Chapter 3

LIPOSOMES AS MODEL CELLULAR SYSTEMS

Liposome formation and applications in microfluidics

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Abstract:

Key words: liposomes, vesicles, hydrodynamic focusing, nanoparticles, mixing

1. INTRODUCTION

The discovery that phospholipids spontaneously form fluid-filled bubbles when exposed to aqueous solution was first made by Alec Bangham in the early 1960's while studying the effects of phospholipids on blood clotting in the Babraham Institute in England.[1] These tiny spherical fluid-filled bubbles became known as liposomes, a term derived from the Greek word, *lipos*, meaning fat. In forming liposomes, amphipathic phospholipids self-associate to create a bilayer membrane with a hydrophobic interior (where the long-chain carbon tails aggregate) and hydrophilic exteriors (where the polar headgroups assemble on both the inner and outer surfaces of the membrane) as shown in Figure 1. A cellular membrane is similarly composed of a phospholipid bilayer membrane but with additional components that include cholesterol and proteins. Therefore, the liposome membrane is the simplest model cell system and can be used to evaluate cellular characteristics such as permeability, stability, and elasticity.[2]

Soon after their discovery, it was observed that liposomes can surround and encapsulate chemical species that are diluted in the aqueous solution in which they form. These two scientific discoveries – the spontaneous assembly of phospholipids into cell-like liposomes and the ability to

randomly encapsulate molecular species – prompted researchers to include liposomes in their theories on the origin of life.[3] The fact that liposomes could be used to stably encapsulate a wide range of biologically active and therapeutically important molecules also led to their use as nanoparticles for targeted drug delivery.[4] Liposomes are now used to encapsulate a wide range of analytes for diverse applications as food additives, cosmetic

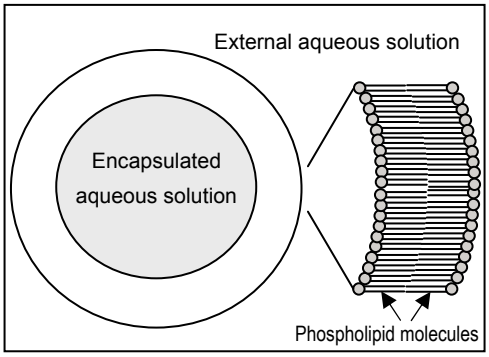

Figure 1. Schematic of a cross section of a spherical liposome.

products, and novel drug formulations. Due to their biomimetic nature, liposome products are readily adapted for human consumption or usage.

We were one of the first research groups to report on the use of liposomes as analytical reagents in the 1980's.[5] We reported that liposomes could be used as fluorescent labels in clinical immunoassays for drugs and hormones in a flow injection format[6-10] or in a planar waveguide optical sensor format.[11] For these studies, liposomes were prepared containing high concentrations of fluorescent dyes encapsulated within their aqueous interiors. The liposome membrane was also coated with antibodies or antigens so that the liposomes could participate in molecular recognition events, leading to their use as detection markers in assays. These liposome formulations were injected into a flow stream and once bound to the compound of interest would fluorescently mark it. One advantage of using liposomes as fluorescent labels for molecular recognition assays (immunoassays, hybridization assays, etc.) is the ability to amplify the result: a single liposome can encapsulate approximately one million fluorescent molecules and therefore magnifies the signal associated with a single binding event as compared to typical fluorescent labeling strategies where one or a few fluorophores are detected per binding event. Due to their ability to amplify the signal, liposome immunoassays compete well with enzyme immunoassays (another common signal amplification strategy) in

terms of assay sensitivity.[9] A second advantage associated with liposomes is that the apparent affinity constant of the liposome reagent can be significantly altered by changing the concentration of the binding molecule (antibody, antigen, DNA) on the liposome membrane.[12] This provides an effective way to alter the binding and dissociation kinetics that are important for the molecular recognition event, thus modifying assay sensitivity and dynamic range. More recently, we have extended this work to the microfluidic format by demonstrating that liposomes can be used as fluorescent labels in immunoassays[13] and DNA hybridization[14] assays (with a collaborator at Cornell University, R.A. Durst) in microfluidic systems. In the past few years, we also have been exploring other applications of liposomes as analytical reagents for microfluidic assays. In particular, in this chapter we will describe the use of liposomes for reagent delivery and mixing in a microfluidic device.

Our interest in using liposomes for a variety of analytical applications has led us to focus some of our research efforts on the development of methods for the automated production of liposomes with encapsulated reagents. Because of the importance of liposomes in many fields, formulation methods that can improve their quality and shelf life or that can simplify their production are valuable. In this chapter, we will describe our new method for automated liposome production in a microfluidic system and point out advantages associated with this novel technique.

2. LIPOSOME FORMATION IN MICROFLUIDIC SYSTEMS

Liposomes can be produced by several different methods resulting in liposome populations that vary in average size, size distribution, average number of lamellae (bilayers) per liposome, and encapsulation efficiency. Encapsulation efficiency refers to the amount of analyte that is entrapped in the liposome upon formation and is often expressed as a percentage from the ratio of analyte concentration inside the liposomes versus the analyte concentration in the bulk solution in which the liposomes form. The characteristics of the liposome population play an important role in determining their suitability for a particular application. For instance, when liposomes are used to encapsulate drugs for therapeutic drug delivery, it is critical that the liposomes are homogeneous in all respects - size, size distribution, lamellarity, and encapsulation efficiency – in order to ensure that precise dosages are being administered with each aliquot of the liposome solution. It is also critical that liposome populations be highly homogeneous for analytical applications such as reagent packaging and

reagent delivery to guarantee accurate and reproducible results. In this section, the characteristics of liposomes formed in a microfluidic system will be compared to liposome populations formed using other bulk production techniques focusing on size, size distribution, and encapsulation efficiency.

2.1 Traditional Liposome Formation Methods: Bulk Techniques

In the past 40 years, many methods have been introduced for the production of liposomes using bulk techniques.[15] These include film hydration,[1] reverse phase evaporation,[16] alcohol injection,[17] detergent removal,[18] and electroformation.[19] Some of these techniques produce heterogeneous populations of larger liposomes that can be further processed to decrease size, increase population homogeneity, and increase encapsulation efficiency. Some liposome post-processing techniques that are commonly used include freeze-thaw cycling,[20] sonication,[21] and membrane extrusion.[22] Liposomes that are formed from all techniques can be classified as large multilamellar vesicles (MLV, ~400 nm to 3500 nm in diameter), large unilamellar vesicles (LUV, ~100 nm to 1000 nm in diameter) or small unilamellar vesicles (SUV, ~20 nm to 200 nm in diameter). Unilamellar vesicles have one phospholipid bilayer comprising the membrane, and multilamellar vesicles have more than one phospholipid bilayer comprising the membrane. A few of the most common approaches to liposome formation by bulk techniques will be described in more detail here.

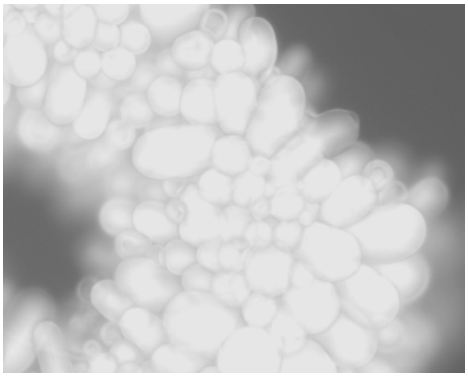

Figure 2. Liposomes prepared by the film hydration method. A droplet of phosphate buffer containing carboxyfluorescein (a fluorescent dye) was added to a dried phospholipid film. Upon liposome formation, the dye was encapsulated. Excess unencapsulated dye was rinsed away with fresh phosphate buffer after the liposomes were formed.

Some of the first methods that were described in the literature involve liposome formation at a solid-liquid interface where a dried film of phospholipid molecules is placed in contact with a droplet of aqueous solution. Under these conditions, liposomes spontaneously form and appear to peel off from the dried film as the phospholipids become hydrated. This process was first described mechanistically by Lasic et al. [23] Figure 2 shows a population of liposomes prepared in our lab by the film hydration method and that encapsulate a solution of fluorescent dye for imaging by fluorescence microscopy. It should be noted that these liposomes are not uniform or spherical. Liposomes prepared by the film hydration method are generally large, highly multilamellar with thick membranes (MLVs), inhomogeneous with respect to both size and shape, and with lower encapsulation efficiencies. Liposomes prepared in this manner often exhibit a broad distribution of sizes ranging in diameter from hundreds of nanometers to several micrometers. Therefore, without further processing or modification, these liposomes are not typically suited for use in analytical or therapeutic applications.

The reverse phase evaporation technique is one of the most common liposome preparation methods and involves liposome formation at a liquid-liquid interface. In this technique, the phospholipids are first dissolved in an organic solvent that is immiscible in water, such as chloroform. The phospholipid solution and an aqueous solution containing the dissolved analyte are added to a flask. The organic solvent is then removed under reduced pressure at a controlled temperature using a rotoevaporator. Liposomes prepared in this manner are generally large with either one (LUVs) or a few lamellae comprising the membrane. A bright field image of two large multilamellar liposomes prepared at NIST using the reverse phase evaporation method is shown in Figure 3. The liposomes shown in the

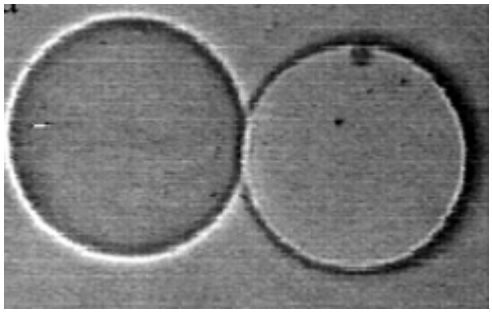

Figure 3. Multilamellar liposomes prepared by the reverse phase evaporation method and imaged using bright field microscopy. Liposome diameter is approximately 20 μm.

figure are approximately equal in size; however, the population of liposomes prepared in batch using this technique in our lab ranged in size from 50 nm to 50 μm.[24] As with the film hydration method, most of the liposomes prepared by reverse phase evaporation require post-processing for use in analytical applications for microfluidics and other assays.

Liposome populations that are more homogeneous with higher encapsulation efficiencies can be prepared using liquid-liquid formation techniques such as alcohol injection. In the alcohol injection method, phospholipids are thoroughly dried into a film from a solution in chloroform or a similar solvent. The dried film is then redissolved in a small amount of alcohol, a solvent that is miscible in water. The phospholipid solution in alcohol is injected via syringe into a rapidly vortexing aqueous solution containing the dissolved analyte. Liposomes formed in this manner in our lab are relatively homogeneous and unilamellar (SUVs) with population size distributions that range from 70 nm to 200 nm as determined by laser light scattering.[7] These liposomes are generally too small to view by bright field microscopy but can be imaged using fluorescence microscopy with encapsulated fluorophores. These liposomes are sufficiently uniform to use in many of our analytical applications without further processing; however, more homogeneous populations would further increase the reproducibility of assays that employ liposomes as reagents.

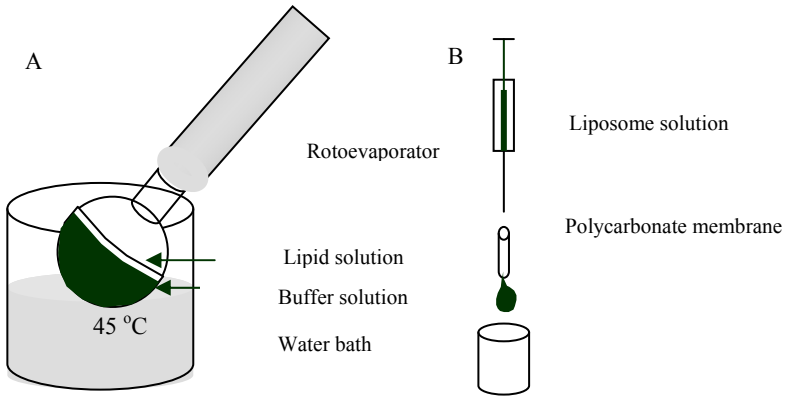

Figure 4. Liposomes prepared by the A) reverse phase evaporation method are processed after preparation by B) membrane extrusion.

As mentioned previously, liposomes are often subjected to post-processing techniques to decrease the average size of the population, improve population homogeneity, or increase encapsulation efficiency. Frequently, we use membrane extrusion to reduce size and increase the

homogeneity of liposomes that have been prepared using other techniques. Figure 4 shows the two-step process wherein liposomes are formed by reverse phase evaporation and then post-processed using membrane extrusion. In membrane extrusion, the solution containing liposomes is placed into a syringe that is used to press the liquid through a polycarbonate filter as shown in the figure. The liposome solution is often passed sequentially through many different polycarbonate filters of varying pore size. In a typical procedure, liposome preparations are passed once through 3 μm and 0.4 μm filters, then five times through 0.2 μm filters. After the extrusion process, the resulting liposomes ranged in size from 145 nm to 565 nm.[25]

Another liposome post-processing technique involves sequential iterations of flash freezing the liposome preparation in liquid nitrogen followed by rapid thawing in a 90 ºC water bath. The freeze-thaw cycling technique is typically used immediately after the liposomes are formed by another method. This technique is relatively simple and rapid to implement and has, in our laboratory, resulted in a two-fold increase in the encapsulation efficiency of common fluorophores into diacyl phosphatidylcholine liposomes formed via the film hydration method. After post-processing by freeze-thaw cycling, liposomes prepared using the film hydration method ranged in size from 2.07 μm to 3.27 μm in diameter as determined by laser light scattering.[26] The population homogeneity of these liposomes is significantly better than that of the original preparation (see results above from the film hydration method).

2.2 Liposome Formation in Microfluidic Systems

Recently, we reported on the development of new methods for the formation of liposomes in a microfluidic system.[27] The two bulk methods that we investigated as potentially useful for implementation in a microfluidic format were the film hydration method and the alcohol injection method. In attempting to reproduce the film hydration method in a microchannel, we were not successful in forming liposomes; however we were able to form other stable lipid structures such as microtubes and nanotubes. Initial observations of lipid microtubes made in this manner were briefly described at the MicroTAS 2003 conference[28] and will be further reported in a future manuscript. We were, however, successful in developing methods for automated liposome formation in a microfluidic system based on the alcohol injection method.

A microfluidic system for liposome formation experiments was developed using a simple double cross design as shown in Figure 5. To approximately mimic the alcohol injection method in a microfluidic format,

an alcohol solution containing dissolved phospholipids and fluorescent dye was introduced into the center inlet (inlet 1) in the first cross. Aqueous buffer solutions were introduced into the two side inlets, inlets 2 and 3, in the first cross. Two syringe pumps were used to control the fluid flow and were connected via capillary tubing to the microfluidic device using Nanoports™ (Upchurch Scientific, Oak Harbor, WA) to interface capillaries to the microfluidic channels. The alcohol solution was pumped into inlet 1 by one syringe pump, and the buffer solution was pumped into inlets 2 and 3 using a second syringe pump. The fluid flowed out of cross 1 and into the center output channel that eventually intersected with cross 2. Outputs 4 and 5 were waste channels, and output 6 was the liposome collection channel. A photograph of the assembled device, fabricated in silicon and glass, is also shown in Figure 5.

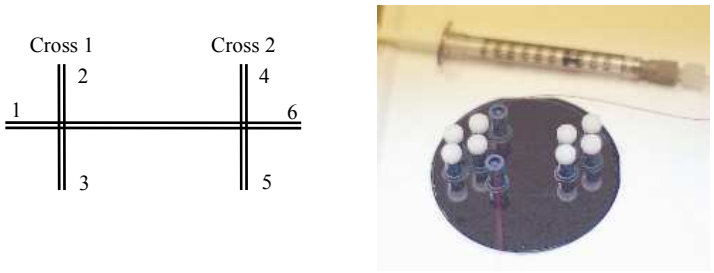

Figure 5. Shematic of a double cross injector shown on the left and an assembled microfluidic device fabricated in silicon and glass is shown on the right with ports and capillary tubing attached.

The buffer solutions in the side inlet channels (inlets 2 and 3) were used to focus the alcohol fluid flowing in the center inlet (inlet 1) based on the principles of hydrodynamic focusing. For most experiments, the flow velocity of the alcohol solution was held constant while the flow velocity of the buffer solution in the side inlet channels was varied. When the flow velocity of the buffer in the side inlet channels was equal to the flow velocity of the alcohol solution in the center inlet channel, the resulting output stream was composed of three stripes of fluid that were approximately equal in width. As the flow velocity in the side inlet channels was increased, the width of the alcohol stream was reduced and thus could be focused down to a very thin stripe (less than1 μm wide) in the middle of the channel as shown in Figure 6. By reducing the width of the center alcohol stream, we were

able to control the dilution rate of the alcohol into the surrounding buffer - an important parameter for liposome formation.

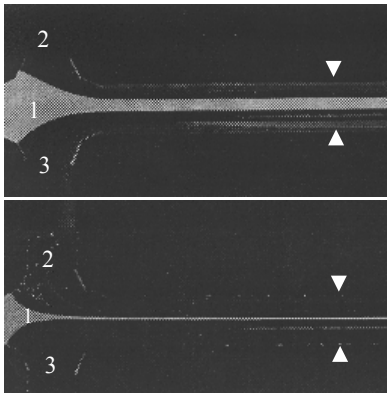

Figure 6. Fluorescence images of microfluidic device with flow rates in inlets 2 and 3 (top) equal to inlet 1; and (bottom) faster than inlet 1 showing the effect of hydrodynamic focusing. For these images, the alcohol solution contains a fluorescent dye and appears as the bright solution in the image; the buffer solution contains no dye and appears dark in the image. White arrows indicate the locations of the edges of the microfluidic channels.

To prepare the phospholipid solution in alcohol, an aliquot of phospholipid solution in chloroform was placed into a small glass test tube along with an aliquot of solution containing 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate ($DiIC_{18}$). $DiIC_{18}$ is a molecule that is composed of a fluorescent dye attached to a long chain carbon tail that inserts into the liposome membrane as it forms. Upon intercalation into the membrane, the quantum efficiency of the fluorescent dye increases and the fluorescence signal is greater. The solution containing phospholipid and fluorescent dye was initially dried down under nitrogen to form a film that was further dried overnight in a vacuum desiccator. The film was then redissolved in an aliquot of dry isopropyl alcohol, and this was used as the stock phospholipid solution for all microfluidic experiments.

The phospholipid molecules are very soluble in isopropyl alcohol but are not soluble in water. In the microfluidic experiment described above, as the concentration of the isopropyl alcohol is decreased by diffusion and dilution into the surrounding aqueous buffer, the phospholipid solubility decreases. Liposome formation occurs when the concentration of the isopropyl alcohol is sufficiently low that the formation of the more stable and soluble liposome structure is energetically favored. This first occurs in our experiment at the buffer-isopropyl alcohol interfacial region. An increased fluorescence is observed at the interface of the two fluids that provides evidence of liposome

formation as the $DiIC_{18}$ inserts into the liposome membrane as shown in Figure 7. Individual liposomes cannot be seen in this figure because the liposome size is below the resolution of the optics.

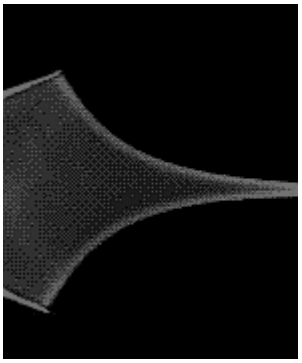

Figure 7. Fluorescence image taken at Cross 1 demonstrating liposome formation at the buffer-isopropyl alcohol interface.

The center stream remained tightly focused as it progressed down the microfluidic channel for collection via output 6, shown in Figure 8. The observation that the fluorescent stream stayed tightly focused provided further evidence of liposome formation since, under these conditions of flow rate and channel length, a nanoparticle (liposome) would not have sufficient time to diffuse and dilute into the surrounding buffer. On the other hand, a small molecule, with a diffusion coefficient that is 2-3 orders of magnitude less than that of the liposome nanoparticle, would diffuse and dilute significantly under the same conditions.

To form liposomes in the microfluidic system, the flow velocity of the phospholipid solution in isopropyl alcohol was held constant at 2.4 mm/sec. The buffer flow velocity was increased between 2.6 mm/sec and 60 mm/sec.

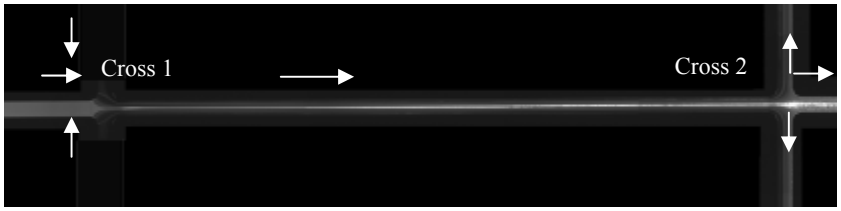

Figure 8. Fluorescence image of microfluidic device showing that the liposome solution remains focused in the microchannel (starting at Cross 1) until the solution reaches Cross 2. At Cross 2, the solution flows from a single channel into three separate channels of equal dimension thus significantly slowing the flow rate and causing dispersion of the focused liposome suspension. Arrows show flow direction.

The ratio of the buffer flow velocity to the isopropyl alcohol solution flow velocity was defined as the flow ratio. Liposomes formed in the microfluidic device at six different flow ratios were collected and analyzed using laser light scattering to assess average size and population homogeneity. In general, liposomes formed at lower flow ratios were less homogeneous with an average liposome diameter that was larger. Liposomes formed at higher flow ratios where the phospholipid solution was more tightly focused were more homogeneous with an average liposome diameter that was smaller. At the lowest flow ratio of 1.08, the resulting liposomes ranged in size from 160 nm to 300 nm (average size = 230 nm, RSD = 30 %). At the highest flow ratio of 25, the resulting liposomes ranged in size from 80 nm to 135 nm (average size = 108 nm, RSD = 25 %). Note that both of these liposome populations are more homogeneous than those prepared using the traditional alcohol injection technique (with the same phospholipid mixture) where the average size was 135 nm with an RSD of 48 %.

All analytical applications of liposomes require the encapsulation of analytes and reagents inside the aqueous interior of the liposomes and the separation of unencapsulated analytes from liposome-encapsulated analytes (clean-up). Therefore, it was critical to determine whether the microfluidic method described here could be used to automate the encapsulation and clean-up processes. In this set of experiments, a water-soluble fluorescent dye, carboxyfluorescein, was dissolved into the buffer solution and introduced into the microfluidic device through inlets 2 and 3. Liposomes were formed as described previously and collected through output 6. These liposomes were then dialyzed in buffer to remove any unencapsulated analyte. Using confocal fluorescence microscopy, we determined that the liposomes had an interior aqueous volume that contained carboxyfluorescein. The second cross in the microfluidic channel network was designed as an initial attempt to partially separate liposomes containing encapsulated analyte from the unencapsulated analyte. The fluid flow in the center channel was equally distributed to 3 different channels through this cross with all liposomes directed through output 6 (Figure 8). Therefore, at least two-thirds of the solution containing the unencapsulated analyte was directed to waste through outputs 4 and 5. This served to concentrate the liposomes but was obviously not successful in separating liposomes from all unencapsulated analyte. New microdevice designs are being tested to improve this aspect of the work.

2.3 Conclusions and Future Directions: Microfluidic Liposome Formation

Investigations are underway in our laboratory to allow us to understand why liposome formation is more reproducible in microfluidic methods than in bulk techniques, and to elucidate the mechanism for controlling the size of the liposome population by controlling the flow ratio between the two co-flowing liquids. We postulate that both effects are due to the fact that the stable laminar flow in the microchannel device can be used to precisely control the distribution of chemical conditions and mechanical forces at the liquid-liquid interface so that they are constant and controllable on the length scale of the liposome. Further experiments and fluid modeling should help us to determine which chemical and/or mechanical effects (*e.g.*, temperature, shear force, viscosity, chemical concentration, etc.) are the most important in dictating size and population homogeneity.

3. ANALYTICAL APPLICATIONS OF LIPOSOMES IN MICROFLUIDICS: MICROFLUIDIC REAGENT DELIVERY AND MIXING

3.1 Introduction

Nature uses liposome-like vesicles for the controlled localization, delivery, mixing, and activation of chemical species within the micro-scale environment of the biological cell. In many cases, cell and tissue function is regulated by the segregation of certain species in vesicles with subsequent controlled delivery of these vesicles to a particular functional unit of the cell or to an entirely separate cell. This is an elegant motif that nature has evolved over several billions of years for precise reagent delivery that allows for exquisite control of mixing and chemical interaction in systems that function on the micrometer length scale.

Commonly, delivery and mixing of chemical species in the biological cell can be accomplished with a characteristic time on the order of milliseconds, whereas mixing of reagents in a typical microfluidic system can require seconds to accomplish. Currently, reagent mixing is one of the limiting aspects of the microfluidic systems as mixing can often require more time and device area than is ideal due to the fundamental physics of fluid flows in microscale geometries. By using reagent-filled liposomes in microfluidic devices, we can simulate cellular vesicle-mediated mixing in microfluidic systems to accomplish mixing on a time scale of several

milliseconds approaching that of evolutionarily optimized biological systems.

Liposome-mediated mixing in microfluidic systems relies on two characteristics of liposomes to function effectively: (1) the liposomes' ability to encapsulate a chemical species and thereby prevent it from interacting with aqueous species that reside outside the liposome; and (2) the ability to render the liposomes permeable at a controlled time and location. With these two characteristics, the liposomes can be used to encapsulate and sequester reagents until their controlled and programmed release from the liposomes allows them to participate in a chemical reaction outside of liposome membrane in the microfluidic channel. This technique is particularly applicable to biological reactions as the liposome mimics both the size and chemical characteristics of the biological cell wall; thus, biological molecules are highly compatible with liposome-mediated mixing approaches.

3.2 Liposome-Mediated Mixing

Mixing in microfluidic systems, in its most simple embodiment, occurs by merging two separate fluidic channels into a single channel and allowing Fickian diffusion to mix the species in the two co-flowing streams. (Turbulent mixing is typically not accessible in microfluidic systems owing to the low Reynold's numbers of these systems.) Thus, mixing occurs through diffusion and is a relatively slow process, particularly for large biomolecules, since the rate of diffusion is inversely proportional to molecular weight. Several novel techniques have been introduced in the past few years to improve the microfluidic mixing process including adding surface features to the microchannel wall to passively mix the fluids[29-32] and incorporating components into the device to actively mix the fluids.[33] To date, these techniques all function by advecting certain fluid elements into other fluid elements thereby decreasing the length that a reagent must diffuse in order to be mixed.

Liposome-mediated mixing is implemented by uniformly dispersing reagent-filled liposomes within a given region of a microfluidic device. This can be done using pressure-driven fluid flow, electroosmotic flow (although care must be give to not electroporate the lipid membrane), and optical tweezer manipulation among other methods. At the appropriate place and time, the liposomes are permeabilized causing the contents to diffuse out from the liposomes' interior volume. Permeabilization can be actuated through a variety of triggers including temperature,[34] electric potential,[35] and pH modulation.[36] Mixing in this case is still driven by diffusion; however, since the liposomes are uniformly dispersed in the microchannel at

the point where mixing and reaction occur, mixing is extremely rapid when compared to other common diffusive mixing approaches. This is due to the fact that the length scales for the diffusive mixing are markedly shorter in a liposome-mediated mixing technique. For example, typical microchannels are on the order of 50 μm in diameter, thus the length scale for diffusion when two fluids are introduced through a 't' junction into a co-flowing stream is 25 μm. In liposome-mediated mixing, the length scale is determined by the liposome membrane thickness (<10 nm) plus the average distance between liposomes (on the order of 1 μm to 5 μm), which to first order is determined by the concentration of the liposomes in suspension.

We have demonstrated liposome-mediated microfluidic mixing using temperature to control reagent release[26]. A given liposome population can be characterized by the phase transition temperature, T_m, defined as the temperature at which the membrane undergoes a gel-to-liquid-crystalline phase transition. The liposome is impermeable to ionic aqueous species when in either the gel or liquid-crystalline phases; however, liposomes become permeable at their characteristic T_m. At the T_m, both gel and liquid-crystalline phases are present in a given liposome producing grain boundaries between the two phases. The portion of the liposome membrane at the grain boundaries is permeable allowing for the passage of ionic species both into and out of the liposome for reaction.

An example of liposome-mediated microfluidic mixing is shown in Figure 9. For this study, liposomes were prepared encapsulating the water-soluble fluorophore, carboxyfluorescein (CF), at a high concentration where the fluorescence is largely self-quenched. The liposomes then flow through a microfluidic channel that has a lateral temperature gradient.[37] As the liposomes travel through the channel they experience progressively higher temperatures. At a unique point in the channel (a point where $T_{channel} = T_m$), they begin to phase transition from the gel to liquid-crystalline phase. At this point, the encapsulated CF diffuses out from the liposome cavity and dilutes into the liquid in the microchannel. When the CF is sufficiently diluted, it is no longer self-quenched as is evidenced by the increase in microchannel fluorescence at the temperature where the phase transition occurs. In this example, mixing is complete in approximately 200 μm. At the given flow velocity, this mixing distance corresponds to a mixing time of 110 msec, which is the time required for the CF to diffuse out of the liposome and reach a steady state concentration in the fluid in the microchannel. This is substantially more rapid than the 5 seconds that would be required to accomplish diffusive mixing between two co-flowing streams in the same microchannel.

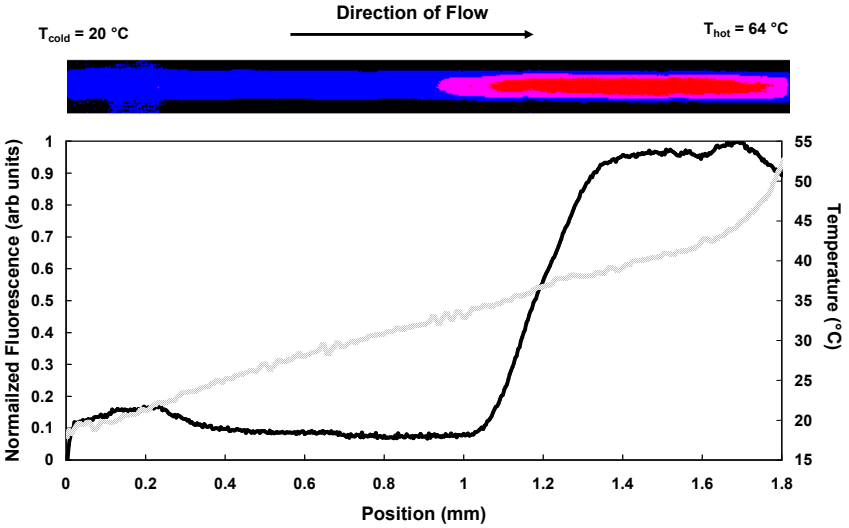

Figure 9. A fluorescence micrograph of a solution of 95 mol% 1,2-Dipalmitoyl-sn-Glycero-3-Phosphocholine (DPPC), 5 mol% cholesterol liposomes in Tris buffer encapsulating self-quenched 200 mM CF flowing through a microfluidic channel under an applied temperature gradient of 20 °C to 64 °C over a 2 mm distance at a flow rate of 5 µL / h. The increase in fluorescence down the channel is caused by the controlled thermal permeabilization of the liposomes. The graph shows a plot of temperature (dotted) and fluorescence (solid) in the channel as a function of lateral position. From reference 26.

The position in the microchannel where the reagent release occurs is controlled by simply adjusting the temperature at the channel termini to change the temperature gradient. Figure 10 demonstrates the movement of this "mixing" point through modulation of the temperature gradient by changing the temperature at one channel terminus. In this example another fluorophore, sulforhodamine B (SRB), was encapsulated in liposomes at a concentration of approximately 10 mM. As in Figure 9, the fluorophore is largely self-quenched inside the liposomes. After thermal permeabilization, self-quenching is eliminated by reagent release and dilution, leading to a large increase in fluorescent signal. In this example the temperature at the microchannel outlet is increased incrementally in each panel of the figure, therefore, the phase transition temperature shifts left towards the inlet end of the microchannel. By simply changing the temperature of the channel terminus, the position where release and mixing occur can be accurately controlled.

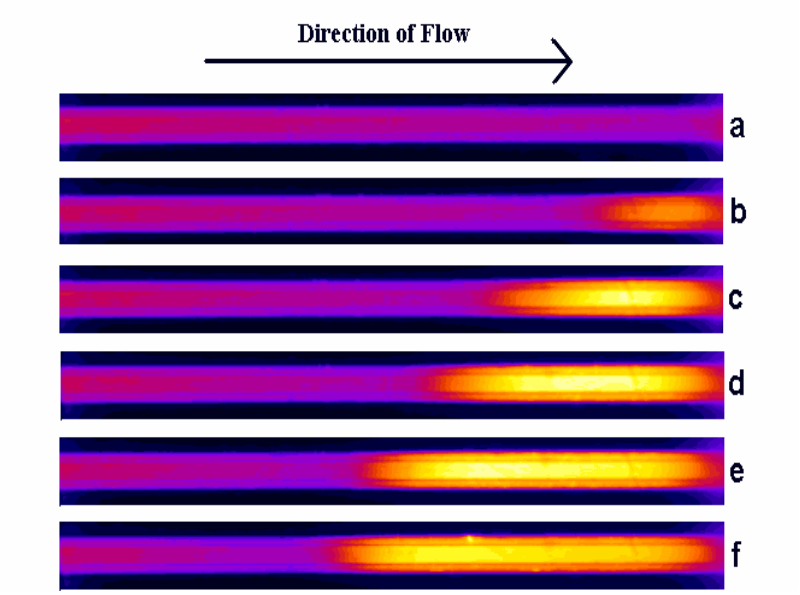

Figure 10. Fluorescence images of solution of 97 mol% DPPC, 3 mol% cholesterol liposomes encapsulating self-quenched 100 mM sulforhodamine B in 0.5 M Tris buffer flowing through a polycarbonate microfluidic channel at a flow rate of 5 μL / h. The temperature gradient applied to the microchannel in each case is as follows; a) no temperature gradient; b) 20 °C to 45 °C; c) 20 °C to 50 °C; d) 20 °C to 55 °C; e) 20 °C to 60 °C; f) 20 ^{o}C to 65 ^{o}C. From reference 26.

It should be noted that temperature actuated liposome-mediated mixing is applicable for small molecules, but would be less effective in releasing large molar mass biomolecules from within the liposomes. The "cracks" generated by grain boundaries between regions of gel phase and liquid-crystalline phase are too small to allow for efficient release of large biomolecules into the aqueous space outside of the liposome. For mixing of molecules with larger molar mass, other options may be more appropriate depending on the precise nature of the application. For instance, the species of low molecular mass can be loaded into the liposome, and the large species (biomolecule) can reside in the bulk fluid in the microfluidic channel. Permeabilization would allow release of the smaller molecule for efficient mixing with the biomolecule in the bulk solution as we have demonstrated in previous work. If both species to be intermixed are large molecules, then a permeabilization technique that is more disruptive to the membrane should be employed. For instance, membrane destabilization can be light activated by the incorporation of *trans* to *cis* photoisomerizable lipids into the liposome membrane.[38] When the liposome is exposed to light of an

appropriate wavelength, isomerization of the hydrocarbon phospholipids tails will allow for poration and the release of large, low diffusion molecules.

3.3 Controlled Sequential Mixing and Reagent Interaction

If multiple formulations of liposomes, each containing a unique species, are designed so that mutually independent triggers can permeabilize them, then multiple species can interact with the microfluidic system in a sequential manner to perform a series of chemical reactions. We have demonstrated this type of sequential reaction system using thermally actuated release of common fluorophores. For this study, two formulations of liposomes are prepared, one with a lower phase transition temperature and one with a higher phase transition temperature, each encapsulating different fluorophores. The lower T_m liposome contains self-quenched CF, and the higher T_m liposome contains self-quenched SRB. The liposome suspension flows through a microchannel with a lateral temperature gradient by pressure-driven flow as described above. In the initial low temperature section of the channel, both fluorophores are encapsulated in their respective liposomes. At the release point for the CF-filled liposomes, the green fluorescence in the microchannel begins to increase as the CF diffuses from the liposome cavity and is diluted in solution to the point where it is no longer self-quenched (Figure 11).

The green fluorescence in the microchannel is then high and relatively constant up to the point where the SRB-loaded liposomes become permeable. At this point, the SRB diffuses from the higher T_m liposomes, and the red fluorescence in the channel begins to increase. Interestingly, at the same point that the red fluorescence (SRB) begins to increase, the green fluorescence (CF) begins to decrease. This is due to the fluorescence resonance energy transfer interaction between the two fluorophores resulting in the lower green fluorescence, and it demonstrates the ability to perform sequential reagent release and reaction.

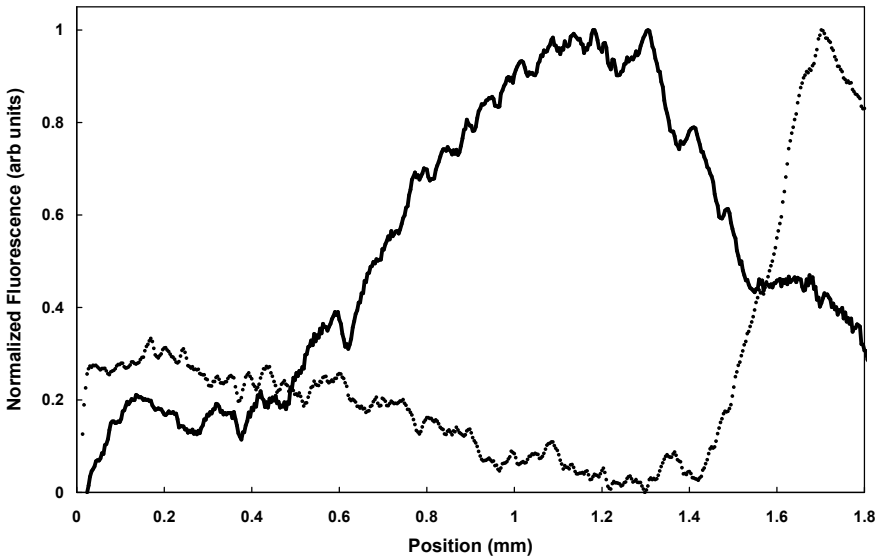

Figure 11. Fluorescence of a solution of liposomes (90 mol% DPPC, 10 mol% cholesterol) encapsulating self-quenched CF and liposomes (75 mol% DPPC, 15 mol% DSPC, and 10 mol% cholesterol) encapsulating self-quenched SRB flowing through a polycarbonate microfluidic channel. Applied temperature gradient = 20 °C to 44 °C; flow rate = 5 μL / hour. The graph shows the fluorescence intensity versus lateral position in the microchannel with the solid line indicating CF fluorescence and the dashed line indicating SRB fluorescence. From reference 26.

3.4 Modulating Liposome Formulations to Control Liposome-Mediated Mixing Reactions

Liposome formulations can be prepared with a wide range of phase transition temperatures to perform programmed chemical reactions in a microfluidic system. Each phospholipid molecule has a characteristic T_m, and liposomes that are composed of only one type of phospholipid will have the same T_m as the phospholipid molecule. The T_m of the phospholipid is dependent on the characteristics of the hydrocarbon tail and the polar headgroup of the molecule. The dependence of the T_m on hydrocarbon tail length (carbon number) is shown in Table 3-1. Liposome formulations that are composed of several different phospholipids will have a composite T_m that is influenced by all individual lipids in the bilayer membrane. For instance, a liposome composed of two types of phospholipids with T_m's of –1 ^oC and 23 ^oC (from the table) will have a T_m that is between those two temperatures. The T_m of the liposome formulation can also be modified by

the addition of other components such as cholesterol. Therefore, to perform liposome-mediated microfluidic mixing, we first choose the desired T_m of the liposome population based on the microfluidic system design and the properties of the chemical or biochemical reactants. Then, the appropriate liposomes are engineered based on knowledge of the phase transitions for all potential phospholipid components and chemical additives. The ability to design the liposomes with the desired T_m provides a great deal of flexibility to our approach.

Table3-1. Phase Transition Temperature as a Function of Tail Group Length

Tail Group (Common Name)	Carbon Number	T_m (^oC)
Lauroyl	12	-1
Myristoyl	14	23
Palmitoyl	16	41
Stearoyl	18	55

3.5 Conclusions and Future Directions: Liposome-Mediated Microfluidic Mixing

For the implementation of this technique in our laboratory, the temperature of the microfluidic system was always modulated by applying a temperature gradient across a single microfluidic channel. A limitation associated with this approach, particularly when we want to perform a series of sequential reactions, is that the T_m of the liposome populations must be very close (often within a few degrees of one another), making their formulation a little more difficult. Previously, silicon micromachined heating elements[39,40] and other resistive heaters[41-43] have been integrated into microfluidic systems in order to achieve temperature control for chemical reaction. By integrating microheating elements into our system for liposome-mediated mixing, a series of complex chemical reactions could be initiated at specific points in the device, thus simplifying the timing of each chemical reaction by individually programming each microheater. Further control of reaction timing and sequence is then provided by the microchannel system design (channel length between each reaction) and the flow rate. This approach potentially provides even greater flexibility to the liposome-mediated microfluidic mixing technique.

4. CONCLUSIONS

We have demonstrated several potential applications for liposomes in a microfluidic format focusing here on their formation and use for reagent

packaging and delivery. As cell-like structures, liposomes are well adapted for microfluidic applications, mimicking the movement of cells in the circulatory system. In fact, large liposomes can deform and flow around corners and obstructions in a microfluidic system without disrupting the membrane in a manner similar to the way that red blood cells deform in the body as they move through small capillaries.

The ability to package analytes and markers (fluorescent dyes and electrochemical markers) in high concentrations inside the liposome cavity makes them very useful as analytical reagents for a wide variety of potential applications that are only beginning to be realized in a microfluidic format. Formulations of liposomes with encapsulated analytes have been made that have a long shelf life of over a year when stored at room temperature; therefore they can also be robust reagents.[44] The stability of the liposome makes it useful as a nanovial or nanocontainer;[24] however, controlled permeabilization actuated by temperature, pressure, light, etc., extends its use to the performance of elegantly controlled nanoscale chemical reactions.

The ability to form high quality liposome reagents on-demand in the microfluidic format should enhance their use as analytical reagents. To date, we have not combined liposome formation and application in the same microfluidic device. Future work in our laboratory will be directed toward achieving this goal.

NOTES

Certain commercial equipment, instruments, or materials are identified in this report to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Dr. M. Richter of the Fraunhofer Institute Zuverlassigkeit und Mikrointegration, Mikromechanik Aktorik und Fluidik for supporting the guest researcher opportunity for Mr. Andreas Jahn at NIST. The authors also acknowledge support from the National Research Council/NIST Fellowship Program for Dr. Wyatt Vreeland and the NIST Director's Competence Program for funding (Project title: 'Single Molecule Manipulation and Measurement").

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