amount of SAdiCbr20. The experimental trend is that as the amount of SAdiCbr20 increases, an increase in diameter is observed. The PDI values associated with these measurements are large (0.235-0.365), indicating the wide distribution of aggregate size. Since the shape parameter of the lipid mixture of 2% SAdiCbr20 is 1.02, which is over shape parameter limit of forming vesicles, another set of experiment was performed with the shape parameter value in each experiment was less than 1. Figure 3-6 showed the sizes of samples with constant POPC at 90%, the sum of cholesterol and SAdiCbr20 was 10% with variable amount of SAdiCbr20. The experimental results presented that addition of high shape parameter lipid of SAdiCbr20
to the system led to increase in vesicle size, which was expected since the higher shape parameter resulted in larger S for the mixture so a larger diameter, in addition, the PDI values of these measurements were increasing from 0.238 to 0.391 as the amount of SAdiCbr20 increased from 2% to 8%.

Figure 3-5: Vesicle diameter from the NanoAssemblr® microfluidic mixer for lipid mixture with increasing proportion of SAdiCbr20. Lipid composition: 25% cholesterol, 3% DSPE-mPeg, POPC = (72% -SAdiCbr20), lipid concentration 10 mg/ml

Figure 3-6: Vesicle diameter from the NanoAssemblr® microfluidic mixer for lipid mixture with increasing proportion of SAdiCbr20. Lipid composition: 90% POPC, cholesterol = (10% -SAdiCbr20), lipid concentration 10 mg/ml
Notably, in the previous figures, the x-axis at “0” represented the same lipid composition (25% chol, 3% DSPE-mPeg, 72% POPC), but produced vesicles with very different sizing. One possibility would be that there is a batch-to-batch difference of microfluidic chips that resulted in the same lipid composition vesicles having different sizes. Figure 3-7 shows that the vesicles made by different chips have various diameters although they have the same lipid composition. The results clearly showed that chips had an effect on vesicle size.

![Figure 3-7: The reproducibility of microfluidic cartridges. Lipid composition: 72% POPC, 25% cholesterol 3% DSPE-mPeg. Lipid concentration 10 mg/ml](image)

To sum up, all the results presented above show that lipid composition has a profound effect on aggregate sizing, which is consistent with the geometric argument of the shape parameter; hence, this suggests that the structures formed are mainly vesicles. All sets of experimental work show the same trends that the diameters of vesicle samples increased with increasing the shape parameters of the systems, the value of PDI of the system gets larger when the value of the shape parameter of the system is close to 1. As shape parameter of the system approaches 1, the curvature of the vesicle decreases, and a flat bilayer is formed. On the other hand, the predicted values for binary systems are larger than the experimental measurements. A better fit of the predicted results with experimental results might be obtained by considering the shrinkage effect on the vesicle size by the Peg-lipid. The difference between experimental results and predicted results might also suggest that there were not only vesicles but also some smaller aggregates or
nanoparticles formed during microfluidic mixing process. A given chip exhibits good reproducibility on the sequential use on the same lipid composition; however, the different chips produce vesicles with different sizes although the same lipid composition is used.
3.2 Are vesicles formed? Cyro-TEM imaging

The goal of this part of the work is to find out what the microfluidic mixing device makes by direct cryo-TEM imaging. The combination of electron microscopy with the cryo-sample preparation method provides a simple and convenient way to directly observe biological tissues or other aggregate structures without disturbing hydrated structures. The principle of cryo-sample preparation is to cool samples fast enough that nanoparticles are trapped in glassy ice. The Scanning Transmission Electron Holography Microscope (STEHM), which is located at the University of Victoria, is the highest resolution microscope ever constructed and has a suitable cryo-probe for these experiments.

The vesicle sample consisting of molar percentage of 59% POPC, 40% cholesterol, and 1% DSPE-mPeg with an initial lipid concentration of 10 mg/ml was prepared. The phosphate buffer contained 0.1 M NaI instead of NaCl to increase the contrast on the microscope. The vesicle sample was dialyzed against buffer solution with 0.1 M NaI over 18 hours to completely remove ethanol. That the potassium dichromate orange color remained after adding a vesicle sample indicates there is no ethanol in vesicle sample. After dialysis, the vesicle sample had the size of 45.2 nm with PDI of 0.213.

In order to prepare the cryo EM sample, 4 µL of 5 nm gold nanoparticle standard solution was added to 12 µL of vesicle sample to get enough solution for four grid samples. After mixing the solution, 3.8 µL of sample was placed on an EM grid, which is held at the bottom of a plunger piston by a tweezer. Under the control of the cryo-sample instrument, a piece of filter paper is pressed against the sample for 4 seconds to wick out the extra solution, followed by the immediate drop of the plunger with the grid sample into a liquid ethane chamber, which is inside a liquid nitrogen reservoir. This quickly freezes the water in the sample to a glass and prevents the formation of cubic ice. The temperature of liquid nitrogen reservoir should be always kept at approximately -190 °C. Once the grid is cool, the sample grid is gently and quickly touched with a piece of filter paper on the wall of liquid nitrogen reservoir to remove ethane, and then the grid is transferred to a grid box under liquid nitrogen. At the microscope the grid is transferred at liquid nitrogen temperatures to the cryo-probe.
Both images in Figure 3-8 show an obvious clump from vesicle samples, which are similar to the majority of images observed. The aggregates have a size larger than 200 nm, which was far from the results obtained from dynamic light scattering, and indicated that the vesicles were damaged in the sample preparation. One possibility is that the hydrophobic lacey carbon grid attracts and binds the vesicles to the grid to form large aggregates. Another possibility could be that the long wicking process led to vesicle dehydration and the formation of large aggregates as a result of concentrating the solution.

Figure 3-9 demonstrates more clearly that vesicle-like aggregates are associated on the lacey carbon grid. These are larger than 200 nm in diameter, which indicates that hydrophobic vesicles were damaged by the hydrophobic lacey carbon grid when migrating to the grid. However, in other regions of this same sample, there were small vesicles present with small diameters. Figure 3-10 shows a few intact vesicles with general spherical structure having size of 50 nm, and the vesicle size indicated by scale bar on the image was consistent with the result from dynamic light scattering.
A survey of the samples showed that there were not only vesicles but also other aggregates with the same lipid composition existing in the mixture. Many images showed the large aggregates rather than individual vesicles having size about 50 nm, which indicated that the majority of vesicles were unstable and destroyed as lipids migrated to the grid and clumped together. Due to this situation, the cryo-TEM method based on
lacey carbon grids is not a useful analysis tool to indicate what the intact particles were like in solution. The sample preparation method used breaks up the sample, but the leftovers look like they were vesicles once.
3.3 Efficiency of incorporation of lipids into vesicles
The goal of this part of the work is to investigate the efficiency of incorporation of different lipids into vesicles, which in turn requires determining the amount of POPC, SAdiCbr20 and SAmonoC18:1 in vesicle fractions. The vesicle samples with different lipid compositions were prepared, and the vesicle mixture was separated by size exclusion chromatography. The vesicles fractions were identified by dynamic light scattering (DLS), and the lipids were detected and quantified by HPLC.

3.3-1: phospholipid analysis
The vesicle sample composed by POPC/ Cholesterol/DSPE-mPeg in the molar ratio of 59%: 40%: 1%, was prepared by the NanoAssmblr ® microfluidic mixer in the flow rate of 12 ml/min with lipid to buffer flow rate ratio of 1:3. The diameter of sample was 42.8 nm. The sample was dialyzed against HEPES buffer to remove EtOH, completely remove of EtOH was verified by remaining orange color after the addition of the potassium dichromate, and the vesicle size was 45.7nm after dialysis. Separation of vesicle mixture was achieved by size exclusion chromatography, which separates different species based on the size of molecules. Species with large molecular weight are eluted first, and small molecules are eluted in later fractions. In order to achieve effective separation, the pores of the gel should be in a range that allows some molecules enter while large molecules are unable to enter any of the pores. Due to exclusion from the pores, large particles come out from the column first, and smaller particles permeate pores, and elute in the reverse order of size.

The chromatogram was recorded at 200 nm-600 nm by a photodiode array detector in the time period of 30 minutes (Figure 3-11). The fractions were collected in 0.5 ml/tube at the buffer (0.01 M Na3PO4, 0.1 M NaCl, pH= 6.4), flow rate of 1 ml/minute. The diameter of each fraction was measured by DLS, and the results are presented in Figure 3-11. The upper panel in Figure 3-11 shows the apparent absorbance over all wavelengths (200-600 nm), and the bottom is the diameter for each fraction measured by DLS. The peak at time about 3.5 minutes in the chromatogram is not due to absorbance, since the components do not have a significant chromophore, but is due to scattering by the aggregates which are assumed to be vesicles in the effluent from the column. The DLS shows a few fractions with large diameters at the beginning, then, the minimum
diameters for the vesicle fractions, and bigger diameters particles to follow. This is contrary to expectation from the gel permission column of small species coming out last and could be explained as a few: large species with very low concentration that were coming out of the column first. Due to the low concentration they scattered light at very low intensity so did not appear in the upper panel. This was followed by the small size vesicles with high concentration being eluted from the column. The single molecule-lipids come along in the later fractions, and they were too small to scatter the light as they pass the detector. However once out of the column, they can sit in the tube, and form poorly defined structures that might not be very stable at all but could be detected by DLS as large scattering particles. On the other hand, if they were forming big aggregates on the column with stable structures, they could scatter light and should have given a peak on the chromatogram. DLS, which studies the fluctuation in light intensity, is able to detect material at very low levels; the detector on the gel column is relatively less sensitive to scattering and is optimized for transmission.

A common and simple method to determine the phospholipid content is the formation of a phosphomolybdate complex by the addition of molybdate (“chromogenic acid”), following with reduction by mercury to yield a light absorbing species, which could be read at 710 nm by UV-visible spectrophotometer. The system obeys Beer-Lambert law at the low concentration range, so the intensity of the blue color is proportional to the amount of phospholipid present. A calibration curve was obtained by plotting absorbance as a function of phospholipids concentration. The procedure use for phospholipids was developed by previous graduate student in Dr. Fyles’group at the University of Victoria.

The analysis of the total lipids was done on the tubes collected. Destruction of the vesicles in each tube was achieved by evaporating the HEPES buffer by a passing air flow. The dried vesicle samples then were re-dissolved in 1ml of chloroform to completely disturb the vesicle structure. Undissolved solid HEPES was filtered out by gravity filtration. Chromogenic acid solution was added to the disturbed vesicle fractions, and then the sample in each fraction was heated for 1 minute at 100 °C. Chloroform was added to each sample after the sample cooled to room temperature. The samples were shaken for 30 seconds and after 30 minutes the absorbance at 710 nm was recorded (Table 4).
Figure 3-11: Fraction of a vesicle sample by gel filtration chromatography. The upper panel shows the chromatogram detected from 200 nm-600 nm for vesicle samples composed of POPC/Cholesterol/DSPE-mPeg in the molar ratio of 59%: 40%: 1%. The lower panel presented the diameters for collected fractions measured by light scattering. See the text for a discussion.
There is a volume lost due to starting and stopping the flow that results in a loss of material. Only 77% of the initial material is present in the collection tube from the microfluidic mixer; hence, the total amount of POPC collected from the microfluidic mixer was 2.772 mg in 1.55 ml. From 1.55 ml of collected sample, 1 ml of the sample was injected to size exclusion chromatography; thus, the amount of phospholipid (POPC) injected to size exclusion chromatography was 1.85 mg and the amount recovered in the vesicle fraction was 1.022 mg, so the efficiency of POPC incorporation in this sample could be calculated as 55%. The less than 100% efficiency indicated that there was a loss during the vesicle preparation and vesicle separation processes. One possibility for lipid loss is the formation of very large aggregates, another possibility could be some lipids do not incorporate into vesicles; hence, they eluted from column as single lipids.

### 3.3-2: Sulfonate lipid analysis

The previous discussion demonstrated that there was a small amount phospholipid lost during vesicle preparation and separation processes. The investigation continues to find the loss of sulfonate lipids. The vesicle sample having size of 65.2 nm was prepared in the lipid composition of POPC/cholesterol/DSPE-mPeg/SAdiCbr20 in the molar ratio of 53%:40%:1%:6%. The sample was separated by size exclusion chromatography, and the chromatogram was recorded at 200 nm-600 nm by a photodiode array detector in the time period of 30 minutes. The fractions were collected in 0.5 ml/tube at the solvent flow rate of 1 ml/minute. Vesicle fractions about 3.5 minutes were identified as above by a strong peak due to scattering. The diameters were measured and the data is presented in Figure 3-12. These vesicle fractions were combined, and destruction of the vesicles was achieved by evaporating the phosphate buffer and re-dissolved the dried sample in 50% MeOH and 50% ACN mixture solvent. Quantitative analysis of sulfonate lipid in vesicle

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Diameters (nm)</th>
<th>Absorbance</th>
<th>Amount (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tube 9</td>
<td>44.7</td>
<td>0.375</td>
<td>0.251</td>
</tr>
<tr>
<td>Tube 10</td>
<td>66.2</td>
<td>0.419</td>
<td>0.282</td>
</tr>
<tr>
<td>Tube 11</td>
<td>68.9</td>
<td>0.460</td>
<td>0.308</td>
</tr>
<tr>
<td>Tube 12</td>
<td>65.2</td>
<td>0.269</td>
<td>0.181</td>
</tr>
</tbody>
</table>
fractions was achieved by isocratic HPLC on a C18 column with mobile phase of 90% ACN and 10% water with both solvent having 1% TFA. The signal was detected at the wavelength of 222 nm (Figure 3-12). There was no chromophore on the other co-formulated lipids in the vesicle; hence, the peak at 2.05 minutes in the chromatogram was the peak for SAdiCbr20. A blank sample of 90% ACN and 10% water was injected to confirm that the negative peak showing right before the SAdiCbr20 peak was not from either vesicle sample or standard solutions. The calibration curve for SAdiCbr20 was prepared (Appendix C). The total SAdiCbr20 used for the sample preparation was 0.44 mg. There is a volume lost due to starting and stopping the flow that results in a loss of material. Only 77% of the initial materials present in the collection tube from the microfluidic mixer. The lipid collected from the microfluidic mixer was 0.385 mg in 1.55 ml, so the concentration of SAdiCbr20 was 0.248 mg/ml from the mixer. Based on the integration from the chromatogram and the calibration curve, the final concentration of SAdiCbr20 was 0.123 mg/ml; hence, the approximate efficiency of SAdiCbr20 incorporation into vesicle was 50%. The less than 100% efficiency suggests there was loss of SAdiCbr20 during the processes.

Figure 3-12: HPLC Chromatogram of the combined vesicle fractions after gel filtration showing the presence of SAdiCbr20:0 as detected at 222 nm. The vesicle sample was composed of POPC/cholesterol/DSPE-m-Peg/SAdiCbr20 in the molar ratio of 53%:40%:1%:6% with initial lipid concentration of 10 mg/ml.
The HPLC separation of SAdiCbr20 in water and ACN mixed solvent is broad and makes it difficult to accurately determine the efficiency of this sulfonate lipid incorporation into vesicles. The vesicles were prepared in the lipid composition of POPC/cholesterol/DSPE-mPeg/SAmonoC18:1 in the molar ratio of 72%:10%:3%:15% with initial lipid concentration of 10 mg/ml, and the vesicle having size of 32.8 nm. As previously, the vesicle sample was separated by size exclusion chromatography, and the sizes of collected fractions were measured by dynamic light scattering, the vesicle fractions were combined, and the amount of sulfonate lipid was determined by HPLC. The signal was detected at the wavelength of 254 nm (Figure 3-13). SAmonoC18:1 had the retention time of 3.29 minutes, which was confirmed by the calibration curve of SAmonoC18:1 generated on the same conditions. The total amount of SAmonoC18:1 used for the vesicle preparation was 0.492 mg, and the amount collected from microfluidic mixer was 0.381 mg in 1.55 ml, thus, the concentration of SAmonoC18:1 in the sample could be calculated as 0.246 mg/ml. Based on the integration from the chromatogram and the calibration curve generated, the concentration of SAmonoC18:1 in the vesicle fractions could be determined as 0.0621 mg/ml; thus, the efficiency for SAmonoC18:1 incorporation into vesicle was 25%.

![Figure 3-13](image.png)

**Figure 3-13:** HPLC Chromatogram of the combined vesicle fractions after gel filtration showing the presence of SAmonoCbr18:1 as detected at 254 nm. The vesicle sample was composed of POPC/cholesterol/DSPE-m-Peg/SAmonoC18:1 in the molar ratio of 72%:10%:3%:15% with initial lipid concentration of 10 mg/ml.
To sum up, the vesicle sample was successfully separated from other aggregates by size exclusion chromatography, and dynamic light scattering results implied the presence of small vesicles. The lipids of POPC, SAdiCbr20, and SAdiC18:1 were able to incorporate into vesicle fractions, while less than 100% incorporation efficiency indicated there was loss during preparation and purification processes.
3.4: Dye entrapment

Previous sections have exhibited that vesicle size could be controlled by variation of lipid compositions, and the efficiency of microfluidic mixing device on making vesicles could be as high as 55%. In this part, the experimental work will be continued to find out the entrapment efficiency, in terms of determining the total amount of a compound initially in either the buffer or ethanol fractions that gets incorporated into the vesicle fractions. In order to study entrapment efficiency, the first question is if the dyes were entrapped, then a quantitative analysis of entrapment efficiency was explored.

3.4-1: Were dyes entrapped?
Awareness that colored dyes are easy to detect and quantify leads to the idea of using hydrophobic and hydrophilic dyes as surrogates for drugs to explore how the microfluidic mixer behaves in making different types of vesicles with different loads. In this part three dyes -neutral red, HPTS and Nile red- are used as examples of hydrophilic and hydrophobic compounds to investigate entrapment efficiency; the structures of these dyes are illustrated in Figure 3-14.

![Neutral red—basic form](image)

![Neutral red—acidic form](image)

![Nile red](image)

![HPTS](image)

Figure 3-14: structures of dyes
3.4-1-1: Dye entrapment—neutral red

Neutral red has $pK_a = 6.81$ and acts as a sensitive pH indicator in the pH range of 6-8 due to the intense color changes from red to yellow as neutral red is deprotonated in basic solution$^{38}$. Neutral red shows a maximum absorbance at about 530 nm in acidic solution (molar extinction coefficient, $\varepsilon = 2.75 \times 10^4$), and the maximum absorbance at 452 nm in basic solution (molar extinction coefficient, $\varepsilon = 1.39 \times 10^4$)$^{38}$. In a buffer containing 0.1 M NaCl and 0.01 M Na$_3$PO$_4$ at pH=6.4, neutral red exists in the solution predominantly as the protonated cationic species. Different concentrations of neutral red were obtained by dissolving a specific amount of neutral red in buffer, and then, the vesicle samples were formulated by microfluidic mixing of the lipid mixture and buffer solutions containing different concentration of neutral red. The lipid mixture was prepared with the composition of 53% POPC, 40% Cholesterol, 1% DSPE-mPeg and 6% SAdiCbr20 with an initial lipid concentration of 10 mg/ml. The diameters for vesicles samples were measured by dynamic light scattering, and the results were presented in table 5.

The external dye was removed by size exclusion gel chromatography. The chromatogram was recorded using a photodiode array detector at wavelengths from 200 nm to 600 nm (Figure 3-15). The advantage of the photodiode array acquiring data at a wide range of wavelengths is that single wavelength data can be also pulled from the signal. The chromatograms at the single wavelengths of 536 nm and 285 nm are of interest since neutral red and SAdiCbr20 show maximum absorbance at these wavelengths respectively. Fractions coming out of the column were collected at the rate of 0.5 min/tube in the solvent flow rate of 1 ml/min and the diameter for each fraction was measured by dynamic light scattering (data not shown; small diameter vesicles are associated with the peak at about 3.5 minutes).
Table 5: The diameters for vesicles containing different concentrations of neutral red dyes

<table>
<thead>
<tr>
<th>concentration of neutral red in buffer</th>
<th>0.05 mM</th>
<th>0.1 mM</th>
<th>0.2 mM</th>
<th>0.3 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>diameters</td>
<td>64.5 nm</td>
<td>61.2 nm</td>
<td>67.5 nm</td>
<td>65.7 nm</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>0.71</td>
<td>1.3</td>
<td>0.38</td>
<td>0.95</td>
</tr>
</tbody>
</table>
Figure 3-15: Chromatogram of vesicles prepared using different concentrations of neutral red in the initial aqueous buffer detected at 536 nm. The vesicle samples were composed of POPC/Cholesterol/DSPE-PEG/SAdicbr20 at molar ratios of 53%: 40%: 1%: 6%
Figure 3-15 shows the chromatograms for vesicles prepared with neural red concentration varied from 0.05 mM to 0.3 mM. Neutral red was incorporated into what appear to be the vesicle fractions at about 3.5 minutes up to 0.2 mM initial concentration but at initial concentration of 0.3 mM the dye was not associated with the early part of the chromatogram but eluted much later as if it was associated with a large species.

Figure 3-16 and Figure 3-17 show the results for vesicle samples with 0.2 mM and 0.3 mM of neutral red respectively that allow this issue to be explored more fully. In Figure 3-16 (0.2 mM neutral red initially), the peak in the chromatogram at a time about 3.5 minutes as detected at 536 nm is also found in the total chromatogram (200-600 nm) and in the chromatogram detected at 285 nm. The diameter of each fraction showed that the small diameter fractions assumed to be vesicles correspond to the fractions that collected at time about 3.5 minutes. The chromatogram detected at 285 nm showed that SAdiCbr20 was incorporated into vesicle fractions. In order to verify that the species detected at 285 nm was the SAdiCbr20, electrospray mass spectrometry in negative mode was used to monitor the presence of SAdiCbr20 (molecular weight of the anionic form is 806 g/mol) in different fractions. In these samples a constant amount of sodium 1-octanesulfonate (molecular weight of the anionic form is 193 g/mol) was added to every fraction as an internal standard to quantitatively trace the amount of SAdiCbr20 in the fractions. The ESI-mass spectrometry result was presented in Figure 3-16 as a ratio of the SAdiCbr20/octanesulfonate ion intensities and showed that the anionic lipid was well incorporated into the vesicle fractions, and there was no anionic lipid observed in the later fractions. All of these observations exhibited that microfluidic mixer was able to generate vesicles containing hydrophilic dye neutral red when neutral red initial concentration was 0.2 mM.

At a higher neutral red concentration (0.3 mM), the total chromatogram showed the scattering peak at a time about 3.5 minutes (Figure 3-17), which was consistent with the chromatogram obtained from the vesicle samples with 0.2 mM of neutral red. The results from DLS showed the small aggregates diameters on the fractions collected at a time about 3.5 minutes, which were assumed to be vesicles. The peak on the chromatogram detected at 285 nm indicated SAdiCbr20 was associated with the vesicle fractions. All the observations suggested that the vesicles were able to form with the buffer containing 0.3
mM of neutral red. However, the later shifted peak on the chromatogram detected at 536 nm implied neutral red was not associated with the vesicles. One possibility could be that at 0.3 mM the neutral red solubility was exceeded in the combined ethanol and buffer mixing solution. In this case, during mixing the dye precipitated and when the mixture was transferred to the column it re-dissolved and ran only in the later fractions.
Figure 3-16: Fractionation of samples prepared at an initial neutral red concentration of 0.2 mM. Panel A= chromatogram detected at 536 nm, panel B= chromatogram detected at 285 nm, panel C= total chromatogram detected from 200 nm- 600 nm, panel D= DLS analysis of the diameters of the aggregates in different fractions. Panel E= the ESI-MS analysis of SAdiCbr20 in different fractions reported as a ratio of the molecular ion relative to octasulfonate as internal standard. The vesicle sample was prepared in the composition of 53% POPC, 40% Cholesterol, 1% DSPE-mPeg and 6% SAdiCbr20.
Figure 3-17: Fractionation of samples prepared at an initial neutral red concentration of 0.3 mM. Panel A= chromatogram detected at 536 nm, panel B= chromatogram detected at 285 nm, panel C= total chromatogram detected from 200 nm- 600 nm, panel D= DLS analysis of the diameters of the aggregates in different fractions. Panel E= the ESI-MS analysis of SAdiCbr20 in different fractions reported as a ratio of the molecular ion relative to octasulfonate as internal standard. The vesicle sample was prepared in the composition of 53% POPC, 40% Cholesterol, 1 %DSPE-mPeg and 6% SAdiCbr20.
3.4.1-2: Dye entrapment—Nile red

Nile red is widely used in staining lipids for histology to estimate the content of lipids in cellular structures\(^{39,39b}\). Nile red is a lipophilic dye that exhibits solvatochromism; that is, its absorbance maximum wavelength varies with a change of solvent polarity\(^{39a}\). The dye is soluble in a wide range of organic solvents; however, it has low solubility in aqueous medium, as a result, selectively binding to lipids will occur when applying Nile red to vesicle samples in buffer solution. Nile red shows strong binding to phospholipids, cholesterol, and cholesteryl esters\(^{39,40}\), and it is not only soluble in organic solvents, but also exhibits strong fluorescence. Previous publications suggest that the excitation and emission wavelengths for Nile red are 549 nm and 628 nm respectively in aqueous solution\(^{41}\). The vesicle samples generated by the microfluidic mixer were in the medium of 75% buffer and 25% ethanol, and the UV-visible spectrum showed the maximum wavelength of 549 nm when Nile red is in the medium of 75% buffer and 25% ethanol.

Nile red solutions with different concentrations were prepared in ethanol, and then, a specific amount of Nile red was added to a constant lipid mixture in ethanol. The concentration range of Nile red from 0.1-0.3 mM in ethanol was achieved with the lipid mixture of 53% POPC, 40% Cholesterol, 1% DSPE-mPeg and 6% SAdiCbr20 at an initial lipid concentration of 10 mg/ml. The vesicle samples were prepared by the microfluidic mixer at the total flow rate of 12 mg/ml with the flow rate ratio of lipid mixture to buffer of 1:3. The diameters of vesicles with different Nile red concentrations were measured, and the results were shown on Table 6. The fractions were collected in 0.5 ml/min in the solvent flow rate is 1 ml/min, and the diameter for each tube was measured by dynamic light scattering. The unbound dye was removed by size exclusion chromatography, the chromatogram was recorded from 200 nm to 600 nm and the chromatograms at 549 nm and 285 nm were selected. Figure 3-18 and Figure 3-19 give the results of the vesicle sample with 0.2 mM Nile red and 0.1 mM Nile red respectively.

**Table 6:** Vesicles’ diameters with different concentration of Nile red

<table>
<thead>
<tr>
<th>Nile red concentration</th>
<th>0.1 mM</th>
<th>0.2 mM</th>
<th>0.3 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vesicle’s diameter</td>
<td>47.9 nm</td>
<td>43.0 nm</td>
<td>48.4 nm</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>2.3</td>
<td>2.6</td>
<td>1.6</td>
</tr>
</tbody>
</table>
Figure 3-18: Fractionation of samples prepared at an initial Nile red concentration of 0.2 mM. Panel A= chromatogram detected at 549 nm, panel B= chromatogram detected at 285 nm, panel C= total chromatogram detected from 200 nm- 600 nm, panel D= DLS analysis of the diameters of the aggregates in different fractions. Panel E= the ESI-MS analysis of SAdiCbr20 in different fractions reported as a ratio of the molecular ion relative to octasulfonate as internal standard. The vesicle sample was prepared in the composition of 53% POPC, 40% Cholesterol, 1% DSPE-mPeg and 6% SAdiCbr20.
The results from DLS showed the small aggregates diameters on the fractions collected at a time about 3.5 minutes, which were assumed to be vesicles. The chromatogram at 549 nm showed a peak at time about 3.5 minutes, which suggested that Nile red was able to stain the vesicle samples made by the microfluidic mixer; furthermore, the peak at time about 3.5 minutes on the chromatogram detected at 285 nm indicated that anionic lipid SAdiCbr20 was incorporated into vesicles. However, quantitative analysis by ESI mass spectrometry indicated that there was only a small amount of sulfonate anionic lipid in the vesicle fractions, which implied that hydrophobic compounds expelled the anionic lipid from the vesicle fractions. The anionic lipid was not detected in the later fractions, but was expected to be at low levels in most of the later fractions and is therefore undetectable by the method used.
Figure 3-19: Fractionation of samples prepared at an initial Nile red concentration of 0.1 mM. Panel A= chromatogram detected at 549 nm, panel B= chromatogram detected at 285 nm, panel C= total chromatogram detected from 200 nm- 600 nm, panel D= DLS analysis of the diameters of the aggregates in different fractions. Panel E= the ESI-MS analysis of SAdiCbr20 in different fractions reported as a ratio of the molecular ion relative to octasulfonate as internal standard. The vesicle sample was prepared in the composition of 53% POPC, 40% Cholesterol, 1% DSPE-mPeg and 6% SAdiCbr20.
In order to confirm that hydrophobic compounds expelled the anionic lipid from the vesicle fractions, Figure 3-19 summarized the results of the vesicle samples with lower concentration (0.1 mM) of Nile red entrapped. The results from DLS showed the presence of vesicles on the fractions collected at a time about 3.5 minutes. That Nile red was able to incorporate into vesicle bilayers was confirmed by the peak at a time about 3.5 minutes in chromatogram detected at 549 nm. ESI- mass spectrometry result verified that SAdiCbr20 was not able to incorporate into vesicle fractions even at very low concentration of Nile red. To conclude, hydrophobic compound Nile red expelled sulfonate lipid from the vesicle fractions.

3.4-1-3: Dye entrapment—HPTS
Another hydrophilic dye HPTS (8-Hydroxypyrene-1,3,6-Trisulfonic Acid, Trisodium Salt) was used to further investigate how the microfluidic mixer handles more highly charged hydrophilic compounds. HPTS is a well-studied water-soluble dye for vesicle entrapment. The protonated and deprotonated forms of HPTS absorb at 403 nm and 455 nm respectively in aqueous medium. Beside the absorbance property, HPTS is also widely applied to investigate the stability of vesicles for its pH sensitive fluorescence property. HPTS can be self-quenched at high concentration, but at low concentration the protonated and deprotonated species of HPTS have excitation wavelengths at 403 nm and 460 nm respectively, and both species emit at 510 nm.

Different concentrations of HPTS in phosphate buffer solution were prepared. The vesicle samples were composed of POPC/ Cholesterol/ DSPE-mPeg/ SAdiCbr20 with the molar ratio of 53%: 40%: 1%: 6% in the initial lipid concentration of 10 mg/ml. The vesicle samples were prepared by microfluidic mixer at the total flow rate of 12 mg/ml with the flow rate ratio of lipid mixture to buffer of 1:3. The diameters for vesicles samples with different initial amount of HPTS were presented in Table 7. The removal of free HPTS dye and separation of aggregates with different sizes were achieved by size exclusion chromatography. Figure 3-20 presents the vesicle sample with the HPTS dye concentrations from 0.05-0.3 mM. The chromatogram at 455 nm was selected since HPTS shows the maximum absorbance at this wavelength in the 1:3 ethanol: buffer medium. As the concentration of HPTS increases, apparently only portion of the dye was entrapped and free HPTS was seen in all preparations. By doing a ratio of the two peak
areas, the entrapment efficiency could be directly estimated. In the presence of 0.05 mM of HPTS, the ratio between the area of vesicle fractions and the areas of non-entrapped dye is 1.2:0.8; however, as dye concentration increases to 0.1 mM, the integration area of non-entrapped dye is larger than the integration area of entrapped dye, which indicates that the entrapment efficiency might be decreased at high HPTS concentration.

Figure 3-21 presents the more detailed analysis of vesicle sample with 0.2 mM HPTS. The results from DLS showed the presence of vesicles on the fractions collected at a time about 3.5 minutes, and the peak at a time about 3.5 minutes on the chromatogram detected at 455 nm indicated that only small portion of HPTS was entrapped. In addition, the chromatogram at 285 nm showed the small absorbance at a time after 6 minutes, however, ESI mass spectrometry was unable to shown that there was anionic lipids was detected at that time range. This implied that HPTS absorbed at 285 nm as well.

**Table 7: Diameters of vesicles with different initial concentration of HPTS**

<table>
<thead>
<tr>
<th>HPTS concentration</th>
<th>0.05 mM</th>
<th>0.1 mM</th>
<th>0.2 mM</th>
<th>0.3 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vesicle diameter</td>
<td>70.7 nm</td>
<td>72.9 nm</td>
<td>70.3 nm</td>
<td>66.4 nm</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>2.2</td>
<td>1.7</td>
<td>1.5</td>
<td>1.2</td>
</tr>
</tbody>
</table>
Figure 3-20: Chromatogram of vesicles prepared using different concentrations of HPTS in the initial aqueous buffer detected at 455 nm. The vesicle samples were composed of POPC/Cholesterol/DSPE-mPeg/SAdiCbr20 at molar ratios of 53%: 40%: 1%: 6%
Figure 3-21: Fractionation of samples prepared at an initial HPTS concentration of 0.2 mM. Panel A= chromatogram detected at 455 nm, panel B= chromatogram detected at 285 nm, panel C= total chromatogram detected from 200 nm-600 nm, panel D= DLS analysis of the diameters of the aggregates in different fractions. Panel E= the ESI-MS analysis of SAdiCbr20 in different fractions reported as a ratio of the molecular ion relative to octasulfonate as internal standard. The vesicle sample was prepared in the composition of 53% POPC, 40% Cholesterol, 1% DSPE-mPeg and 6% SAdiCbr20.
3.4-2: Entrapment efficiency
The previous section has demonstrated that sulfonate lipid SAdiCbr20 could be associated in vesicle fractions for the vesicle sample made by microfluidic mixer. However, the question about the efficiency of the dye being entrapped inside the vesicles is unknown. For the purpose of quantifying the entrapment efficiency of the dyes, the HPLC analysis was performed.

The vesicle sample was prepared in the composition of 53% POPC, 40% Cholesterol, 1% DSPE-mPeg and 6% SAdiCbr20 with 0.2 mM of Nile red. The sample was separated by the size exclusion chromatography, and the vesicle fractions were combined and analysed as in previous cases. HPLC quantification was achieved in the mobile phase of 70% ACN and 30% MeOH. The chromatogram recorded is presented on Figure 3-22. The calibration curves of SAdiCbr20, neutral red, and Nile red were presented in Appendix C.

![Figure 3-22: The chromatogram detected at 254nm for combined vesicle fractions prepared from 53% POPC, 40% Cholesterol, 1%DSPE-mPeg and 6%SAdiCbr20 with initial concentration of 0.2mM Nile red entrapped. The initial lipid concentration is 10mg/ml](image)

The small peak having retention time at 2.45 minutes is SAdiCbr20 peak, and the peak at 2.294 minutes is the peak for Nile red. The total amount of Nile red injected was 0.0315 mg, and only 77% material is collected in the collection tube; thus, the amount of Nile red collected from the microfluidic mixer was 0.024 mg. Since the total volume of
1.55 mL was collected, the concentration of Nile red in the sample was 0.0155 mg/ml. Based on the integration area on the chromatogram of vesicle sample and the calibration curve of Nile red, the concentration of Nile red in vesicle sample was 0.0017 mg/ml. The efficiency could be determined as 11% for Nile red entrapment by using microfluidic mixer.

The low intensity for SAdiCbr20 shown on the chromatogram indicated that little SAdiCbr20 was incorporated into vesicle fractions in the presence of Nile red, which was consistent with the result obtained from ESI mass spectrometry that SAdiCbr20 was expelled from vesicle fractions with Nile red present.

Another set of analyses was carried out with the vesicle sample composed by 53% POPC, 40% Cholesterol, 1% DSPE-mPeg and 6% SAdiCbr20 with initial concentration of 0.2 mM neutral red entrapped. The vesicle sample was separated using size exclusion chromatography, and size of each fraction was measured by DLS. The vesicle fractions were combined and the buffer was evaporated by a passing air flow, and then the dried sample was re-dissolved in 50% MeOH and 50% ACN. The quantitative analysis was achieved with the mobile phase of 90% ACN and 10% water with both solvents having 1% of TFA. The signal was detected at 254 nm. The chromatogram is presented in Figure 3-23.

Figure 3-23: The chromatogram detected at 254 nm for combined vesicle fractions prepared from 53% POPC, 40% Cholesterol, 1%DSPE-mPeg and 6%SAdiCbr20 with initial concentration of 0.2 mM neutral red entrapped. The initial lipid concentration is 10 mg/ml
Neutral red has the retention time of 1.28 minutes, and SAdiC20 appears at 2.32 minutes. The initial concentration of neutral red used was 0.0578 mg/ml. The initial 0.2 mM of neutral red in 1.5 ml of buffer solution was diluted to a total vesicle sample volume of 2 ml; thus, the concentration of neutral red in the vesicle sample was 0.0433 mg/ml. The final concentration of neutral red was 0.00628 mg/ml, which was calculated based on the integration area on the vesicle sample chromatogram and the calibration curve for neutral red. Thus, the entrapment efficiency for neutral red was 14.5%.
3.5: Permeability assays and Multilamellarity determination

The previous discussion demonstrated that vesicles are likely formed by the microfluidic mixer, and they are able to entrap dyes and stable enough to pass through gel filtration chromatography; hence, the purpose of this section is to focus on the properties of the bilayer membranes. Can the vesicles bear a pH gradient across the membrane? Can the usual types of transport experiments be conducted using these vesicles? Do the vesicles containing the synthesized lipids show similar stability compared to the vesicles made from natural lipids? Is the vesicle sample unilamellar?

3.5-1: Permeability assays with ion transporters gramicidin and valinomycin in vesicles without sulfonate lipids

A closed bilayer membrane acts as a semi-permeable barrier which effectively separates a cell and its surrounding environment. Generally, some solutes such as small lipophilic molecules can pass through the membrane easily by diffusion; however, polar and big molecules such as amino acids, ions, and sugars cannot pass through the membrane by themselves; as a result, they need be transported by transporters. Ion channels and ion carriers acting as transporters are able to facilitate ions passing across a bilayer membrane. Ion channels are large trans-membrane proteins embedded in membranes that permit rapid diffusion of ions or big molecules through membranes. Gramicidin, which is produced by a soil bacterium Italic Bacillus brevis, is a linear pentadecapeptide with alternative L- and D- amino acids with molecular weight of ~1900. In spite of its small size, gramicidin is a well-studied efficient channel for alkali cations and protons. When embedded in bilayer membranes, two gramicidin molecules form a helical dimer by head to head dimerization to initialize a transport activity. The interior of the gramicidin channel is formed by the polar peptide backbone and the nonpolar side-chains point outward in contact with the fatty acid chains in lipids. The dimer is stabilized by intermolecular hydrogen bonds and intramolecular hydrogen bonds; as a result, the channel is gated by associating and dissociating of the monomers. The diameter of the gramicidin channel pore is about 4 Å, which makes it as an excellent candidate for monovalent cationic ion transportation.
Previous sections have successfully investigated the properties of vesicles, which included controlling of vesicle size and dye entrapment efficiency; in this part of the study, the permeability and stability of vesicles will be investigated, and the comparison of vesicles’ behaviors between the vesicles made by extrusion method, and the vesicles made by Microfluidic mixer will be carried out. Permeability assays via ion channels and fluorescent dyes are a standard and an effective approach to investigate the stability of vesicles. HPTS is a pH sensitive fluorescent dye that can be used to monitor pH gradients across vesicle bilayer membrane. The protonated and deprotonated forms are excited at 403 nm and 460 nm respectively; both species emit at 510 nm. Entrapment of HPTS inside vesicles permits to monitor the small change of proton concentration in both sides of membrane by establishing pH gradient across the membrane and by monitoring the gradient collapse in the presence of ion transporters.

The vesicle sample composed of 59% POPC, 40% cholesterol, 1% DSPE-mPeg with 0.05 mM HPTS entrapped was prepared by the microfluidic mixer in an initial lipid concentration of 10 mg/ml with the total flow of 12 ml/min in the flow rate ratio of buffer to lipid of 3:1. The size of vesicle was 54.0 nm with PDI of 0.248. Figure 3-24 shows the different transport activity of the vesicle sample without/with gramicidin. All figures in this part shows processed data, which raw data for fluorescence intensity of 460:510 nm and fluorescence intensity of 403:510 nm was combined to give a ratio, the ratio of the data was normalized to the span of values prior to base addition (set as 0) to after Triton lysis of the vesicles (set to 1). Addition of base at t= 60 s aimed to establish a pH gradient across the membrane, and the transport activity was barely started in the absence of gramicidin, although there was pH gradient across the membrane. Proton transport activity was initialized by adding gramicidin (t= 150 s), and the transport activity was facilitated by increasing the concentration of gramicidin. Collapse of pH gradient confirmed the transport activity, then, the vesicles were lysed by Triton at t= 540 s.

To attempt to understand the factors that affect the stability of vesicles, the relationship between initial concentration of lipid mixtures and vesicles stability was investigated. A vesicle sample composed by 59% POPC, 40% cholesterol, 1% DSPE-mPeg with 0.05 mM HPTS entrapped was prepared by the microfluidic mixer in initial lipid concentration
of 25 mg/ml with the total flow of 12 ml/min in the flow rate ratio of buffer to lipid of 3:1. The size of vesicle was 78.3 nm with PDI of 0.145. The sample was treated with NaOH to establish a pH gradient, and then addition of the ion channel gramicidin to initialize the proton transport activity, and the vesicles were lysed by Triton (Figure 3-25). These two sets of vesicle samples with different initial lipid concentrations presented similar results. Vesicles were able to bear basic external environment, and proton transport activity was observed by adding gramicidin, and transport activity was accelerated by increasing gramicidin concentration.

**Figure 3-24:** Normalized extent of transport as a function of time for vesicles in contract with variable concentration of gramicidin. Vesicle samples were composed of POPC/Cholesterol/DSPE-mPeg at molar ratios of 59%: 40%: 1% with initial lipid concentration of 10 mg/ml (Raw data could be found at Appendix C-6)
Figure 3-25: Normalized extent of transport as a function of time for vesicles in contract with variable concentration of gramicidin. The vesicle samples were composed of POPC/Cholesterol/DSPE-mPeg at molar ratios of 59%: 40%: 1% with initial lipid concentration of 25 mg/ml (Raw data could be found at Appendix C-7)

Figure 3-26: Normalized extent of transport as a function of time for vesicles in contract with variable concentration of gramicidin. The vesicle samples were prepared by Extrusion method; the lipid mixture was composed of POPC/Cholesterol/DSPE-mPeg at molar ratios of 59%: 40%: 1% with initial lipid concentration of 50 mg/ml (Raw data could be found at Appendix C-8)
In order to study if vesicle samples behave in the same manner when prepared by different methods, a vesicle sample was made by the extrusion method in the same lipid compositions of POPC/Cholesterol/DSPE-mPeg in the molar ratio of 59%:40%:1%. The initial concentration of 50 mg/ml of lipid mixture in chloroform was evaporated to a lipid thin film, and the film was hydrated with the internal buffer (0.05 mM HPTS, 0.1 M NaCl, and 0.01 M Na₃PO₄). The suspension was disrupted by 5 freeze-thaw cycles, and the resulting multilamellar vesicle suspension was disrupted by a probe tip sonicator. The vesicle mixture was then extruded back and forth through a 0.1 µm Nucleopore membrane 19 times. The external HPTS was removed by a disposable gel permeation chromatography (Sephadex G10). The size of vesicle was 130.9 nm with PDI of 0.154. Figure 3-26 shows the permeability assay with gramicidin and the vesicles made by extrusion method. The pH gradient was established by the addition of NaOH, followed by adding gramicidin to start proton transport. Collapse of pH gradient after addition of gramicidin confirmed the proton transport activity.

Figure 3-24, Figure 3-25 and Figure 3-26 presented the proton transport results for vesicle samples with initial lipid concentration of 10 mg/ml, 25 mg/ml and 50 mg/ml respectively. In order to further understand the stability of these vesicles, the comparison between the extent transport activity and gramicidin to vesicles ratio in each sample is required. Example for calculating the ratio of gramicidin to lipid in the vesicle sample with initial lipid concentration of 10 mg/ml is given and the calculation results for other two vesicle samples are listed in Table 8. Assuming all the vesicles are unilamellar with the bilayer thickness of 4 nm. In the sample with vesicle diameter of 54 nm, the volume of the bilayer per vesicle could be calculated as 1.36×10⁵ nm³. Assuming the density of the sample is 0.9 g/cm³, so the mass of lipid per vesicle is 1.22×10⁻¹⁶ g. Since the weighted average molecule weight of the lipids in the vesicle sample is 608 g/mol, the molar amount of lipid per vesicle is 2.0×10⁻¹⁹ moles. Although the initial lipid concentration was 10 mg/ml, the finial concentration of lipids from the microfluidic mixer was 5 mg/ml (7.75 mg in 1.55 ml), so the total moles of lipids in the 1 ml sample was 8.22×10⁶ mol. 200 µL of sample was placed in 2 mL buffer solution for analysis, so the concentration of vesicle in buffer was 3.0×10¹⁵ vesicles/L. At the concentration of 1.5
nM of gramicidin in the vesicle sample, the total number of gramicidin molecules present was \(9 \times 10^{14}/L\). Hence, the ratio of gramicidin to vesicle can be determined as 0.42. The same calculation processes were applied; thus, the gramicidin to vesicle ratio in 25 ml/mg and 50 ml/mg samples were calculated, and the results are presented on Table 8. The table showed the vesicle sample made by extrusion method has the lowest gramicidin to vesicle ratio, which is 0.27; the vesicle sample with the lowest initial concentration has the highest gramicidin to vesicle ratio, which is 0.42.

<table>
<thead>
<tr>
<th>Initial lipid conc.</th>
<th>Dia. (nm)</th>
<th>Number of vesicles/L buffer</th>
<th>Gramicidin molecules/L buffer</th>
<th>Gramicidin to vesicle ratio</th>
<th>Extent of transport</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mg/ml</td>
<td>54.0</td>
<td>(2.0 \times 10^{15}/L)</td>
<td>(9 \times 10^{14})</td>
<td>0.42</td>
<td>78%</td>
</tr>
<tr>
<td>25 mg/ml</td>
<td>78.3</td>
<td>(2.4 \times 10^{15}/L)</td>
<td>(9 \times 10^{14})</td>
<td>0.38</td>
<td>80%</td>
</tr>
<tr>
<td>50 mg/ml</td>
<td>130.9</td>
<td>(3.3 \times 10^{15}/L)</td>
<td>(9 \times 10^{14})</td>
<td>0.27</td>
<td>60%</td>
</tr>
</tbody>
</table>

The three vesicle samples demonstrated the ability to transport protons through the pathway that gramicidin provided. The vesicle sample made by extrusion method appears to have the lowest gramicidin to vesicle ratio, which is consistent with the observation that this set of sample has the lowest extent of transport. The possibility could be that all vesicle samples were assumed as unilamellar for the estimation; however, the vesicle sample made by different methods might have different uni-to multi-lamellar proportion. The interaction between the vesicles and the ion channel is another factor affects transport activity, and the vesicles made by extrusion method might have low affinity to gramicidin.

In order to confirm there was a stable barrier between the outside and the inside of the vesicles produced by microfluidic mixing, another permeability assay using the ion transporter valinomycin was performed. Valinomycin is a cyclic dodecadepsipeptide containing D-valine, D-\(\alpha\)-hydroxyvaleric acid, L-valine, and L-lactic acid in the sequence. It is an ion carrier to catalyze the exchange of K\(^+\) and H\(^+\) through the membrane\(^{47}\). Valinomycin is highly selective for K\(^+\) as compared to Na\(^+\). In a polar solvent, valinomycin exposes the backbone to the solvent; however, in a nonpolar
solvent, the conformation changes with the isopropyl group located on the exterior, thus, valinomycin is positioned in the surface of the membrane to capture $K^+$ from the solvent. When $K^+$ is captured inside the cyclic dodecapeptide, the valinomycin molecule locks the conformation with shielding of the $K^+$ by the carbonyl groups of the backbone\textsuperscript{47b}. The complex moves from the membrane lipid surface, and dissolves in the lipid core using the non-polar isopropyl group on the exterior of the complex. The complex diffuses to the opposite face of the membrane, and then, $K^+$ is released to the other side of membrane via the reverse process\textsuperscript{47}.

Since valinomycin is acknowledged to catalyze the exchange between $K^+$ with $H^+$ across a lipid membrane, the pH-sensitive dye HPTS could be applied for monitoring the small change in the concentration of $H^+$ across a membrane. The lipid mixture was composed by POPC, cholesterol and DSPE-mPeg in the molar ratio of 59\%:40\%:1\% in the initial lipid concentration of 10 mg/ml. The vesicle sample was formulated with phosphate buffer containing 0.05 mM HPTS and 0.1 M KCl, and then diluted in the medium containing 0.1 M NaCl to establish a transmembrane potassium gradient. The vesicle sample has the diameter of 42.2 nm with PDI of 0.221. By addition of NaOH, a pH gradient was established, the proton flux was observed by the change in the fluorescence intensity. In the absence of valinomycin, there is no proton being transported (Figure 3-27). The transport activity is initialized by adding valinomycin. When the concentration of valinomycin was below 250 nM, proton transport activity was slow. Valinomycin produced a remarkable transport activity at the concentration was 375 nM, where the ratio of valinomycin to vesicle is 3.36. The significant increase in fluorescence intensity at the valinomycin concentration of 375 nM indicated that the ratio of valinomycin to vesicle of 3.36 is able to provide adequate pathway for proton/potassium exchange across the membrane. On the other hand, the transport activity was decreased when valinomycin concentration was 500 nM, which suggests that the high concentration of valinomycin might induce detergent-like lysis in the vesicle.
Figure 3-27: Normalized extent of transport as a function of time for vesicles in contact with variable concentration of valinomycin. The vesicle samples were composed of POPC/Cholesterol/DSPE-mPeg at molar ratios of 59%: 40%: 1% with initial lipid concentration of 10 mg/ml (Raw data could be found at Appendix C-9).

As presented above, these observation supported the conclusion that vesicles made by the microfluidic mixer behaved in the same manner as the vesicles made by extrusion methods.

3.5-2: Permeability assays with ion transporters gramicidin and valinomycin in vesicles with sulfonate lipids

The study on lipid incorporation efficiency (section 3.4) has demonstrated that SAdiCbr20 lipid was able to incorporate into vesicles; furthermore, dye entrapment efficiency showed that the vesicles so-formulated with sulfonate lipid SAdiCbr20 could entrap HPTS dye. In order to investigate the stability of anionic vesicles, permeability assays were performed with the vesicle samples co-formulated with different sulfonate lipids. The same experimental method was applied as in previous cases.
Figure 3-28: Normalized extent of transport as a function of time for vesicles in contact with variable concentration of gramicidin. Gramicidin assay with the vesicle samples were composed of POPC/ Cholesterol/ DSPE-mPeg/SAdiCbr20 at molar ratios of 53%: 40%: 1%:6% with initial lipid concentration of 10 mg/ml (Raw data could be found at Appendix C- 9)

The sample was prepared in the same lipid compositions as described in Section 3.4 (POPC/ Cholesterol/ DSPE-mPeg/SAdiCbr20: molar ratios of 53%: 40%: 1%:6%, microfluidic mixing method). Figure 3-28 showed that a significant change in the fluorescence intensity after addition of NaOH, which indicated the proton leakage in the vesicle sample. The fluorescence intensity was little changed after adding gramicidin, which suggested that there was no proton transport activity. The small fluorescence intensity increased after addition of Triton further confirmed that the vesicles are depleted before addition of Triton. Comparing the results presented on Figure 3-24 and Figure 3-28, the anionic lipid SAdiCbr20 appeared to disrupt the bilayer membrane structure making it more permeable to protons.

Another set of vesicle sample was made by extrusion method with the lipid composition of POPC/ Cholesterol/ DSPE-mPeg/SAdiCbr20 at the molar ratio of 53%: 40%: 1%:6% with initial lipid concentration of 50 mg/ml (Figure 3-29). In contrast to previous observation (Figure 3-28), addition of base to the vesicle sample led to small change in
fluorescence intensity, which indicated the vesicles were stable in the basic environment. The transport activity was initialized by the addition of gramicidin. The fluorescence intensity is increased as the concentration of gramicidin increases.

Figure 3-29: Gramicidin assay with the vesicle samples were made by extrusion method; lipid mixture composed of POPC/ Cholesterol/ DSPE-mPeg: SAdiCbr20 at molar ratios of 53%: 40%: 1%:6% with initial lipid concentration of 50 mg/ml (Raw data could be found at Appendix C- 10)
Figure 3-30: Gramicidin assay with the vesicle samples were made by extrusion method; lipid mixture composed of POPC/ Cholesterol/ DSPE-mPeg/ SAmonoCbr20 at molar ratios of 50%: 40%: 1%:9% with initial lipid concentration of 50 mg/ml (Raw data could be found at Appendix C-11)

In order to further study how sulfonate lipids behave in vesicles, the vesicle sample was prepared in the presence of SAmonoC20 by the extrusion method. Addition of NaOH resulted in little change in fluorescence intensity, and the transport activity was facilitated by increasing the concentration of gramicidin. Triton was added to lyse all vesicles (Figure 3-30).

The comparison of vesicle’s permeability between the vesicles made by extrusion method and vesicles made by the microfluidic mixer with the same lipids composition (Figure 3-28 and Figure 3-29) showed that the vesicles made by the microfluidic mixer were more permeable. Comparing the vesicle samples prepared by conventional extrusion method with different lipid compositions (Figure 3-29: SAdiCbr20; Figure 3-30: SAmonoCbr20) showed that the vesicles co-formulated with SAmonoCbr20 were more preamble than vesicles co-formulated with SAdiCbr20; on the other hand, it also implied that in SAmonoCbr20 vesicle system, there must be some sulfonate lipid incorporated in the vesicles; hence, sulfonate lipids as a class are not excluded under the extruder method.
To sum up, the microfluidic mixer was able to make stable vesicles, and both types of vesicles demonstrated the similar proton transport behaviour in the presence of ion carries. However, vesicles that were co-formulated with anionic lipids SAdiCbr20 or SAmonoCbr20 were more permeable than the vesicles made by natural lipids.

3.5-3: Are the vesicles unilamellar?

Melittin, a peptide from bee venom, has high binding affinity to lipid membranes, so it is known to induce a change of integrality of the membrane and the deformation of vesicles. Although the mechanism of the lytic activity of melittin remains incomplete, a particular aspect of melittin and other membrane-active peptide is useful. Due to the lytic property which acts only on the outmost bilayer membrane of a multilamellar vesicle, addition of melittin to vesicles enables estimation of the proportions of multilamellar and unilamellar vesicles in a sample. The vesicle sample was prepared with HPTS entrapped, and the addition of base established a pH gradient. The lytic activity of melittin was verified by monitoring the increase of fluorescence intensity after addition of melittin. Successive addition of melittin and finally complete lysis by Triton can be used to determine the multilamellar proportion in vesicles as those vesicles that are resistant to melittin but not Triton.

Figure 3-31 shows the vesicle sample made by the microfluidic mixer with the composition of POPC/ Cholesterol/ DSPE-mPeg at molar ratios of 59%: 40%: 1% with the initial lipid concentration of 10 mg/ml, with added melittin in low concentration aliquots, e.g. 100 nM to give the change of 100, 200, 300 nM for mellitin concentration after consecutive second and third injections, produces successive changes in the fluorescence. When aliquots that produced larger incremental concentration changes were injected, the fluorescence intensity jumped up directly to a constant level, e.g. injection of melittin of 500 nM. After adding a total mellitin concentration of about 500 nM or higher in one step, successive aliquots made little change. Addition of Triton led to increase in the fluorescence intensity, which indicated residual entrapped acidic buffer. The result is indicated in Figure 3-31, and it is consistent with a unilamellar fraction of 80% with 20% of vesicles having more than 2 bilayers on the outside.
Another vesicle sample was prepared in the same lipid composition of POPC/Cholesterol/DSPE-mPeg at molar ratios of 59%: 40%: 1% with the initial lipid concentration of 50 mg/ml. To the vesicle sample prepared by the extrusion method was added melittin in low concentration, e.g. 100 nM to give the change of 100, 200, 300 nM, and successive change in the fluorescence intensity was observed (Figure 3-32). When larger concentration aliquot was added, the fluorescence intensity jumped in one step (e.g. 500 nM), and successive addition of melittin at large concentration made little change, but still show residual entrapped acidic buffer on Triton addition. The results presented that the vesicle sample made by extrusion method had a unilamellar proportion of 70% and 30% of vesicles sample are multilamellar. The vesicle sample made by microfluidic mixer has lower population of multilamellar vesicles, which might due to the quick mixing process between the lipid mixture and buffer.

![Figure 3-31: Extent of transport as a function of added melittin added in aliquots of increasing final concentration. The vesicle samples were composed of POPC/Cholesterol/DSPE-mPeg at molar ratios of 59%: 40%: 1% with the initial lipid concentration of 10 mg/ml (Raw data could be found at Appendix C-12)](image-url)
Figure 3-32: Extent of transport as a function of added melittin added in aliquots of increasing final concentration. The vesicle samples were made by exclusion method; lipid mixture composed of POPC/ Cholesterol/ DSPE-mPeg at molar ratios of 59%: 40%: 1% with initial lipid concentration of 50 mg/ml (Raw data could be found at Appendix C-13)
Chapter 4 Conclusion and future work

The goal of this project was to investigate the properties of vesicles prepared by the NanoAssmblr ® microfluidic mixer in turn uncovers the answers to the questions posed in the introductory section. In order to achieve the goal, single chain and double chain sulfonate anionic lipids having shape parameters from 0.7-2.1 were synthetized successfully; and, the vesicle samples were formulated with synthetized and natural lipids with various lipid compositions; then, the study about vesicle properties were performed. The most basic question about what type of aggregates the microfluidic mixing device makes has been answered by showing the ability to control and predict the vesicle size. The predicted values were large than experimental results. One possibility for that could be that microfluidic mixing device not only make vesicles, but also some small nanoparticles. Although the size control via changing lipid composition is not ideal, the trend of increasing shape parameters of systems leading to an increase in vesicle size has been observed. The facts that addition of SAdiCbr20 to lipid mixture led to increase in vesicle size, and addition of SAmonoC18:1 to vesicle sample resulted in reducing vesicle size indicates that sulfonate lipids were able to incorporate into vesicle sample. On the other hand, in spite of majority of vesicle sample was damaged during preparation process, cryo-TEM images still showed that there were vesicles present in the vesicle sample.

The question about the efficiency of lipid incorporation in to the vesicles after microfluidic mixing also has been resolved. Phospholipid analysis and quantitative analysis by HPLC determined the incorporation efficiency of POPC (55%) and sulfonate lipids (50% incorporation efficiency for SAdiCbr20; 25% incorporation efficiency for SAmonoC18:1). Lipid lost during preparation and separation process suggested there might be some small micelles formed in vesicle sample, and which was confirmed by showing two columns size distribution in MSD in dynamic light scattering. The entrapment efficiency was investigated via entrapping of three different dyes (neutral red, Nile red, and HPTS). At low dyes concentrations, the dyes concentrations barely affect the size of vesicle. Semi-quantitative analyses were performed by size exclusion
chromatography, and chromatograms were recorded from 200 nm to 600 nm. Dynamic light scattering was used to measure size of each fraction collected, and ESI-mass spectrometry to confirm the incorporation of SAdiCbr20 into vesicles. Neutral red was entrapped by vesicle at low concentration (less than 0.3 mM). There was not neutral red entrapped as the concentration of neutral red increased to 0.3 mM, which due to the low solubility of neutral red in aqueous solution. Further quantitative analysis by HPLC demonstrated that the entrapment efficiency could be up to 11%-15% (Entrapment efficiency for neutral red at initial concentration of 0.2 mM was 14.5%; entrapment efficiency for Nile red at initial concentration of 0.2 mM was 11%). The stability of the aggregates was studied by permeability assays with ion transporters and the fluorescent dye HPTS. The increase in fluorescence intensity after applying the ion transporters gramicidin exhibited that vesicle samples were able to transport protons; which demonstrated that the vesicles made by microfluidic method have the same behaviour as and the vesicles made by extruder method. However, in the presence of sulfonate lipids, the vesicles made by microfluidic method were more permeable than vesicles made by extruder method, and the vesicle size is larger in both cases. The different behaviour in these two set of vesicles indicates that microfluidic method has the ability to incorporate all types of lipids into vesicles, while the same conclusion could not be drawn for extruder method. Finally, a melittin assay showed that the vesicle sample prepared by the microfluidic mixer had higher unilamellar proportion than the vesicles made by the extrusion method.

In spite of many questions related to the project that have been addressed, there is still some ambiguity that needs to be cleared. Cryo-TEM showed there were some vesicles present in the sample generated from the microfluidic mixer, while the majority of samples were damaged, which suggests different methods are required to obtain better images. For example, other types of grids instead of hydrophobic lacey carbon grids could be used to prevent vesicle decomposition. Perhaps the wicking process can be done in shorter time and at lower pressure, so that the sample is preserved better before freezing. Due to majority images showed the concentrated aggregates, the less amount of material might be used for sample preparation; alternatively, vesicles could be prepared in lower initial lipid concentration.
Although the ability to control vesicle size was demonstrated, the size control was not good enough since the samples had high polydispersity. The vesicle size could be controlled better by different lipid mixtures, for example, using lipids with higher transition temperature. The microfluidic mixing device currently used was operated at ambient temperature, which limits the choice of that lipids could be applied. Heating the chips would be useful since it would give access to use other lipids. Lipids with higher transition temperature are expected to be important in the formulation of sample for biological studies. It is worth to mention that the vesicle produced by the microfluidic mixer has smaller size compared the vesicles made by the extrusion method in the same lipid composition, which suggests the mixer is better in making vesicles since the mixer can force the components together better. Vesicle is thermodynamic unstable, and its size distribution strong depends on the energy input. Previous studies showed longer sonication time and strong sonication power led to small vesicle size. Microfluidic mixing making vesicle requires higher energy input compared to the convectional method, as a result, the vesicle made by microfluidic mixer has the small size then the vesicle made by extruder method. Microfluidic mixing device could be used for drug entrapment to overcome the low drug loading efficiency and time consuming in conventional drug loading process. Although the anionic vesicles presented a similar behaviour to the vesicles made by natural lipids, the low stability associated with anionic vesicles limits their application in drug delivery systems.
Bibliography


46. PHD THESIS.


Appendix A: Synthesis Details

General procedure for synthesis
The chemicals and solvents were used as received from known suppliers. Compounds are purified by column chromatography using silica gel 60 and Amberlite IRA410 (Cl) anionic exchange resin. $^1$H-NMR spectra were collected on 300MHZ Bruker instrument. ESI Mass spectra were recorded on a Waters MicroMass Q-TOF instrument running in a negative mode.

Synthetic details:
Nucleophilic substitution of alcohol to bromide by Appel Reaction

\[
\text{R}^{\text{OH}} + \text{1.2 equiv CBr}_4 + \text{1.3 equiv PPh}_3 \rightarrow \text{R}^{\text{Br}}
\]

\[
\text{DCM, 0^\circ C} \rightarrow \text{rt}
\]

\[1-2(c-e) \rightarrow 1-3(c-e)\]

\[c: R= \]
\[d: R= \]
\[e: R= \]

Scheme A-1: Appel Reaction for synthesis of compounds 1-3c, 1-3d, 1-3e

General procedure for the synthesis of alkyl bromide (Scheme A-1)
To a solution of 1 equivalent of the alcohol in DCM in a round bottom flask, 1.2 equivalent of CBr$_4$ and 1.3 equivalent of PPh$_3$ were added to the solution in portions at 0 °C, and the solution was stirred for 40 minutes at room temperature. DCM was removed under reduced pressure by rotary evaporator, and then hexane was added to the residue to precipitate PPh$_3$ and the oxide. The crude product was purified by column chromatography on silica gel by DCM and hexane solvent mixture.
Synthesis of 1-Bromooctadecane (1-3c)
Prepared according to the general procedure: octadecan-1-ol (1-2c) (2.7049 g, 10 mmol, 1 equiv) was stirred in 30 mL of DCM, CBr₄ (3.9809 g, 12 mmol, 1.2 equiv), and PPh₃ (3.4071 g, 13 mmol, 1.3 equiv) were added in portions. The crude product was purified by column chromatography on silica gel by DCM: hexane 9:1 to give 3.0551 g (92%) of 1-3c as colorless liquid. H-NMR (300MHz, CDCl₃) δ: 3.407 (t, 2H, J = 7.0 Hz), 1.857 (q, 2H, J = 7.1 Hz), 1.418 (m, 2H), 1.259 (s, 26H), 0.882 (t, 3H, J = 6.60 Hz).

Compared to known compound¹:
H-NMR (200 MHz, CDCl₃) δ: 3.41 (2H, t, J = 7.0 Hz), 1.85 (2H, q, J = 7.0 Hz), 1.46-1.37 (2H, m), 1.25 (26H, s), 0.88 (3H, t, J = 6.6 Hz)

Synthesis of 1-bromo-(2-octyl)dodecane (1-3d)
Prepared according to the general procedure: 2-octyldodecan-1-ol (1-2d) (1.75 mL, 5 mmol, 1 equiv) was stirred in 30 mL DCM, CBr₄ (1.658 g, 6 mmol, 1.2 equiv), and PPh₃ (1.7036 g, 6.5 mmol, 1.3 equiv) were added slowly. The crude product was purified by column chromatography on silica gel by DCM: hexane 9:1 to give 1-3d 1.7531 g (97%) as colorless liquid. H-NMR (300 MHz, CDCl₃) δ: 3.444 (d, 2H, J = 4.8 Hz, CH₂-Br), 1.75 (1H, bs, CH-CH₂-Br), 1.26 (32H, m, CH₂ alkyl), 0.87 (6H, t, J = 6.9 Hz, CH₃).

Compared to known compound²:
H-NMR (400 MHz, CDCl₃) δ: 3.42 (2H, d, J = 4.8 Hz, CH₂-Br), 1.75 (1H, bs, CH-CH₂-Br), 1.26 (32H, m, CH₂ alkyl), 0.87 (6H, t, J = 6.9 Hz, CH₃).

Synthesis of Z-1-bromooctadec-9-ene (1-3e)
Prepared according to the general procedure: oleyl alcohol (1-2e) (1.58 mL, 5 mmol, 1 equiv) was stirred in 30 mL DCM, CBr₄ (2.073 g, 6 mmol, 1.2 equiv), and PPh₃ (1.7036 g, 6.5 mmol, 1.3 equiv) were added slowly. The crude product was purified by column chromatography on silica gel by DCM: hexane 9:1 to give 1-3e of 1.5165 g (90%) as colorless liquid. H-NMR (CDCl₃) δ: 5.421 (m, 2H), 3.415 (t, 2H, J = 7.3 Hz), 2.005 (m,
4H), 1.861 (q, 2H, J=7.0 Hz), 1.325 (m, 22H), 0.882 (t, 3H, J=6.5 Hz).

Compared to known compound:¹

H-NMR (200 MHz, CDCl₃) δ: 5.41-5.28 (2H, m), 3.40 (2H, t, J=7.0 Hz), 2.08-1.97 (4H, m), 1.85 (2H, q, J= 7.0 Hz), 1.41-1.27 (22H, m), 0.88 (3H, t, J= 6.6 Hz).

**Ester coupling from alkyl bromides with 5-sulfoisophthalic acids**

![Chemical structure](image)

**Scheme A-2:** Synthesis of SAdiC14:0 (1-4a), SAdiC16:0 (1-4b), SAdiC18:0 (1-4c), SAdiCbr20:0 (1-4d), SAdiC18:1 (1-3e)

**General procedure for the synthesis of benzenesulfonate diesters (Scheme A-2):**

SAdiC14:0 (1-4a), SAdiC16:0 (1-4b), SAdiC18:0 (1-4c)

To a solution of 2.2 equivalent of related alkyl bromide in DMSO, 1 equivalent of 5-sulfoisophthalic acid (sodium salt), 2.2 equivalent of cesium carbonate, and 0.2 equivalent of sodium iodide were added to the solution, and the reaction mixture was heated at 100 °C for 3 hours. The reaction was quenched by adding 20 mL chloroform at room temperature. The reaction mixture was stored in a freezer for 24 hours to get more precipitate, and the resulting precipitate was filtered by suction filtration. The excess of
alkyl bromide was washed out by chloroform, and inorganic salts were removed by washing the precipitate with water to afford the final product.

Synthesis of SAdiC14:0 (Sodium salt) (1-4a)
Prepared according to the general procedure: 1-bromotetradecane (0.65 mL, 2.2 mmol, 2.2 equiv) in 20 mL DMSO was added to the mixture of 5-sulfoisophthalic acid (sodium salt)(1-1)(0.2682 g, 1 mmol, 1 equiv), cesium carbonate (0.7172 g, 2.2 mmol, 2.2 equiv), and sodium iodide(0.031 g, 0.2 mmol, 0.2 equiv). Standard work-up yielded 0.4627 g (70%) of 1-4a as white solid. H-NMR (300 MHz, DMSO-d6) δ: 8.403 (t,1H, J=1.7 Hz), 8.362 (d, 2H, J=1.5 Hz), 4.306 (t, 4H, J= 6.5 Hz), 1.718 (q, 4H, J=6.6 Hz), 1.215 (s, 44H), 0.835 (t, 6H, J= 6.6 Hz).

Synthesis of SAdiC16:0 (Sodium salt) (1-4b)
Prepared according to the general procedure: 1-bromohexadecane (0.67 mL, 2.2 mmol, 2.2 equiv) in 20 mL DMSO, 5-sulfoisophthalic acid (sodium salt)(1-1)(0.2682 g, 1 mmol, 1 equiv), cesium carbonate (0.7172 g, 2.2 mmol, 2.2 equiv), and sodium iodide (0.032 g, 0.2 mmol, 0.2 equiv). The pure product is white solid compound 1-4b with 0.4661 g (65% yield). H-NMR (300 MHz, DMSO-d6) δ: 8.404 (t,1H, J=1.4 Hz), 8.362 (d, 2H, J= 1.4 Hz), 4.306 (t,4H, J= 6.6 Hz), 1.723 (q, 4H, J= 7.0 Hz), 1.214 (s, 52H), 0.837 (t, 6H, J= 6.9 Hz).

Synthesis of SAdiC18:0 (Sodium salt) (1-4c)
Prepared according to the general procedure: 1-3c (0.3667 g, 1.1 mmol, 2.2 equiv) in 10 mL DMSO, 5-sulfoisophthalic acid (sodium salt) (1-1)(0.1341 g, 0.5 mmol, 1 equiv), cesium carbonate (0.3584 g, 1.1 mmol, 2.2 equiv), and sodium iodide (0.0151 g, 0.2 mmol, 0.2 equiv), the final product is white solid 1-4c with 0.3017 g (78%). H-NMR (300 MHz, DMSO-d6) δ: 8.404 (t,1H, J=1.4 Hz), 8.369 (d,2H J=1.4 Hz), 4.305 (t, 4H, J= 6.5 Hz), 1.720 (q, 4H, J=7.1 Hz), 1.213 (s, 66H), 0.838 (t, 6H, J=6.8 Hz).
General procedure for the synthesis of benzenesulfonate diesters (Scheme 2)  
SAdiC20:0 (Sodium salt) (1-5d) and SAdiC18:1 (Sodium salt) (1-4e)

To a solution of 2 equivalent of related alkyl bromide in DMSO, 1.2 equivalent of 5-sulfoisophthalic acid (sodium salt), 2 equivalent cesium carbonate, and 0.2 equivalent of sodium iodide were added to the solution, and the reaction mixture was heated at 100 °C for 12 hours. The reaction was quenched by adding 20 mL chloroform at room temperature. The reaction mixture was stored in a freezer for 24 hours, and the unreacted salts were filtered by suction filtration. A large amount of water was added to the filtrate, and the mixture was stirred at room temperature for 30 minutes. Slow rotation and evaporation under reduced pressure by a rotary evaporator formed white particles, which were collected by suction filtration to afford the products.

Synthesis of SAdiCbr20:0 (Sodium salt) (1-4d)

Prepared according to the general procedure: 5-sulfoisophthalic acid (sodium salt) (I-1) (0.1615 g, 0.6 mmol, 1.2 equiv), 1-3d (0.615 g, 1 mmol, 1 equiv) cesium carbonate (0.3258 g, 1 mmol, 2 equiv), and sodium iodide (0.0151 g, 0.1 mmol, 0.2 equiv) were dissolved in 20 mL DMSO at 100 °C for 12 hours. The white gel-like compound 1-4d of 0.1134 g was obtained (25% yield). H-NMR (300 MHz, DMSO-d6) δ: 8.415 (t, 1H, J=1.6 Hz), 8.377 (d, 2H, J=1.6 Hz), 4.227 (d, 2H, J=5.2 Hz), 1.751 (s, 1H), 1.197 (s, 52H), 0.814 (m, 12H).

Synthesis of SAdiC18:1(Sodium salt)(1-4e)

Prepared according to the general procedure: 1-3e (0.2736 g, 1 mmol, 2 equiv), 5-sulfoisophthalic acid (sodium salt)(I-1) (0.1332 g, 0.6 mmol, 1.2 equiv), cesium carbonate (0.2726 g, 1 mmol, 2 equiv), and sodium iodide(0.0151 g, 0.1 mmol, 0.2 equiv) were dissolved in 20 mL DMSO at 100 °C for 12 hours. The white gel-like compound 1-4e of 0.0697 g was obtained (17% yield). H-NMR (300 MHz, DMSO-d6) δ: 8.41 (t, 1H, J=1.6 Hz), 8.37 (d, 2H, J=1.5 Hz), 5.31 (m, 4H), 4.30 (t, 4H, J=6.2 Hz), 1.96 (m, 8H), 1.72 (q, 4H, J=7.3 Hz), 1.21 (s, 44H), 0.83 (t, 6H, J= 7.0 Hz).
Monoesters formed from the reaction of alkyl bromides with 4-sulfobenzoic acid (potassium salt)

Scheme A-3: Monoester coupling for synthesis of SAmonoC14 (I-7a), SAmonoC16 (I-7b), SAmonoC18 (I-7c), SAmonoCbr20 (I-7d), SAmonoC18:1(I-7e)

General procedure for synthesis of monoester of SAmonoC14 (I-7a), SAmonoC16 (I-7b), SAmonoC18 (I-7c) (Scheme A-3)
A mixture of 1 equivalent of 4-sulfobenzoic acid(potassium salt), 1.1 equivalent of cesium carbonate, and 0.2 equivalent of sodium iodide were added to an oven-dried round bottom flask equipped with a stirred bar. 1.1 equivalent of related alkyl bromide in DMSO was added to the mixture through a syringe. The mixture was stirred at 100 °C for 3 hours. Once complete, the reaction mixture was diluted by chloroform. The resulting precipitate was filtered by suction filtration. The excess of alkyl bromide was removed by washing the precipitate with chloroform, and the inorganic salts were removed by washing the mixture with water to give monoester.
Synthesis of SAmonoC14:0 (Sodium salt) (I-7a)
Prepared according to the general procedure: 4-sulfobenzoic acid (potassium salt)( 1-6) (0.2403 g, 1 mmol, 1 equiv), 1-bromotetradecane (0.325 mL, 1.1 mmol, 1.1 equiv), cesium carbonate (0.3584 g, 1.1 mmol, 1.1 equiv), and sodium iodide (0.0151 g, 0.2 mmol, 0.2 equiv) were stirred in 20 mL DMSO at 100 °C for 3 hours. The product was isolated to give 0.218 g (50%) of I-7a as white solid. H-NMR (300 MHz, DMSO-d₆) δ: 7.93 (d, 2H, J=8.4 Hz), 7.73 (d, 2H, J=8.3 Hz), 4.26 (t, 2H, J=6.6 Hz), 1.70 (q, 2H, J=6.7 Hz), 1.24 (s, 23H), 0.85 (t, 2H, J= 6.3 Hz).

Synthesis of SAmonoC16:0 (Sodium salt) (I-7b)
Prepared according to the general procedure: 4-sulfobenzoic acid (potassium salt)( 1-6) (0.2403 g, 1 mmol, 1 equiv), 1-bromohexadecane (0.34 mL, 1.1 mmol, 1.1 equiv), cesium carbonate (0.3584 g, 1.1 mmol, 1.1 equiv), and sodium iodide (0.0151 g, 0.2 mmol, 0.2 equiv) were stirred in 20 mL DMSO at 100 °C for 3 hours. The product was isolated to give 0.2459 g (53%) of I-7b as white solid. H-NMR (300 MHz, DMSO-d₆) δ: 7.93 (d, 2H, J=8.4 Hz), 7.73 (d, 2H, J=8.4 Hz), 4.28 (t, 2H, J=6.6 Hz), 1.73 (q, 2H, J=6.8 Hz), 1.23 (s, 26H), 0.85 (t, 2H, J=6.7 Hz).

Synthesis of SAmonoC18:0 (Sodium salt) (I-7c)
Prepared according to the general procedure: 4-sulfobenzoic acid (potassium salt)( 1-6) (0.2403 g, 1 mmol, 1 equiv), 1-3c (0.3667 g, 1.1 mmol, 1.1 equiv) cesium carbonate (0.3584 g, 1.1 mmol, 1.1 equiv), and sodium iodide (0.0151 g, 0.2 mmol, 0.2 equiv) were stirred in 20 mL DMSO at 100 °C for 3 hours. The product was isolated to give 0.2903 g (59%) of I-7c as white solid. H-NMR (300 MHz, DMSO-d₆) δ: 7.92(d, 2H, J=8.4 Hz), 7.73(d, 2H, J=8.4 Hz), 4.26(t, 2H, J=6.7 Hz), 1.68(q, 2H, J=6.8 Hz), 1.23(s, 30H), 0.85(t, 3H, J=6.9 Hz).
General procedure for synthesis of monoester of SAmonoCbr20:0(1-7d), SAmonoC18:1(1-7e)

A mixture of 1.1 equivalent of 4-sulfobenzoic acid (potassium salt), 1 equivalent of cesium carbonate, and 0.2 equivalent of sodium iodide were added to an oven-dried round bottom flask equipped with a stirred bar. 1 equivalent of the alkyl bromide in DMSO was added to the mixture through a syringe. The mixture was stirred at 100 °C for 12 hours. Once complete, the reaction mixture was diluted by chloroform. After storage of the reaction mixture in a freezer for 24 hours, the reaction was filtered to remove unreacted residues. A large amount of brine was added to the filtrate. The organic layer was separated, and the aqueous layer was extracted with chloroform 6 times. The combined organic layers were concentrated under reduced pressure to yield a crude product, which was purified by anion exchange chromatography using Amberlite IRA410 (Cl) anionic exchange resin. Remove unreacted bromide by MeOH as elute, and then switch to NaI acetone solution as eluent. Evaporation acetone under reduced pressure afforded the mixture of product and NaI, the mixture was then dissolved in chloroform. NaI solid was removed by filtration. Evaporate chloroform under reduced pressure to give the pure products.

**Synthesis of SAmonoC20:0 (Sodium salt) (1-7d)**

Prepared according to the general procedure: 4-sufobenzoic acid (potassium salt)( 1-6) (0.2403 g, 1 mmol, 1 equiv), 1-3d (0.615 g, 1 mmol, 1 equiv), cesium carbonate (0.3258 g, 1 mmol, 1 equiv), and sodium iodide (0.0151 g, 0.2 mmol, 0.2 equiv) were stirred in 20 mL DMSO at 100 °C for 12 hours. Standard work-up, and purified by ion exchange chromatography to give 0.1089 g (21%) of 1-7d as colorless gel-like compound.

**Synthesis of SAmonoC18:1 (Sodium salt) (1-7e)**

Prepared according to the general procedure: 4-sufobenzoic acid (potassium salt)( 1-6) (0.2403 g, 1 mmol, 1 equiv), 1-3e (0.2736 g, 1 mmol, 1 equiv), cesium carbonate (0.3258 g, 1 mmol, 1 equiv), and sodium iodide (0.0151 g, 0.2 mmol, 0.2 equiv) were stirred in 20 mL DMSO at 100 °C for 12 hours. Standard work-up, and purified by ion exchange chromatography to give 0.1235g (25%) of 1-7e as colorless gel-like compound. H-NMR
(300 MHz, DMSO-d$_6$) $\delta$: 7.92 (d, 2H, $J$=8.4 Hz), 7.73 (d, 2H, $J$=8.4 Hz), 5.33 (m, 2H), 4.25 (t, 2H, $J$=6.4 Hz), 1.97 (m, 4H), 1.70 (q, 2H, $J$=6.4 Hz), 1.23 (m, 23H), 0.84 (t, 3H, $J$=6.2 Hz).

Converting sodium and potassium salts to tetramethylammonium salts

\[ \text{Scheme A-4: Converting sodium salts to tetramethylammonium salts} \]
General procedure for converting sodium salts to tetramethylammonium salts
(Scheme A-4)
To a suspension of 1 equivalent of benzenesulfonate diesters (sodium salt) or benzenesulfonate monoester (potassium salt) acetone in an oven-dried round bottom flask equipped with a stirred bar, 1.3 equivalent of NMe₄Cl was added. The reaction mixture was stirred at room temperature for 24 hours. The reaction mixture was filtered to remove unreacted residues, and acetone was removed under reduced pressure by rotary evaporator to yield final products.

Synthesis of SAdiC14:0 (tetramethylammonium salt) (1-5a)
Prepared according to the General procedure for converting sodium salts to tetramethylammonium salts: SAdiC14:0 (Sodium salt) (1-4a)(0.0791 g, 0.12 mmol, 1 equiv) and NMe₄Cl (0.0172 g, 0.155 mmol, 1.3 equiv) in 10 mL acetone was stirred for 24 hours. The product was obtained to give 0.0427 g (50% yield) of SAdiC14:0 (tetramethylammonium salt) (1-5a) as white solid. H-NMR (300 MHz, CDCl₃) δ: 8.745 (d, 2H, J=1.5 Hz), 8.676 (t, 1H, J=1.7 Hz), 4.329 (t, 4H, J=6.5 Hz), 3.472 (s, 12H), 1.772 (q, 4H, J=6.6 Hz), 1.269 (s, 44H), 0.885 (t, 6H, J=6.6 Hz). ¹³C-NMR (75.5 MHz, DMSO-d₆) δ: 164.6, 149.7, 130.2, 130.1, 129.3, 65.2, 54.4, 28.6, 25.4, 22.0, 13.9. MS (-ve ESI): Calculated for 1-5a C₃₆H₆₁O₇S= 637.41 amu, obtained for 1-5a = 637.80 amu.

Synthesis of SAdiC16:0 (tetramethylammonium salt) (1-5b)
Prepared according to the General procedure for converting sodium salts to tetramethylammonium salts: SAdiC16:0 (Sodium salt) (1-4b)(0.0868 g, 0.12 mmol, 1 equiv) and NMe₄Cl (0.016 g, 0.15 mmol, 1.2 equiv) in 12 mL acetone was stirred for 24 hours. The product was obtained to give 0.0489 g (53% yield) of SAdiC16:0(tetramethylammonium salt) (1-5b) as white solid. H-NMR (CDCl₃) δ: 8.749 (d, 2H, J=1.4 Hz), 8.676 (t, 1H, J=1.4 Hz), 4.319 (t, 4H, J=6.6 Hz), 3.486 (s, 12H), 1.766 (q, 4H, J=7.0 Hz), 1.257 (s, 52H), 0.879 (t, 6H, J=6.9 Hz). ¹³C-NMR (75.5 MHz, DMSO-d₆) δ: 165.2, 150.5, 130.8, 129.7, 65.6, 55.1, 31.7, 29.3, 28.0, 25.9, 22.5, 14.3. Calculated for 1-5b C₄₀H₆₉O₇S= 693.48 amu, obtained for 1-5b = 693.93 amu.
Synthesis of SAdiC18:0 (tetramethylammonium salt) (1-5c)
Prepared according to the General procedure for converting sodium salts to tetramethylammonium salts: SAdiC18:0 (Sodium salt) (1-4c) (0.108 g, 0.14 mmol, 1 equiv) and NMe₄Cl (0.200 g, 0.18 mmol, 1.3 equiv) in 12 mL acetone was stirred for 24 hours. The product SAdiC18:0 (tetramethylammonium salt) (1-5c) was obtained to give 0.0669 g (58% yield) as white solid.

\[
\text{H-NMR (CDCl}_3\text{) } \delta: 8.654 (d, 2H, J=1.4 Hz), 8.593 (t, 4H, J=6.5 Hz), 3.434 (s, 12H), 2.099 (s, 60H), 0.805 (t, 6H, J=6.8 Hz). 
\]

\[
\text{13C-NMR (75.5 MHz, DMSO-d}_6\text{) } \delta: 165.4, 152.7, 129.6, 128.7, 125.8, 64.7, 54.4, 31.2, 28.9, 25.4, 22.0, 13.9. 
\]

Calculated for 1-5c C_{44}H_{77}O_{7}S= 749.54 amu, obtained for 1-5c= 749.93 amu.

Synthesis of SAdiC20:0 (tetramethylammonium salt) (1-5d)
Prepared according to the General procedure for converting sodium salts to tetramethylammonium salts: SAdiC20:0 (Sodium salt) (1-4d) (0.2012 g, 0.24 mmol, 1 equiv) and NMe₄Cl (0.03162 g, 0.29 mmol, 1.2 equiv) in 20 mL acetone was stirred for 24 hours. The product SAdiC20:0 (tetramethylammonium salt) (1-5d) was obtained to give 0.0676 g (32%) as white solid compound. H-NMR (CDCl₃) δ: 8.742 (d, 2H, J=1.6 Hz), 8.648 (t, 4H, J=6.5 Hz), 4.247 (d, 4H, J=5.2 Hz), 2.485 (s, 12H), 1.778 (s, 1H), 1.263 (s, 52H), 0.886 (m, 12H). \text{13C-NMR (75.5 MHz, DMSO-d}_6\text{) } \delta: 164.5, 149.9, 130.4, 130.1, 128.9, 67.3, 54.4, 36.7, 31.2, 30.8, 29.3, 28.9, 26.1, 22.0, 13.8. Calculated for 1-5d C_{48}H_{85}O_{7}S= 805.60 amu, obtained for 1-5d= 806.07 amu.

Synthesis of SAdiC18:1 (tetramethylammonium salt) (1-5e)
Prepared according to the General procedure for converting sodium salts to tetramethylammonium salts: SAdiC18:1 (Sodium salt) (1-4e) (0.07232 g, 0.1 mmol, 1 equiv) and NMe₄Cl (0.0131 g, 0.12 mmol, 1.2 equiv) in 10 mL acetone was stirred for 24 hours. The product SAdiC18:1 (tetramethylammonium salt) (1-5e) was obtained to give 0.0207 g (25%) as white solid. H-NMR (DMSO-d₆) δ: 8.46 (t, 1H, J=1.6 Hz), 8.4 (d, 2H, J= 1.7 Hz), 5.3 (Q, 4H, J=5.8 Hz), 4.3 (t, 4H, 6.4 Hz), 3.1 (s, 12H), 1.9 (8H, m), 1.7 (4H, Q, J= 6.3 Hz), 1.2 (s, 44), 0.8 (6H, m). \text{13C-NMR (75.5 MHz, DMSO-d}_6\text{) } \delta: 164.6, 149.7,
Calculated for \( I-5e \) \( C_{44}H_{73}O_7S^- = 745.51 \) amu, obtained for \( I-5e \) = 745.93 amu.

**Synthesis of SAmonoC14:0 (tetramethylammonium salt) (1-8a)**

Prepared according to the General procedure: SAmonoC14:0 (Sodium salt) \( I-7a \) (0.0872 g, 0.2 mmol, 1 equiv) and NMe\(_4\)Cl (0.0263 g, 0.24 mmol, 1.2 equiv) in 10 mL acetone was stirred for 24 hours. The product SAmonoC14:0 (tetramethylammonium salt) \( I-8a \) was obtained to give 0.0433 g (50% yield) as white solid. H-NMR (300 MHz, DMSO-d\(_6\)) \( \delta \): 7.93 (d, 2H, \( J=8.4 \) Hz), 7.73 (d, 2H, \( J=8.4 \) Hz), 4.26 (t, 2H, \( J=6.6 \) Hz), 3.09 (s, 12H), 1.70 (q, 2H, \( J=6.7 \) Hz), 1.23 (s, 25H), 0.85 (t, 3H, \( J=6.3 \) Hz). \(^{13}\)C-NMR (75.5 MHz, DMSO-d\(_6\)) \( \delta \): 165.4, 152.7, 129.6, 128.7, 125.8, 64.7, 54.4, 31.2, 30.6, 28.9, 25.4, 22.0, 13.9. MS (-ve ESI): Calculated for \( I-8a \) \( C_{21}H_{33}O_5S^- = 397.21 \) amu; obtained for \( I-8a \) = 397.60 amu.

**Synthesis of SAmonoC16:0 (tetramethylammonium salt) (1-8b)**

Prepared according to the General procedure: SAmonoC16:0 (Sodium salt) \( I-7b \) (0.0462 g, 0.1 mmol, 1 equiv) and NMe\(_4\)Cl (0.0132 g, 0.12 mmol, 1.2 equiv) in 10 mL acetone was stirred for 24 hours. The product was obtained to give 0.0203 g (43%) as White solid. H-NMR (300 MHz, DMSO-d\(_6\)) \( \delta \): 7.92 (d, 2H, \( J=8.4 \) Hz), 7.73 (d, 2H, \( J=8.4 \) Hz), 4.26 (t, 2H, \( J=6.6 \) Hz), 3.09 (s, 12H), 1.70 (q, 2H, \( J=6.8 \) Hz), 1.23 (s, 27H), 0.85 (t, 3H, \( J=6.7 \) Hz). \(^{13}\)C-NMR (75.5 MHz, DMSO-d\(_6\)) \( \delta \): 165.4, 152.7, 129.6, 128.7, 125.8, 64.7, 54.4, 31.2, 30.6, 28.9, 25.4, 22.0, 13.9. Calculated for \( I-8b \) \( C_{23}H_{37}O_5S^- = 425.24 \) amu; obtained for \( I-8b \) = 425.67 amu.

**Synthesis of SAmonoC18:0 (tetramethylammonium salt) (1-8c)**

Prepared according to the General procedure: SAmonoC18:0 (Sodium salt) \( I-7c \) (0.0489 g, 0.12 mmol, 1 equiv) and NMe\(_4\)Cl (0.0132 g, 0.12 mmol, 1.2 equiv) in 10 mL acetone was stirred for 24 hours. The product was obtained to give SAmonoC18:0 (tetramethylammonium salt) \( I-8c \) 0.0216 g (41%) as white solid. H-NMR (300 MHz, DMSO-d\(_6\)) \( \delta \): 7.92 (d, 2H, \( J=8.4 \) Hz), 7.73 (d, 2H, \( J=8.4 \) Hz), 4.26 (t, 2H, \( J=6.7 \) Hz), 3.09 (s, 13H), 1.70 (q, 2H, \( J=6.8 \) Hz), 1.23 (s, 32H), 0.85 (t, 3H, \( J=6.9 \) Hz). \(^{13}\)C-NMR (75.5
MHz, DMSO-d$_6$) δ: 165.4, 152.7, 129.6, 128.7, 125.8, 64.6, 54.3, 28.6, 25.3, 22.0, 13.9.
Calculated for I-8c C$_{25}$H$_{41}$O$_5$S = 453.27 amu; obtained for I-8c = 453.73 amu.

Synthesis ofSAMonoC20:0 (tetramethylammonium salt) (I-8d)
Prepared according to the General procedure: SAMonoC20:0 (Sodium salt) (I-7d)
(0.0571 g, 0.12 mmol, 1 equiv) and NMe$_4$Cl (0.0132 g, 0.12 mmol, 1.2 equiv) in 10 mL
acetone was stirred for 24 hours. The product SAMonoC20:0(tetramethylammonium
salt)(I-8d) was obtained to give 0.0101 g (18%) as white solid. H-NMR (300 MHz, CDCl$_3$) δ: 8.05(d, 2H, J=8.4 Hz), 7.96 (d, 2H, J=8.4 Hz), 4.22 (d, 2H, J=5.7 Hz), 3.43 (s, 12H), 1.75 (s, 1H), 1.26 (s, 34H), 0.87 (t, 6H, J=6.4 Hz). $^{13}$C NMR (75.5 MHz, DMSO-
$^d_6$) δ: 165.4, 152.7, 129.6, 128.7, 125.8, 67.2, 54.4, 36.6, 31.2, 30.6, 29.1, 28.9, 25.9,
22.0, 13.9. Calculated for I-8d C$_{27}$H$_{45}$O$_5$S = 481.30 amu; obtained for I-8d = 481.73 amu.

Synthesis ofSAMonoC18:1 (tetramethylammonium salt) (I-8e)
Prepared according to the General procedure: SAMonoC18:1 (Sodium salt) (I-7e)(0.0481
 g, 0.1 mmol, 1 equiv) and NMe$_4$Cl (0.0132 g, 0.12 mmol, 1.2 equiv) in 10 mL acetone
was stirred for 24 hours. The product SAMonoC18:1(tetramethylammonium salt)(I-8e)
was obtained to give 0.0111 g (21%) as white solid. H-NMR(300 MHz,CDCl$_3$) δ: 7.99
(d, 2H, J=8.4 Hz), 7.87 (d, 2H, J=8.4 Hz), 5.29 (m, 2H), 4.24 (t, 2H, J=6.4 Hz), 3.24 (s,
12H), 1.94 (m, 4H), 1.71 (q, 2H, J=6.4 Hz), 1.22 (m, 24H), 0.83 (t, 3H, J=6.2 Hz). $^{13}$C
NMR (75.5 MHz, DMSO-d$_6$) δ: 165.4, 152.7, 130.0, 129.6, 128.7, 125.8, 64.6, 54.4,
31.2, 29.0, 28.8, 26.5, 25.4, 22.0, 13.9. Calculated for I-8e C$_{25}$H$_{39}$O$_5$S = 451.25 amu;
obtained for I-8e = 451.73 amu.
Appendix B: Spectra

$^1$H-NMR for compound 1-4a

![NMR Spectrum Image]

SAdiC14:0 (Sodium salt)
$^1$H-NMR for compound \textit{1-4b}
$^{1}$H-NMR for compound 1-4c

$1\text{-}4c$

SAdiCl18:0 (Sodium salt)
$^1$H-NMR for compound 1-4d

1-4d
$^1$H-NMR for compound 1-4e
$^1$H-NMR for compound 1-7a

1-7a
$^1$H-NMR for compound $1-7b$

![H-NMR spectrum](image)
$^1$H-NMR for compound 1-7c
$^1$H-NMR for compound 1-7e
$^1$H-NMR for compound 1-5a

1-5a (SAdiC14:0)
$^{13}$C-NMR for compound 1-5a

![Chemical structure of 1-5a (SAdiC14:0)]
$^1$H-NMR for compound 1-5b

![Diagram of compound 1-5b (SAdiC16:0)]
$^{13}$C-NMR for compound 1-5b

![SAdiC16:0](image)
$^1$H-NMR for compound 1-5c

1-5c (SAdiC18:0)
\(^{13}\)C-NMR for compound 1-5c

\[
\begin{align*}
\text{SO}_3\text{NMe}_4^+ \\
\text{O} \\
\text{O} \\
\text{O} \\
\text{SAdiC18:0}
\end{align*}
\]
$^1$H-NMR for compound 1-5d

1-5d (SAdiCbr20:0)
$^{13}$C-NMR for compound \textit{1-5d}

1-5d (SAdiCbr20:0)
$^1$H-NMR for compound 1-5e

1-5e (SAdiC18:1)
$^{13}$C-NMR for compound 1-5e
$^1$H-NMR for compound 1-8a

![](image)

1-8a (SAmonoC14:0)
$^{13}$C-NMR for compound 1-8a
$^1$H-NMR for compound 1-8b

1-8b (SAmonoC16:0)
$^{13}$C-NMR for compound **1-8b**

![NMR spectrum of 1-8b (SAmonoC16:0)]
$^1$H-NMR for compound 1-8c

$\text{SO}_3^- \text{NMe}_4^+$

1-8c (SAMonoC18:0)
$^{13}$C-NMR for compound 1-8c

1-8c (SAmonoC18:0)
$^1$H-NMR for compound 1-8d

![NMR spectrum of 1-8d (SAmonoCbr20:0)]
$^{13}$C-NMR for compound 1-8d

\[ \text{SO}_3^- \cdot \text{NMe}_4^+ \]

1-8d (SAmonoCbr20:0)

\begin{figure}
\centering
\includegraphics[width=\textwidth]{spectrum}
\caption{13C-NMR spectrum for compound 1-8d.}
\end{figure}
$^1$H-NMR for compound 1-8e

1-8e (SAmonoC18:1)
\(^{13}\text{C}-\text{NMR for compound } 1-8e\)
Appendix C: Vesicle Formulations

Material and instruments
Vesicle samples prepared by the extrusion method used a LiposoFast® extruder from Avestin containing Nucleopore 0.1 µm polycarbonate membrane. The sonicator used for disrupting lipid dispersion was Misonix sonicator ultrasonic cell disruptor with temperature control. The NanoAssemblerTM Microfluidic mixer was a beta version of the commercially available instrument from Precision NanoSystems operated under external computer control, and microfluidic mixing chips were purchased from the Precision NanoSystems. Vesicle sizing was done by dynamic light scattering (DLS) on a Brookhaven Instrument by ZetaPALS particle sizing software. Size exclusion chromatography used a hand-packed column (Tricorn Glass Tube 10/100) packed with Sephadex G25/G50 gel size exclusion resin. Fractions were collected by Gilson FC 203B fraction collector from Mandel Scientific Co. Ltd.. Signal was detected by a Waters 996 Photodiode Array Detector; the chromatogram was recorded by MassLynx software. The fluorescence intensity for vesicle assays was measured by PTI QM40 fluorimeter. The molecular ion masses for different compounds were measured by ESI-Mass spectrometry, which is purchased from International Equipment Trading Ltd.. 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), cholesterol, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000](DSPE-mPEG) were purchased from Avanti Polar Lipids, Inc.. Anhydrous ethanol and chloroform were purchased from Sigma/Aldrich. Three dyes (8-hydroxypyrene-1,3,6-trisulfonic acid, trisodium salt(HPTS), Nile red and neutral red), and buffer salts (sodium phosphate, sodium chloride, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid(HEPES)) were purchased from Sigma/Aldrich.

General procedure for vesicle preparation
Extrusion method
This method closely follows published work from this laboratory. In brief, a desired amount of an ethanolic lipid mixture was evaporated under reduced pressure to generate thin film, and trace amount of solvent was removed from the lipid thin film under high
vacuum overnight. The lipid film was hydrated with a known amount of buffer. The suspension was disrupted by 5 freeze-thaw cycles. A probe tip sonicator delivered high energy input to further disrupt the multilamellar vesicle suspension. The multilamellar liposome suspension was submitted to extrusion to force through a polycarbonate filter with 0.1 μm pore size to generate small unilamellar vesicles with diameter in the range of 80-200 nm. The vesicles were separated from other small lipid aggregates by gel filtration on a by Sephadex G-25M disposable size exclusion column, and then the vesicle size was measured by dynamic light scattering.

**NanoAssemblr™ Microfluidic mixer**

Prior to each vesicle sample preparation, the microfluidic mixer cartridge was washed by 3 ml of Millipore water at the left inlet port and 3 ml of EtOH at right inlet port in the flow ratio of 1:1 with total flow rate of 12 ml/min, and the cartridge was primed with 3 ml of buffer and 1 ml of ethanol on left and right inlet sides in the flow ratio of 3:1 with total flow rate of 12 ml/min. The desired amount of lipids were mixed in ethanol. Buffer solution (0.01 M of sodium phosphate and 0.1 M of sodium chloride buffer, or 0.01 M of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 0.1 M sodium chloride buffer) was loaded at the left inlet port, and the lipid mixture was loaded in the right inlet port. 1.55 ml of sample was collected in the flow rate of 12 ml/min with flow rate ratio of buffer to lipid of 3:1, the total volume was set at 2 ml, sample switching time(start) at 1.75 s, and end time at 0.5 s. Following use, the microfluidic mixer cartridge was washed according to the following sequence that the both inlet ports of cartridge were initially washed by 3 ml of EtOH in each inlet port in the total flow rate at 12 ml/min in flow rate ratio of 1:1; and then, 3 ml of EtOH in the right inlet port and 3 ml of Millipore water in the left inlet pore to further clean the cartridge, and the end, both inlet ports were cleaned by 3 ml of Millipore water in each inlet port. The size distribution of the vesicle sample was determined by dynamic light scattering by adding 200 μL of vesicle sample into 2.4 ml of buffer in a disposable cuvette with 10 mm pathlength. Vesicle separation was achieved by Sephadex G-50 size exclusion chromatography, and the fractions were collected by fraction collector at 0.5 min/tube at the solvent flow rate of 1 ml/min, and the vesicle fractions were verified by DLS.
Phospholipid analysis

Working solutions for phospholipid analysis\textsuperscript{24}

Solution 1: 4.000 g ammonium molybdate-$4\text{H}_2\text{O}$ was dissolved in 30.00 ml Millipore water.

Solution 2: 20.0 ml solution 1, 10.0 ml concentrated hydrochloric acid, and 2.5 ml of mercury were mixed and stirred for 30 minutes. On standing for 5 minutes, 3 layers were formed, suction filtration to remove unreacted ammonium molybdate-$4\text{H}_2\text{O}$.

Solution 3: all the filtrate of solution 2, the remaining portion of solution 1, and 50.0 ml of concentrated sulfuric acid.

Chromogenic acid solution: 5 ml of solution 3, 9 ml of methanol, 1 ml of chloroform, 4 ml of water were mixed. The solution was highly reactive, and kept in fridge all the time.

Preparation of phospholipid standard solutions and sample\textsuperscript{24}

Phospholipid stock solution was made by dissolving 0.0500 g of POPC in 1 ml of chloroform (50 mg/ml). Aliquots of 1.5 µL, 2.5 µL, 5 µL, 6.5 µL, 7.5 µL of stock solution were transferred to test tubes to cover the range of 0-0.4 mg phospholipids. To each tube was added 0.4 ml of chloroform and 0.1 ml of the chromogenic solution, the test tubes were heated for 1 minute at 100 °C, and then cooled to room temperature, and 4 ml chloroform was added to each test tube. Each tube was shaken gently, and allowed to sit for 30 minutes to obtain the maximum absorbance value. The blank solution was prepared by 50 µL of chloroform as the solution aliquot. The absorbance for every tube was measured at 710 nm by UV-visible spectrophotometer. The calibration curve was established by plotting absorbance as a function of mass of phospholipid at the wavelength of 710 nm (Figure C-1).
Figure C-1: Calibration curve for POPC concentration determination in the vesicle fractions

Quantitative analysis by HPLC for dyes entrapment experiment

The stock solutions for Nile red were prepared at the concentrations of 0.005 mg/ml, 0.0015 mg/ml, 0.002 mg/ml, 0.004 mg/ml, 0.015 mg/ml, and 0.03 mg/ml in 30% MeOH and 70% ACN. The HPLC analysis was performed on the Ultimate 3000 LC system with C18 column having diameter 250×4.6 mm with pore size of 4 micron. The volume injected to HPLC was 50 µL, and each stock solution was run for 10 minutes with flow rate of 1.5 ml/min and at the pressure of 110 bars in mobile phase of 90% water and 10% ACN with the presence of 1% TFA in both solvents. The signal was detected at 254 nm.

The stock solutions for SAdiCbr20 were prepared at the concentrations of 3 mg/ml, 2 mg/ml, 1.5 mg/ml, 1.2 mg/ml, 1 mg/ml, 0.6 mg/ml, and 0.2 mg/ml in 90% CAN and 10% water. The stock solutions were run on the Ultimate 3000 LC system with C18 column having diameter 250×4.6 mm with pore size of 4 micron. The total volume of 50 µL was injected, and the mobile phase used is 90% ACN and 10% water with both solvents having 1% of TFA. The sample was run for 10 minutes flow rate of 1.5 ml/min and at the pressure of 60 bars. The signal was detected at 222 nm.

The stock solutions for SAmonoC18:1 was prepared at the concentrations of 0.05 mg/ml, 0.1 mg/ml, 0.15 mg/ml, 0.2 mg/ml, 0.25 mg/ml, 0.3 mg/ml, 0.4 mg/ml in 90% ACN and 10% water. The stock solutions were analyzed on the Ultimate 3000 LC system with C18 column having diameter 250×4.6 mm with pore size of 4 micron. The total volume of 50
µL was injected, and the mobile phase is 90% ACN and 10% water with both solvents containing 1% of TFA. The sample was run for 10 minutes with flow rate of 1.5 ml/min and at the pressure of 60 bars, and the signal was detected at 254 nm.

The stock solutions for neutral red was prepared at the concentrations of 0.003 mg/ml, 0.01 mg/ml, 0.015 mg/ml, 0.1 mg/ml and 0.2 mg/ml in 90% ACN and 10% water. The stock solutions were analyzed on the Ultimate 3000 LC system with C18 column having diameter 250×4.6 mm with pore size of 4 micron. The total volume of 50 µL was injected, and the mobile phase is 90% ACN and 10% water with both solvents containing 1% of TFA. The sample was run for 10 minutes with flow rate of 1.5 ml/min and at the pressure of 60 bars, and the signal was detected at 254 nm.

![Figure C-2: Calibration curve for Nile red detected at 254 nm](image)

\[
y = 2396.7x - 1.2815 \\
R^2 = 0.9955
\]
**Figure C-3: Calibration curve for SAdiCbr20 detected at 222 nm**

\[ y = 2681.7x + 362.94 \]

\[ R^2 = 0.987 \]

**Figure C-4: Calibration curve for SAmonoC18:1 detected at 254 nm**

\[ y = 491.69x + 3.9459 \]

\[ R^2 = 0.9906 \]
Procedure for determination of the 806/139 ratio by ESI-Mass spectrometry

The stock solution for sodium octasulfonate at the concentration of $1 \times 10^{-4}$ mol/L was prepared in HPLC grade methanol. 150 µL sample from the vesicle fractions (determined by dynamic light scattering) was added to the 1.5 ml snaplock microtube. To the microtube, 100 µL of sodium octasulfonate internal standard solution was added. The solution was made up to 1 ml by HPLC grade methanol. The samples were analyzed by ESI-Mass spectrometry in negative mode.
**HPTS assay protocol**

**Parameters setting of fluorimeter**

An “Excitation ratio scan” was required. From the acquisition tab, open the acquisition of “HPTS-Exc-ratio-QM40”, and then the parameters are set as following: set the excitation at 403 nm and 460 nm and emission at 510 nm, and the scan time is 600 seconds. Slit size is set at 3 nm.

Under “real time correction tab” use energy correction, change the “gain” (start at 1.10 V) and then go to the excitation wavelengths, the signal should be less than 10 V and the change only at second decimal place. For excitation this is the “defaultExc. corr”, and for emission is “emcorr-energy-most recent daye”. Uncheck the box under “background” tab. Check the following options under “preference”: “always create the new session”, “manual pause acquisition click”, and “reset clock for time based acquisition”.

Add the ratio trace under the “traces” tab, under “derived” choose “excorr 460:510” as source, and choose the function “divide”. “excorr 403:510” was chosen for source 2.

**Sample preparation**

A sample of 100 µL of HPTS-loaded vesicle samples was added to 2 ml of the corresponding external buffer equipped with a stir bar. The fluorimetric cell was placed into the themostated sample holder. During the experiment, 50 µL of 0.5M aqueous NaOH was injected at t=60 s (without pausing the scan). The solution of the compound of interest (Gramicidin, valinomycin, and Mellitin) was added at t=150 s, and the vesicles were lysed by adding 50 µL (5% aq) of Triton at t=540 s (stop the scan before adding triton, and then continue.)

**Cleaning the cells**

All the glassware and syringes should be cleaned before and after the experiments. Syringes can be sonicated in MeOH for 60 minutes, and the fluorimetric cell is cleaned by the solution of 2/3 Nitric acid and 1/3 water, and let it soak overnight, and then rinsed with MeOH.
Formulations for section 3.1 Are vesicles formed by microfluidic mixing?

Size control via molecular parameters

Formulation for Figure 3.1-2

<table>
<thead>
<tr>
<th>Lipids</th>
<th>Mole percentage</th>
<th>Stock solution in EtOH (mg/ml)</th>
<th>Amount needed</th>
</tr>
</thead>
<tbody>
<tr>
<td>POPC</td>
<td>59</td>
<td>10</td>
<td>720µL</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>40</td>
<td>10</td>
<td>250µL</td>
</tr>
<tr>
<td>DSPE-mPeg</td>
<td>1</td>
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<td>30µL</td>
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Formulations for Figure 3.1-3

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<th>Materials</th>
<th>No. 1 Mole percentage</th>
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<th>No. 3 Mole percentage</th>
<th>No. 4 Mole percentage</th>
<th>No. 5 Mole percentage</th>
<th>( \text{d_{measured}} ) (nm)</th>
<th>( \text{d_{predicted}} ) (nm)</th>
<th>PDI</th>
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<td>72</td>
<td>67</td>
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<td>0.265</td>
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<tr>
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<td>3</td>
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Formulations for Figure 3.1-4

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<th>No. 4 Mole Percentage</th>
<th>No. 5 Mole Percentage</th>
<th>( \text{d_{measured}} ) (nm)</th>
<th>( \text{d_{predicted}} ) (nm)</th>
<th>PDI</th>
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<td>72</td>
<td>72</td>
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<td>10</td>
<td>15</td>
<td>20</td>
<td>33.2</td>
<td>32.1</td>
<td>0.232</td>
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<td>40</td>
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<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
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<td>SAdiCbr20</td>
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<td>d&lt;sub&gt;measured&lt;/sub&gt;(nm)</td>
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### Formulations for Figure 3.1-6

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<td>8</td>
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### Formulation for section 3.2 Are vesicles form? Cyro-TEM imaging

### Formulation for section 3.2

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<th>Amount needed (µL)</th>
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<td>POPC</td>
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<tr>
<td>Cholesterol</td>
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<td>250</td>
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<td>DSPE-mPeg</td>
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<tr>
<td>Diameter(nm)</td>
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Formulation for section 3.3 Efficiency of incorporation of lipids into vesicles

Formulation for Figure 3.3-1

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</tr>
<tr>
<td>Cholesterol</td>
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<td>40</td>
<td>250</td>
</tr>
<tr>
<td>DSPE-mPeg</td>
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Formulation for Figure 3.3-3

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<td>Cholesterol</td>
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<td>DSPE-mPeg</td>
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<td>50</td>
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<tr>
<td>EtOH</td>
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<td>---</td>
<td>152</td>
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<tr>
<td>Diameter(nm)</td>
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<td>PDI</td>
<td>0.236</td>
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</table>

Formulation for Figure 3.3-4

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<tbody>
<tr>
<td>POPC</td>
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<td>72</td>
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<tr>
<td>Cholesterol</td>
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<td>10</td>
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<td>DSPE-mPeg</td>
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<td>52.6</td>
</tr>
<tr>
<td>SAmonoC18:1</td>
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<td>15</td>
<td>49.2</td>
</tr>
<tr>
<td>EtOH</td>
<td>---</td>
<td>---</td>
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<td>Diameter(nm)</td>
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</table>
Formulation for section 3.4 Dyes entrapment

Formulation for Figure 3.4-2

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<th>Mole percentage</th>
<th>Amount needed (µL)</th>
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</thead>
<tbody>
<tr>
<td>POPC</td>
<td>53</td>
<td>640</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>40</td>
<td>250</td>
</tr>
<tr>
<td>DSPE-mPeg</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>SAdiCbr20</td>
<td>6</td>
<td>80</td>
</tr>
</tbody>
</table>

The Neutral red dye was dissolved in phosphate buffer (0.01 M of Na₃PO₄, 0.1 M of NaCl at pH=6.4) to achieve concentrations of 0.05 mM, 0.1 mM, 0.2 mM, 0.3 mM, respectively.

Formulation for Figure 3.4-3

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Diameter(nm)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. 1 (initial neutral red concentration 0.05 mM)</td>
<td>61.8</td>
<td>0.158</td>
</tr>
<tr>
<td>No. 2 (initial neutral red concentration 0.1 mM)</td>
<td>61.4</td>
<td>0.200</td>
</tr>
<tr>
<td>No.3 (initial neutral red concentration 0.2 mM)</td>
<td>54.8</td>
<td>0.253</td>
</tr>
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<td>No.4 (initial neutral red concentration 0.3 mM)</td>
<td>65.9</td>
<td>0.204</td>
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</table>

Formulation for Figure 3.4-3

<table>
<thead>
<tr>
<th>Lipids</th>
<th>Stock solution in EtOH (mg/ml)</th>
<th>Mole percentage</th>
<th>Amount needed (µL)</th>
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</thead>
<tbody>
<tr>
<td>POPC</td>
<td>20</td>
<td>53</td>
<td>320</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>10</td>
<td>40</td>
<td>250</td>
</tr>
<tr>
<td>DSPE-mPeg</td>
<td>10</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>SAdiCbr20</td>
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<td>6</td>
<td>80</td>
</tr>
<tr>
<td>Nile red</td>
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<tr>
<td>Diameter(nm)</td>
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<td>PDI</td>
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</table>

100 µL of 2 mM of Nile red was added to 1 ml lipid mixture to achieve 0.2 mM concentration.
Formulation for Figure 3.4-6

<table>
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<th>Stock solution in EtOH (mg/ml)</th>
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<tr>
<td>Cholesterol</td>
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<td>40</td>
<td>250</td>
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<tr>
<td>DSPE-mPeg</td>
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<td>1</td>
<td>30</td>
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<tr>
<td>SAdiCbr20</td>
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<td>6</td>
<td>80</td>
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<tr>
<td>Diameter(nm)</td>
<td>90.3</td>
<td>PDI</td>
<td>0.265</td>
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</tbody>
</table>

*The Neutral red dye was dissolved in phosphate buffer (0.01 M of Na$_3$PO$_4$, 0.1 M of NaCl at pH= 6.4) to achieve concentrations of 0.05 mM, 0.1 mM, 0.2 mM, 0.3 mM, respectively.

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Diameter(nm)</th>
<th>PDI</th>
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</thead>
<tbody>
<tr>
<td>No. 1 (initial HPTS concentration 0.05 mM)</td>
<td>70.7</td>
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<tr>
<td>No. 2 (initial HPTS concentration 0.1 mM)</td>
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<td>0.208</td>
</tr>
<tr>
<td>No. 3 (initial HPTS concentration 0.2 mM)</td>
<td>70.3</td>
<td>0.230</td>
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<td>No. 4 (initial HPTS concentration 0.25 mM)</td>
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<td>No. 5 (initial HPTS concentration 0.3 mM)</td>
<td>66.4</td>
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</table>

Formulation for section 3.5 Permeability assays

The buffer used in Formulation for section 3.5 Permeability assays was phosphate buffer (0.01 M of Na$_3$PO$_4$, and 0.1 M of NaCl at pH= 6.4) with 0.05 mM HPTS

Formulation for Figure 3.5-1

<table>
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<th>Mole percentage</th>
<th>Amount needed (µL)</th>
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</thead>
<tbody>
<tr>
<td>POPC</td>
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<td>59</td>
<td>720</td>
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<tr>
<td>Cholesterol</td>
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<td>40</td>
<td>250</td>
</tr>
<tr>
<td>DSPE-mPeg</td>
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<td>1</td>
<td>30</td>
</tr>
<tr>
<td>Diameter(nm)</td>
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Formulation for Figure 3.5-2<sup>d</sup>

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<th>Amount needed (µL)</th>
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<td>Diameter(nm)</td>
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<sup>d</sup>25 mg lipids in 1 ml EtOH was achieved by combining the amount of lipid mixture, and evaporation of all EtOH to generate a lipid thin firm, and the thin film was dissolved in 1 ml EtOH.

Formulation for Figure 3.5-3

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<tbody>
<tr>
<td>POPC</td>
<td>40</td>
<td>59</td>
<td>900</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>10</td>
<td>40</td>
<td>1250</td>
</tr>
<tr>
<td>DSPE-mPeg</td>
<td>20</td>
<td>1</td>
<td>75</td>
</tr>
<tr>
<td>Diameter(nm)</td>
<td>102.7</td>
<td>PDI</td>
<td>0.109</td>
</tr>
</tbody>
</table>

Formulation for Figure 3.5-4<sup>e</sup>

<table>
<thead>
<tr>
<th>Lipids</th>
<th>Stock solution in EtOH(mg/ml)</th>
<th>Mole percentage</th>
<th>Amount needed (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>POPC</td>
<td>10</td>
<td>59</td>
<td>720</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>10</td>
<td>40</td>
<td>250</td>
</tr>
<tr>
<td>DSPE-mPeg</td>
<td>10</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>Diameter(nm)</td>
<td>102.7</td>
<td>PDI</td>
<td>0.109</td>
</tr>
</tbody>
</table>

<sup>e</sup>0.05 mM of HPTS was prepared in phosphate buffer(0.01 M of Na<sub>3</sub>PO<sub>4</sub>, and 0.1 M of KCl at pH= 6.4)
Formulation for Figure 3.5-5

<table>
<thead>
<tr>
<th>Lipids</th>
<th>Stock solution in EtOH (mg/ml)</th>
<th>Mole percentage</th>
<th>Amount needed (µL)</th>
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</thead>
<tbody>
<tr>
<td>POPC</td>
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<td>53</td>
<td>640</td>
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<tr>
<td>Cholesterol</td>
<td>10</td>
<td>40</td>
<td>250</td>
</tr>
<tr>
<td>DSPE-mPeg</td>
<td>10</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>SAdiCbr20</td>
<td>10</td>
<td>6</td>
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<tr>
<td>Diameter(nm)</td>
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Formulation for Figure 3.5-6f

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<tbody>
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<td>POPC</td>
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<tr>
<td>Cholesterol</td>
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</table>

Formulation for Figure 3.5-8

<table>
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<th>Stock solution in EtOH (mg/ml)</th>
<th>Mole percentage</th>
<th>Amount needed (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>POPC</td>
<td>10</td>
<td>59</td>
<td>720</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>10</td>
<td>40</td>
<td>250</td>
</tr>
<tr>
<td>DSPE-mPeg</td>
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<td>30</td>
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<tr>
<td>Diameter(nm)</td>
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</table>

Formulation for Figure 3.5-9f

<table>
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<th>Stock solution in EtOH(mg/ml)</th>
<th>Mole percentage</th>
<th>Amount needed (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>POPC</td>
<td>40</td>
<td>59</td>
<td>900</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>10</td>
<td>40</td>
<td>1250</td>
</tr>
<tr>
<td>DSPE-mPeg</td>
<td>20</td>
<td>1</td>
<td>75</td>
</tr>
<tr>
<td>Diameter(nm)</td>
<td>135.1</td>
<td></td>
<td>0.126</td>
</tr>
</tbody>
</table>

fVesicle sample was prepared by exclusion method
Figure C- 6: Raw data related to Figure 3- 24: Fluorescence emission at 510 nm for excitation at 403 and 460 nm. Vesicle samples were composed of POPC/ Cholesterol/ DSPE-mPeg at molar ratios of 59% : 40% : 1% with initial lipid concentration of 10 mg/ml; total flow rate was maintained at 12 ml/min with lipids to buffer ratio of 1:3.
Figure C-7: Raw data related to Figure 3-25: Fluorescence emission at 510 nm for excitation at 403 and 460 nm. The vesicle samples were composed of POPC/Cholesterol/DSPE-mPeg at molar ratios of 59%: 40%: 1% with initial lipid concentration of 25 mg/ml; flow rate was maintained at 12 ml/min with lipids to buffer ratio of 1:3.
Figure C-8: Raw data related to Figure 3-27: Fluorescence emission at 510 nm for excitation at 403 and 460 nm. The vesicle samples were prepared by Extrusion method; the lipid mixture was composed of POPC/Cholesterol/DSPE-mPeg at molar ratios of 59%: 40%: 1% with initial lipid concentration of 50 mg/ml.
Figure C- 9: Raw data related to Figure 3- 28: Fluorescence emission at 510 nm for excitation at 403 and 460 nm. The vesicle samples were composed of POPC/ Cholesterol/DSPE-mPeg at molar ratios of 59%: 40%: 1% with initial lipid concentration of 10 mg/ml; flow rate was maintained at 12 ml/min with lipids to buffer ratio of 1:3 in the phosphate buffer with 0.1 M of KCl
Figure C-10: Raw data related to Figure 3-29: Fluorescence emission at 510 nm for excitation at 403 and 460 nm Gramicidin assay with the vesicle samples were composed of POPC/Cholesterol/DSPE-mPeg/SAdiCbr20 at molar ratios of 53%:40%:1%:6% with initial lipid concentration of 10 mg/ml; flow rate was maintained at 12 ml/min with lipids to buffer ratio of 1:3.
Figure C-11: Raw data related to Figure 3-30: Fluorescence emission at 510 nm for excitation at 403 and 460 nm. Gramicidin assay with the vesicle samples were made by extrusion method; lipid mixture composed of POPC/Cholesterol/DSPE-mPeg: SAdiCbr20 at molar ratios of 53%:40%:1%:6% with initial lipid concentration of 50 mg/ml
Figure C-12: Raw data related to Figure 3-31: Fluorescence emission at 510 nm for excitation at 403 and 460 nm. Gramicidin assay with the vesicle samples were made by extrusion method; lipid mixture composed of POPC/Cholesterol/DSPE-mPeg: SAmonoCbr20 at molar ratios of 50%:40%:1%:9% with initial lipid concentration of 50 mg/ml.
Figure C-13: Raw data related to Figure 3-32: Fluorescence emission at 510 nm for excitation at 403 and 460 nm. The vesicle samples were composed of POPC/Cholesterol/DSPE-mPeg at molar ratios of 59%: 40%: 1% with the initial lipid concentration of 10 mg/ml; flow rate was maintained at 12 ml/min with lipids to buffer ratio of 1:3.
Figure C-14: Raw data related to Figure 3-33: Fluorescence emission at 510 nm for excitation at 403 and 460 nm. The vesicle samples were made by exclusion method; lipid mixture composed of POPC/Cholesterol/DSPE-mPeg at molar ratios of 59%: 40%: 1% with initial lipid concentration of 50 mg/ml.