

A novel single step method of synthesizing large unilamellar liposomes for biomedical applications

S.M. Phapal, and P. Sunthar*

Chemical Engineering Department, Indian Institute of Technology Bombay,
Powai, Mumbai-400076, India. *Email: p.sunthar@iitb.ac.in

ABSTRACT

Predictable size control is important to liposomal drug delivery formulations. Mixing phospholipids dissolved in ethanol with an aqueous phase leads to the formation (by coacervation) of a suspension of liposomes of varied size and lamellarity. The nature of the process that leads to this size and size distribution is not well understood. Here we show that the extent of convective mixing of the two miscible phases-ethanol and water-has a significant influence on the size distribution of the liposomes. The formation of liposomes is shown to be influenced by a competition between convective and diffusive transport of lipid molecules followed by its self assembly. In the absence of convection the lipids assembly is independent of bulk lipid concentration. An understanding of this process will help in the design of liposome synthesis devices, which can finely control convective mixing. We expect that this will lead to new designs and scale-up strategies for bulk synthesis.

Keywords: liposomes, drug delivery, coacervation, stationary interdiffusion.

1 INTRODUCTION

Liposomes are spherical capsules (vesicles) made of a phospholipid bilayer membrane enclosing an aqueous compartment. Phospholipids are amphiphilic surfactants which assemble to a bilayer membrane vesicle upon hydration. Liposome size and size distribution is a critical parameter to control and it is very important for application of liposomes in vivo for drug delivery systems as the size influences the clearance rate of drug from body [1]. Batzri *et al* first prepared the liposome using ethanol injection and they mentioned in their pioneering work that small unilamellar vesicles (SUVs–50 nm) can be prepared without sonication [2]. Increasing the lipid concentration increases the liposome size, polydispersity and lamellarity. The optimum ethanol concentration which does not cause any deterioration of the liposomes is about 10% v/v [3]. In the modified cross flow ethanol injection method, the lipid solution was kept in one reservoir and aqueous medium in another and both the phases were brought in contact by a cross flow injection module [4]. Recently one more modification of injection method is Microfluidic liposome synthesis where the flow rates of the two phases are used to

control the size of the liposomes. The size range of liposomes is 50-150 nm [5]. The polydispersity of the liposome suspension increases with the average size of liposomes and the size of the microfluidic channels, both of which are not desirable. In many cases the suspension is also very dilute.

The main drawback of ethanol injection type methods considered so far is that the bulk convective motion has dominated over the diffusive mixing of the phases. This, we believe, has led to a poor understanding of the phenomenon as well as limited the scope of a possible assembly of the lipids into larger sized liposomes. We have therefore attempted to study the influence of convection on the size of liposomes formed.

2 MATERIALS AND METHODS

The phospholipids 1, 2 –dimyristoyl -sn- glycerol-3-phosphocholine (14:0-DMPC) and 1,2-dimyristoyl-sn-glycerol-3-phosphoethanolamine -N- (7-nitro-2-1,3-benzoxadiazol-4-yl) (14:0- NBD PE) were purchased from Avanti Polar lipid. Lipids were in chloroform solution (20mg/ml), appropriate amount was taken in a glass vial and chloroform was evaporated by using nitrogen gas stream. The thin film formed at the bottom of glass vial was kept in vacuum dessicator overnight so as to remove traces of solvents. Ethanol was purchased from Merck.

2.1 Liposome preparation

The phase transition temperature of DMPC is 23°C, so liposome preparation was performed at room temperature (25°C) for all the methods. Different stock concentrations of DMPC were prepared in ethanol viz. 5, 10, 20, and 40 mg/ml. The four methods used to synthesize liposomes are shown in (Figure 1). The four methods employed are: (a) Direct mixing of lipid-ethanol by injection in the aqueous phase under continuous stirring; (b) Mixing in portions: mixing of lipid-ethanol phase stream with aqueous phase stream, each of them pumped using calibrated syringe pumps. Here the flow rate of lipid solution was 10µL/min and aqueous phase was 100 µL/min. (c) co-axial glass capillary microfluidic device was fabricated and liposomes were synthesized by passing lipid-ethanol solution through central channel which was hydrodynamically focused by an aqueous phase through side channel; flow rates were same as in (b) and fourth method is (d) stationary phase interface

mixing of an organic phase (lipid-ethanol) with an aqueous phase. In all the above methods, the ratio of ethanol and aqueous phase is same e.g. 1:10.

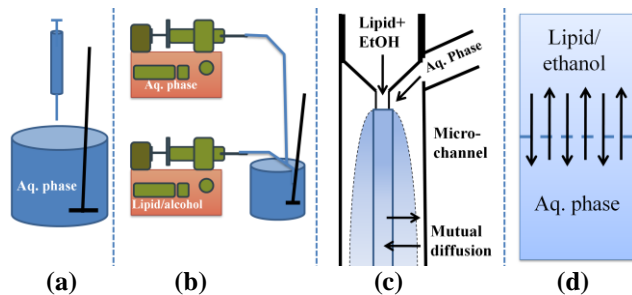


Figure 1: Different methods used to synthesize liposomes (a) injection mixing, (b) mixing in portion, (c) microfluidic mixing and (d) stationary interdiffusion mixing.

2.2 Characterization:

Liposomes synthesized by above methods were subjected to following characterization techniques:

2.2.1 Dynamic light scattering (DLS)/Photon correlation spectroscopy (PCS): size and polydispersity

The resultant liposomes were characterized by DLS or PCS (Zetasizer nano ZS, Malvern, UK) for size and size distribution (polydispersity), without any further processing. The laser used was He-Ne laser with 633 nm wavelength, 4 mW power and light scattering was detected at 173° angle. The size and size distribution was calculated using software DTS 6.1 (supplied by vendor)

2.2.2 Transmission electron microscopy (TEM): size and lamellarity

High resolution TEM with cryo and negative staining mode (phosphotungstic acid 2% w/v) used to determine the lamellarity of the liposomes.

2.2.3 Confocal microscopy: size and aggregation

The fluorescent lipid 14:0 NBD-PE was mixed along with DMPC (0.25 mole %). Liposomes samples prepared by stationary interdiffusion method were examined under confocal microscope (Zeiss LSM 501) for size distribution and aggregation (63X-oil immersion objective, FITC filter-488 nm excitation and 525 nm emission).

3 RESULTS AND DISCUSSION

In the ethanol injection methods, changed solubility conditions around phospholipid molecules leads to phase separation, in one phase they are aggregated (by self assembly), and which is in equilibrium with the other phase where they are dispersed and in a dilute concentration.

The mixing of the two phases is influenced by convective transport of the solute due to bulk motion of the fluids and diffusive transport due to molecular mixing. The relative importance of these two transport mechanisms is captured by the Peclet number Pe and is defined as,

$$Pe = \frac{UL}{D} \quad (1)$$

Where U is a characteristic mean local bulk velocity, L is a characteristic system length scale, and D is the molecular diffusivity of one species in the other medium.

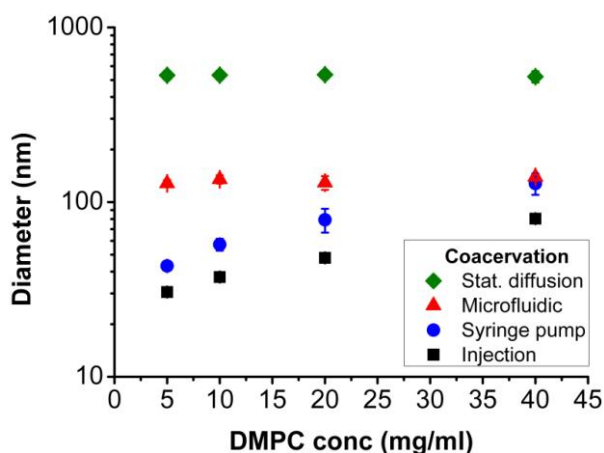


Figure 2: Different ways of mixing the phospholipid-ethanol phase with aqueous phase at the same aqueous-ethanol-phospholipid ratio and its effect on liposome hydrodynamic diameter by DLS.

The conventional direct mixing by turbulence is yielding small vesicles (SUVs), mixing in streams gives slightly larger vesicles (Figure 2). The convections in mixing not allowing bilayer to grow larger, shear caused by turbulence is responsible for the bilayer fragmentations and thus leads to formation of small vesicles. In case of microfluidic mixing, there is fine control over the mixing of two fluids. Rapid diffusive mixing occurs in the focused stream, due to flow in microchannel, the time for formation of bigger intermediate bilayer structure is small so the vesicles formed are smaller than stationary interdiffusive mixing method. In stationary interdiffusion method, as there are no mechanical putrefactions and flow, Peclet number is reduced from 10^3 (microfluidic) to 10^0 , indicating there is pure diffusive mixing. There is sufficient time for bilayer to grow to its maximum size, thus yields much larger vesicles. The first two methods showed a dependence of size on the lipid concentration but in the case

of stationary interdiffusion, the liposome size is independent of lipid concentration. This method is highly reproducible and unlike other methods mentioned in literature, the liposome size is unaffected by ratios of phospholipid-ethanol- water [6]. The new finding from present stationary interdiffusion method is that the way of mixing is major factor responsible for size selection by ethanol injection methods. The present method seems to be an upper bound of the sizes obtainable. Since the process is not significantly affected by convection (bulk fluid motion) or any mechanical or other forces, the resulting sizes are highly monodispersed reproducible (Figure 4).

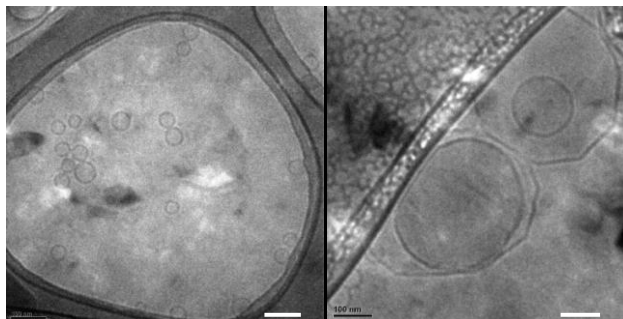


Figure 3: CryoTEM image of DMPC liposomes: (a) SUVs (50 nm) by convective mixing method and (b) LUVs (500 nm) by diffusive mixing method; scale bar- 100 nm.

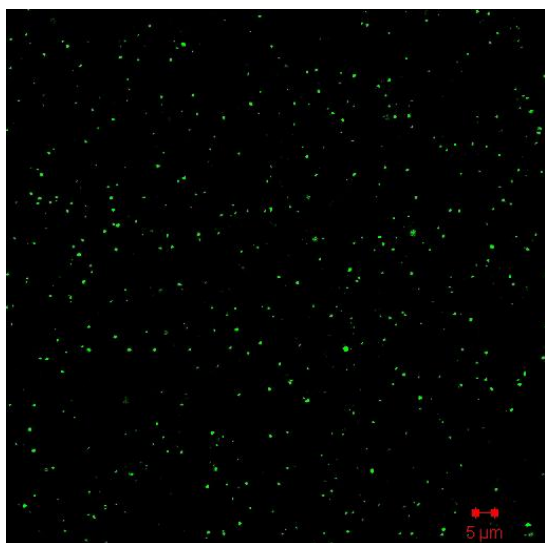


Figure 4: Confocal micrograph of DMPC-NBD liposomes by stationary interdiffusion synthesis, scale 5 μ m.

3.1 Mechanism of liposome formation:

According to theory proposed by Lasic, phospholipids dissolved into water miscible organic solvent, due to change in solubility conditions upon hydration, transform into an intermediate structure called bilayer phospholipid fragment (BPF) [7]. The conventional

injection mixing is turbulent mixing and the BPFs formed are smaller as the shear in turbulence could cause fragmentation of bilayers. Also there is no sufficient contact time for the BPF to grow larger leading to the formation of small vesicles (Figure 3a). As the concentration of the lipid in ethanol increased, it forms bigger, multilamellar vesicles (Figure 2). This is because more lipid molecules are available in the given volume which leads to formation of bigger vesicles. In microfluidic mixing, due to their small size, flow pattern is dominated by viscous forces. There is a stable (miscible) interface between the alcohol phase and the aqueous phase, through which there is a continuous diffusive exchange. This provides a good control over liposome size, at lower velocities, (small Pe), diffusive transport dominates. This leads to formation of large BPFs and thus liposomes, compared to bulk convective methods. In stationary phase interdiffusion method, there is a near zero flow condition which is ideal for purely diffusive, convection free environment ($Pe \cong 0$). This leads to formation of larger BPFs and thus LUVs ~ 500 nm are formed (Figure 3b).

4 CONCLUSION

In summary, we conclude that when the Peclet number (defined in eq (1)) is large, the convective transport dominates leading to smaller time for assembly, whereas for low Pe , when diffusive transport is dominant, the larger time of self assembly leads to the largest size of liposomes at a given condition.

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