Microfluidic hydrodynamic focusing based synthesis of POPC liposomes for model biological systems

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Abstract

Lipid vesicles have received significant attention in areas ranging from pharmaceutical and biomedical engineering to novel materials and nanotechnology. Microfluidic-based synthesis of liposomes offers a number of advantages over the more traditional synthesis methods such as extrusion and sonication. One such microfluidic approach is microfluidic hydrodynamic focusing (MHF), which has been used to synthesize nanoparticles and vesicles of various lipids. We show here that this method can be utilized in synthesis of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) vesicles with controllable size. Since POPC is among the primary constituents of cellular membranes, this work is of direct applicability to modelling of biological systems and development of nano-containers with higher biologic compatibility for pharmaceutical and medical applications.

Keywords: liposomes; microfluidics; model biological systems; drug delivery; artificial cells

1 Introduction

Liposomes or lipid vesicles are globular structures whose aqueous interior is surrounded by a bilayer composed primarily of lipid molecules. Since their discovery in the 1960s [1], liposome application has spanned a wide range of fields. They have been used as gene and drug delivery vectors in the pharmaceutical industry [2-5], as contrast agent containers in enhanced medical imaging [6-8], and as nano-sized crystallization reactors [9, 10]. Similarities in the chemical and physical properties of the lipid bilayers of liposomes and living cells have also led to their use as models in studying cellular membranes [1, 11]. Of particular interest in cell biology and biochemistry is also their morphological similarity to the cell and its organelles, which has inspired application of liposomes as model systems of cells and various cellular compartments, such as the endoplasmic reticulum or Golgi apparatus [12-14]. Similarly, synthetic biology has seen efforts to convert liposomes into minimal artificial cells by loading them with essential transcription and translation machinery needed for DNA replication [15, 16]. The use of lipid vesicles in modelling biological systems has been especially facilitated by the simplicity of their chemical composition and the ability to control it precisely.

Traditional methods for liposome synthesis are based on drying of lipids, which promotes organization of their molecules into sheets of bilayers, and subsequent hydration, which facilitates swelling of sheets and formation of spherical compartments [17]. This approach, however, results in creation of multilamellar structures in which liposome walls consist of multiple concentric bilayers. Most liposome applications, especially drug and gene delivery, and modelling of biological systems, require unilamellar vesicles, whose walls are

built of a single lipid bilayer. In order to create these structures, lipid hydration is normally followed by postprocessing methods, such as sonication [18, 19] or extrusion [20], in which multilamellar vesicles are transformed into unilamellar counterparts through the action of mechanical shear forces. While these techniques successfully disperse the majority of larger liposomes, a not insignificant fraction of them still remains multilamellar. Furthermore, it is often difficult to control the size of vesicles synthesized by these methods, necessitating additional postprocessing steps such as size exclusion chromatography [21-23] to achieve a desired size distribution.

More recently, a new approach based on hydrodynamic focusing in micro-channels [24] has been developed and successfully applied in synthesis of vesicles of various lipids, as well as other types on nanoparticles [25-30]. Microfluidic hydrodynamic focusing (MHF) is conducted by introducing the lipid solution through a central inlet channel of a microfluidic device, and focusing this central stream by the flow of an aqueous solution through two or more side channels [24]. Focusing of the central stream causes controlled removal of organic solvent in which lipid molecules are dissolved by its diffusion into the aqueous phase. As a consequence, water molecules from the aqueous phase replace molecules of the organic solvent around lipid molecules, thus changing their environment from the one in which they are soluble to the one in which they are not. This promotes self-assembly of lipid molecules into bilayers, which then grow and bend until they completely close and form spherical structures [31]. Virtually simultaneous self-assembly into bilayers and deformation of planar bilayers into spherical vesicles ensures that the majority of synthesized liposomes are unilamellar. It was also shown that the size distribution of liposomes can be relatively easily controlled by changing the flow rates of the lipid solution in organic solvent and the aqueous phase [25, 26], thus making it a relatively simple and straightforward method for production of unilamellar liposomes of controllable size distribution. Several studies have also used the MHF approach to encapsulate different molecules in the aqueous interior of liposomes [25-29], demonstrating its applicability in drug delivery and similar pharmaceutical and medical applications.

Despite these advances in the MHF-based synthesis and applications, no effort has been made to apply the method to develop model biological systems or cell models in synthetic biology. The objective of the study reported here is to demonstrate that the MHF approach can be used to synthesize lipid vesicles whose properties will be more similar to those of the cell and its internal compartments. In order to do that, we have for the first time applied the MHF method in synthesis of 1-palmitoyl-2-oleoyl-s*n*-glycero-3-phosphocholine (POPC) liposomes. Phosphatidylcholine (PC) lipids account for more than half of phospholipids of most eukaryotic cellular and subcellular membranes [32-34]. Among them, POPC is the most abundant [35-37] and has been extensively used as a model compound for representation of natural PC mixtures [38]. While the MHF method has been used in synthesis of liposomes with other PC lipids [25-27, 31], these studies have used molecules with two identical saturated fatty acid chains attached to the glycerol backbone, whereas lipid molecules of natural membranes usually contain two different fatty acids [39]. Fatty acid chains of the POPC molecule are different in length with one of them also containing an unsaturated C=C bond which affects fluidity and other physical properties of the lipid bilayer. We show that despite these different properties of POPC membranes, the MHF method is an effective method for POPC vesicles synthesis, thus opening a new venue for its application in cell modelling and synthetic biology. Furthermore, higher biocompatibility of POPC vesicles is expected to improve the applicability of the MHF approach in drug and gene delivery.

We first describe the methodology and experimental procedure used in synthesis and characterization of liposomes at a range of environmental conditions. This is followed by presentation of our experimental outcomes and their analysis. Finally, the conclusions and outlook for future research are provided.

2 Materials and Methods

2.1 Microchannel Specifications

Preparation of liposomes by microfluidic hydrodynamic focusing was conducted in a microchannel, following the procedure developed by Jahn *et al.* [25-27]. The microchannel network was etched in a glass wafer and consisted of three inlet channels and one outlet, as illustrated in Figure 1. Lipid solution was introduced through the central channel. The aqueous phase was flown through the two side inlet channels. Liposomes are formed in the outlet channel and their solution collected at its exit. All channels of the network were 220 μm wide and 50 μm deep.

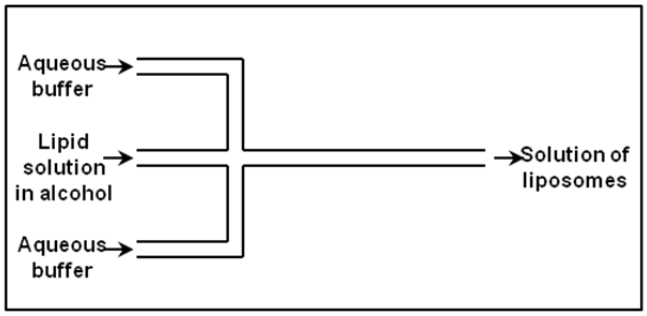

Figure 1

2.2 Preparation of Lipids

Liposomes were formed primarily from 1-palmitoyl-2-oleoyl-s*n*-glycero-3-phosphocholine (POPC), with a set of experiments conducted using 1,2-dimyristoyl-s*n*-glycero-3-phosphocholine (DMPC). For the preparation of lipid solutions, dry lipids in powder form (Avanti Polar Lipids Inc., Alabaster, AL, USA) were first dissolved in 0.5 ml of chloroform (Sigma-Aldrich, St. Louis, MO, USA), which was then evaporated in a fume cupboard and the remaining lipid sample placed in a vacuum desiccator at room temperature for at least 12 hours. Lipids were then dissolved in isopropanol (IPA) (Merck, VIC, Australia) to a desired concentration which varied from 2 to 20 mM. Phosphate buffered saline (PBS) (10 mM phosphate, 2.7 mM potassium chloride, 138 mM sodium chloride, 3 mM sodium azide, pH 7.4) solution was used as the aqueous phase for liposome preparations.

2.3 Liposome Synthesis

Lipid solution and PBS were introduced into the microfluidic device from gastight glass syringes (Hamilton, Reno, NV, USA), which were connected to the microchannel using polyethylene tubing of 0.58 mm internal diameter. Flow rates were controlled using two PHD Ultra pumps (Harvard Apparatus, MA, USA). The flow rate ratio (FRR), defined as the ratio of total volumetric flow rates of the PBS buffer in side channels and the volumetric flow rate of the lipid solution in isopropanol in the central inlet channel, was varied from 4 to 40. The flow rate of the lipid solution was set to 6 μl/min.

2.4 Liposome Detection by Confocal Microscopy

Detection of POPC liposomes was performed by confocal fluorescence imaging [25]. A 10 mM POPC solution in IPA was prepared as described above, with the addition of 1 wt% of the 1mM 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) (Sigma-Aldrich, St. Louis, MO, USA) lipid intercalating dye. POPC liposomes with DiI were synthesized at the flow rate ratio of 10 and samples imaged using a Leica TCS SP5 confocal microscope (Leica Microsystems Pty Ltd, Australia) at the excitation wavelength of 514 nm and 63× magnification.

2.5 Liposome Size Characterization

Liposome size distribution was determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS particle sizer (Malvern Instruments Ltd, UK) with a He/Ne laser of 633 nm wavelength as the light source. The detection of the scattered light was conducted at an angle of 173°. At least three 60-second measurements were performed for each sample. The average liposome diameters, z_{avg}, from the measurements were used to determine an average liposome size and standard deviation thereof.

2.6 Liposome Lamellarity Determination

Liposome lamellarity was determined by thin-section transmission electron microscopy (TEM), following the procedure similar to that used for thin-section TEM tissue analysis [40]. A solution of POPC liposomes prepared using the 10 mM POPC in IPA at the flow rate ratio of 10 was first centrifuged at 7400*g* (SIGMA 1-14, SIGMA Laborzentrifugen GmbH, Germany) for 1 hour. The pellet was dissolved in the primary fixative (4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA), 1.25% glutaraldehyde (ProSciTech, Kirwan, Australia), 4% sucrose (Chem-Supply, SA, Australia) in PBS, pH7.2) and left at 4 °C overnight. The sample was then centrifuged at 7400*g* for 15 min and the pellet dissolved in a heated 3% agarose (Sigma-Aldrich, St. Lous, MO, USA) solution in Milli-Q water. Upon cooling to the room temperature and formation of agarose gel, the gel was sectioned into 1-2 mm sized blocks. Agarose gel blocks with embedded lipid vesicles were washed in 4% sucrose solution in PBS and post-fixed in 2% OsO_4 (Sigma-Aldrich, St. Louis, MO, USA) for 1 hour. Upon removal of OsO_4, the gel blocks were washed twice in 70% ethanol (VWR International S.A.S., France) solution in Milli-Q water for 15 min. This is followed by two more cycles of washing in 90% ethanol solution in water for 15 min and three more washing cycles in 100% ethanol for 15 min. Agarose blocks are extracted by using a 1:1 mixture of ethanol and epoxy resin prepared from Procure-Araldite embedding kit (ProSciTech, Kirwan, Australia) and left in the solution for 2 hours. The ethanol/resin mixture is then replaced by pure resin and left overnight. The resin infiltration is continued by extracting agarose blocks and placing them into the fresh epoxy resin for 6 hours, after which the blocks are extracted again, placed into fresh epoxy resin and polymerized at 70 °C for 24 hours. Polymerized blocks were then sectioned into 30 nm thick slices and imaged by TEM. TEM was conducted on a Philips CM100 instrument operated at 80 kV, with a MegaView II CCD camera (Olympus Soft Imaging Solutions GmbH, Germany).

3 Results

3.1 Effect of Flow Rate Ratio on Liposome Size

The flow rate ratio (FRR) is often used as a means of varying vesicle size, including for DMPC vesicles [25-27, 31]. We have, therefore, investigated here the effect of FRR for POPC liposomes. The variation of the liposome size (expressed as the hydrodynamic diameter measured by the dynamic light scattering) with the FRR at which they were

synthesized is shown in Figure 2; results also obtained by us for DMPC using the same apparatus are also shown in this figure for comparison.

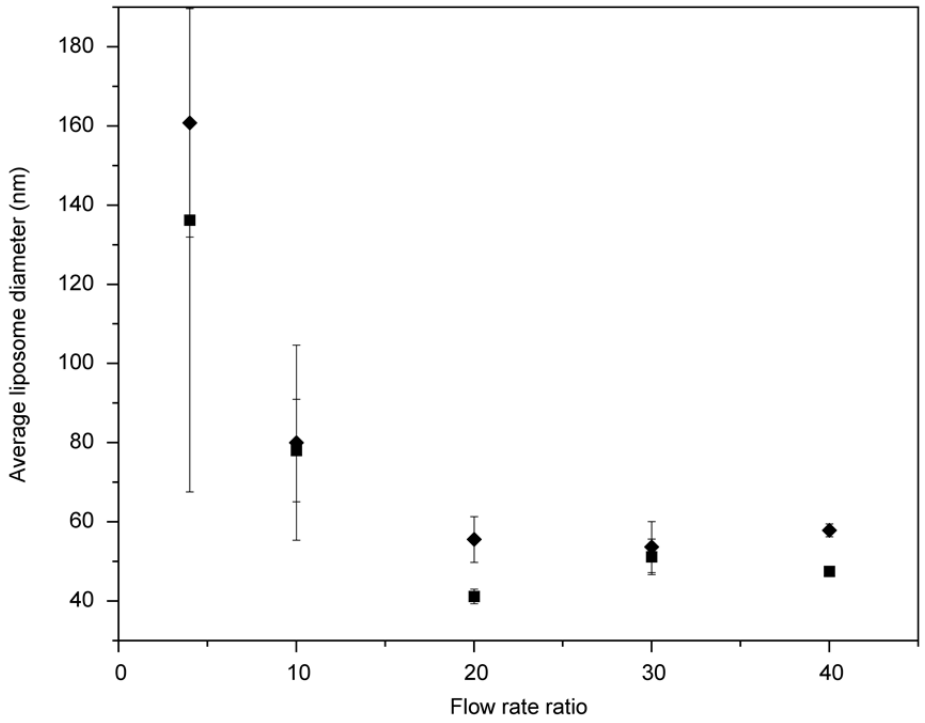

Figure 2

In agreement with earlier studies [25, 26], both the average size and the width of the size distribution of DMPC vesicles decrease with increasing FRR. Although the size of liposomes synthesized here is lower than previously reported for vesicles of a similar composition [25, 26], it should be noted that our liposomes consist of only DMPC rather than the mixture of DMPC with cholesterol that has been used in these previous studies. Presence of cholesterol in the lipid mixture has been shown to increase the bilayer rigidity and, consequently, the liposome size [41].

In line with the DMPC behaviour, the average size of the POPC liposomes decreases with increasing FRR. A somewhat unexpected result is the statistically negligible difference in diameters of POPC and DMPC liposomes synthesized at all FRR values. The temperatures of transition from rippled gel to a more disordered liquid-crystalline phase for POPC and DMPC bilayers are –2.5°C and 23.6°C [42], respectively. Due to the lower transition temperature, POPC membranes were expected to be characterized with higher fluidity that would allow them to sustain greater curvatures and, hence, form smaller vesicles in a process described by Zook and Vreeland [31]. Our experiments were, however, performed at temperatures between 25 and 30 °C, which are above the transition temperatures of both lipids, thus rendering the differences in their fluidity and, consequently, curvature radii of their liposomes negligible.

In order to verify that we indeed did synthesize lipid vesicles, we added DiI into the POPC solution in IPA prior to the vesicle synthesis and then observed the product obtained from the microfluidic-based process under a confocal microscope. As DiI is a lipid intercalating dye whose fluorescence quantum efficiency increases substantially when embedded in a lipid bilayer compared with its aqueous solution [43], a strong fluorescence

should be observed under the microscope if liposomes are formed. Strong fluorescence is indeed seen here as shown in Figure 3, offering further proof that POPC liposomes have been synthesized in the microfluidic process. It should be noted, however, that liposome sizes are below the resolution capabilities of confocal microscopy and, as such, the spatial extent of fluorescence signals in Figure 3 cannot be used to accurately infer the liposome diameters.

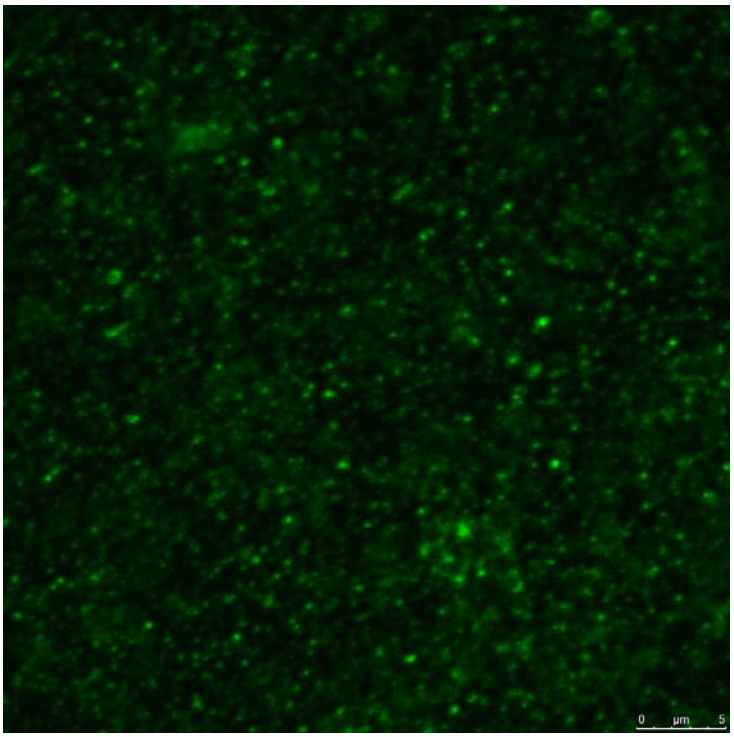

Figure 3

3.2 Effect of POPC Concentration in Isopropanol on Liposome Size

In order to explore the effect of POPC lipid concentration on liposome size, we have performed the synthesis of liposomes for a range of POPC concentrations in isopropanol at a fixed FRR (of 10). The results obtained are shown in Figure 4, which shows the average hydrodynamic diameter of particles as a function of the POPC concentration in isopropanol. With the exception of low concentrations, a general trend of increase in liposome size with lipid concentration is observed. Interestingly, this behaviour is in-line with that obtained for other phosphatydilcholine lipids using injection methods [41, 44]. Previous studies on vesicles of similar lipids have indeed shown that smaller liposomes are more susceptible to coalescence due to their increased surface curvature and consequently reduced geometric stability [45] – this is the most likely explanation for the apparently anomalous increase in the average liposome diameter at the lowest concentrations investigated here.

3.3 Morphology of Liposomes

Prior work by others using cryo-TEM has provided an indication that the microfluidic approach may be used to synthesize vesicles that are predominantly unilamellar, albeit of a different composition from the one in our study [29, 46]. We have applied here the thin-section TEM approach to determine the lamellarity of positively stained POPC vesicles. The samples are fixed in OsO_4, which is used as a positive stain for lipid molecules. As may be seen in Figure 5, the boundaries around liposomes are represented with a single stained layer, which is consistent with a unilamellar morphology.

4 Discussion

Liposome size is a critical characteristic in many contexts, particularly in biological and pharmaceutical applications. For example, the interaction of particles with some cell types is known to vary with particle size [47, 48]. Thus, fine control over liposome size can be exploited to produce optimal drug and gene delivery vectors [2, 5]. Liposome geometry is also expected to play a significant role in their application as model biological systems. For example, some cellular organelles, such as the endoplasmic reticulum (ER), are characterized with highly curved membranes of high specific surface area that directly dictate their protein synthesis efficiency. Membrane curvature may also have a role in proper folding of membrane-bound proteins, thus enabling them to express their biological function [49]. Such high curvature can be emulated in an artificial system via use of liposomes of very small diameters.

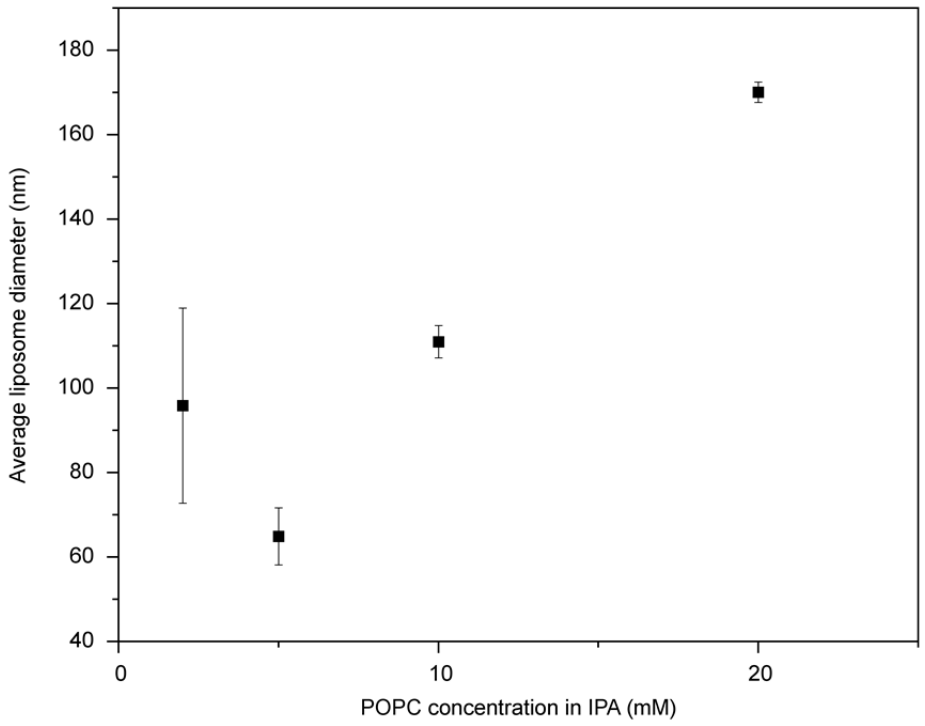

Figure 4

The results presented here offer some insight into the lower range of sizes of POPC vesicles produced by the MHF approach. The average size of liposomes produced in microchannels has been observed to approach a lower limit for FRR values of 30 or more [25, 26]. While it is impossible to perform direct comparison with previous studies due to differences in lipid composition and the microchannel geometry, results for POPC and DMPC vesicles synthesized here follow a similar trend. The average diameter of vesicles of both lipids stabilizes at about 50 nm. Curvature of liposomes of this size is likely to be similar to that of cisternae of the rough endoplasmic reticulum, whose mean diameter was found to be only slightly greater [50].

While dimensions of certain cellular compartments (*e.g.* some tubules of the Golgi apparatus [51]) are somewhat below the *average size* of our vesicles, it should be noted that 50 nm is not the absolute minimum of the vesicle size produced but, rather, the lower limit of their average diameter. The polydispersity index of the POPC vesicles synthesized in our experiments was about 0.3, indicating a relatively wide distribution of particle sizes. An

example of the size distribution of synthesized vesicles obtained by the DLS method is shown in Figure 6 for liposomes synthesized at the flow rate ratio of 10 and using 5 mM POPC solution in IPA. The intensity of the light scattered from a spherical particle is proportional to the sixth power of the particle diameter, which means that the distribution in Figure 6 is skewed towards larger particles and should not be confused with the particle size distribution based on particle number. Nevertheless, it may be used to demonstrate that some of the vesicles in the sample produced here have a hydrodynamic diameter of about 15 nm, well below the average particle size and in-line with the minimal size phosphatydilcholine vesicles produced by other methods [52]. Synthesis of smaller liposomes is, therefore, not limited by the nature of the microfluidic approach, but by the vesicle thermodynamic stability.

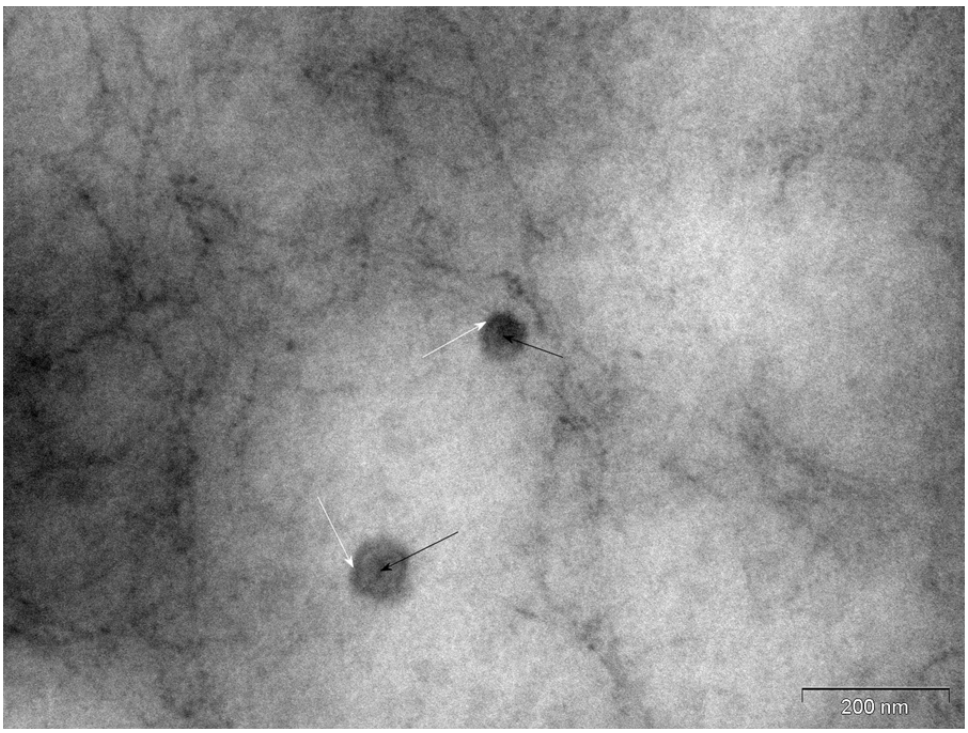

Figure 5

Whereas the lower limit of lipid vesicle sizes is determined by the nature of lipid molecules and cannot be bypassed, the upper limit of liposome diameters appears to be much easier to manipulate. For a synthesis of very large liposomes, however, it is necessary to control the flow rate ratio in combination with other adjustable parameters. Reduction of FRR is characterized by a lower limit below which the amount of aqueous solution is too low to provide efficient mixing with the alcohol phase. This is reflected in an asymptotic approach of the average vesicle size to a maximum when the FRR value is reduced below 10, as seen in Figure 1 here and in previous studies of other liposomes [25]. One possible approach to circumvent this limitation is through the change in lipid concentration in the alcohol solution, as we have shown here. Other factors, such as the channel geometry or presence of cholesterol, have also been shown to facilitate synthesis of larger liposomes in different settings [27]. Indeed, through a combination of strategies, an approach based on the MHF method has been used to produce lipid vesicle in the μm range, capable of encapsulating entire cells [53]. Our results here as well as these results of others indicate a great flexibility of the MHF method in adjusting geometric parameters of POPC lipid vesicles.

In addition to the size requirements, application of liposomes as model biological systems and drug and gene delivery vectors places constraints on their internal structure. Most cellular organelles consist of a single lipid bilayer which separates their inner domain or lumen from the cytosol. Many membrane proteins, such as ion channels and pumps, are not able to express their biological function in a multilamellar environment. It is, therefore, vital for synthesized liposomes to display similar morphology. As we have shown above, the MHF approach provides viable means for the synthesis of unilamellar POPC liposomes, making it ideal for producing model biological systems.

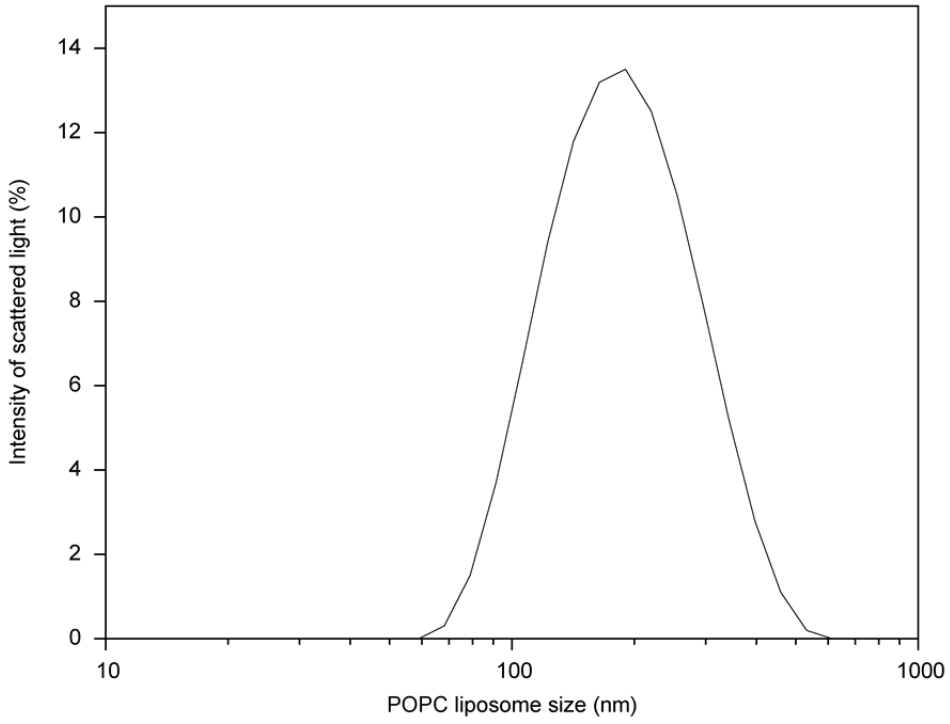

Figure 6

Utilization of liposomes as model biological systems often requires them to be loaded with sets of proteins, nucleic acids and other biological molecules [12]. Earlier studies have demonstrated the ability of vesicles synthesized by the microfluidics approach to encapsulate hydrophilic molecules including DNA fragments [25, 29, 54]. It has also been shown previously [25, 30, 43] and in this study that hydrophobic dye molecules can be incorporated in the vesicle lipid bilayer. All of these results show capability of the MHF approach to synthesize unilamellar lipid vesicles loaded with a versatile set of hydrophilic and hydrophobic molecules that can be used in simplified models of cellular compartments.

5 Conclusions

Microfluidic hydrodynamic focusing (MHF) has previously been developed as a simple and efficient method for synthesis of liposomes and other vesicles. It has been successfully used to synthesize vesicles of various compositions and in a range of sizes, primarily with pharmaceutical and materials science applications. This study demonstrates that the MHF method can also be used to synthesize POPC vesicles whose major area of application will be in modelling of artificial cells and cellular organelles, as well as in increase in efficiency of drug and gene delivery where a high degree of transfer vector biocompatibility is required.

As previously observed with vesicles of other lipids, the most straightforward approach to controlling the size of POPC liposomes was shown to be the ratio of flow rates of organic solvent and lipid-insoluble aqueous phase through the microfluidic device. Whereas the lower limit of the liposome size is defined by the lipid bilayer nature, we have shown that its upper limit may be manipulated through the concentration of the lipid in the organic solvent phase.

Liposomes synthesized by the MHF approach satisfy geometric and morphological requirements for models of cellular compartments. Our work and numerous other studies have also shown the ability of the method to load liposomes with various smaller molecules. Further studies will be required to investigate the efficiency of the approach in encapsulating water soluble biomolecules and embedding membrane proteins in lipid bilayers of POPC vesicles so as to increase their effectiveness in pharmaceutical and biomedical applications.

Acknowledgments

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