Spontaneously Forming Unilamellar Nano-Sized Vesicles – Polydispersity, Size, Shape And Stability

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ABSTRACT

Unilamellar vesicles (ULV) made up of biologically relevant materials can serve as carriers for drug delivery or gene therapy. Compared to extrusion and sonication, methods traditionally used for producing ULV, spontaneous formation offers a cost-effective alternative for ULV mass production. Moreover, the fact that these ULV are most likely, thermodynamically stable assures that the final product will have an extended shelf life. Here we report recent work on ULV formed from long- and short-chain phospholipids (i.e., di 14:0 and di 6:0 phosphatidylcholine), and address some of the important factors that affect their polydispersity, size, shape and stability.

Keywords: unilamellar vesicles, phospholipids, small angle neutron scattering, polydispersity, anti-cancer drug delivery, nano particles, DMPC, DHPC

INTRODUCTION

Phospholipids are a major constituent of cell membranes. Because of their biocompatibility and capability of forming stable unilamellar vesicles, ULV, phopholipid vesicles have found applications in encapsulation and drug delivery, in particular, as carriers for anti-cancer drugs, e.g., PLD, NPLD, and DNX [1]. One class of phospholipids commonly used for anti-cancer drug delivery are long chain (acyl chain length > 9 carbons) phosphatidylcholine (PC), which are zwitterionic and form bilayered multilamellar vesicles (MLV) when placed in water. Traditionally, ULV are produced from multi-stage extrusion or sonication of MLV. Hence, their production is labor intensive adding to the cost of the final product . We have successfully developed low-polydispersity, spontaneously formed PC ULV using PC lipid mixtures (di-14:0, DMPC and di-6:0, DHPC) doped with di-14:0 phosphatidylglycerol (DMPG) [2-4]. We found these ULV to be stable over extended periods of time (weeks to months). Here we discuss the parameters, which modulate the size, polydispersity and stability.

MATERIALS & METHODS

All lipids (DMPC, DHPC, DMPG) were purchased from Avanti Polar Lipids. D_2O (99.9%, Chalk River Lab.) is used as a solvent to enhance the neutron scattering contrast between lipid and water. Small angle neutron scattering (SANS) was employed to characterize the ULV structure and experiments were conducted at the 30-m SANS instrument (NG7) located at the NIST Center for Neutron Research (Gaithersburg, MD, USA). 2-D SANS raw data were corrected for empty-cell scattering and background, and then circularly averaged, producing a 1-D intensity (I) versus scattering vector (q, defined as $\frac{4\pi}{\lambda}\sin\frac{\theta}{2}$) which was then placed on an absolute scale using the flux of the incident neutron beam. [5].

RESULTS & DISCUSSION

There are several important physical parameters which influence the size and polydispersity of ULV. Low-polydispersity ULV were found in dilute lipid solutions (i.e., total lipid concentration, c_{lp}< 2 wt.%) and high T (> 30 ^{o}C). Moreover, the molar ratio of long- to short-chain lipid, ([DMPC]+[DMPG])/[DHPC], Q, is needed to be in the

range between 2.5 and 4. In the following subsections, we will discuss the effects of charge, Q, lipid concentration and sample preparation protocol on the formation and stability of ULV.

Effect of Charge

Zwitterionic lipid mixtures of DMPC and DHPC (Q > 3 and T > 45 °C) form MLV, exhibiting an interlamellar repeat distance, d ~ 65 Å and coexist with a population of ULV [6,7]. This is explained in terms of the equilibrium that results from a combination of repulsive and attractive forces (i.e., Van der Waals, hydration and steric forces) [8]. For charged lipids, the complete unbinding of MLV is possible as the repulsive electrostatic force overwhelms the attraction forces [9]. By modulating the amount of charged lipid (i.e., DMPG in our case), we can break-up MLV whose fragments reform into low polydispersity ULV.

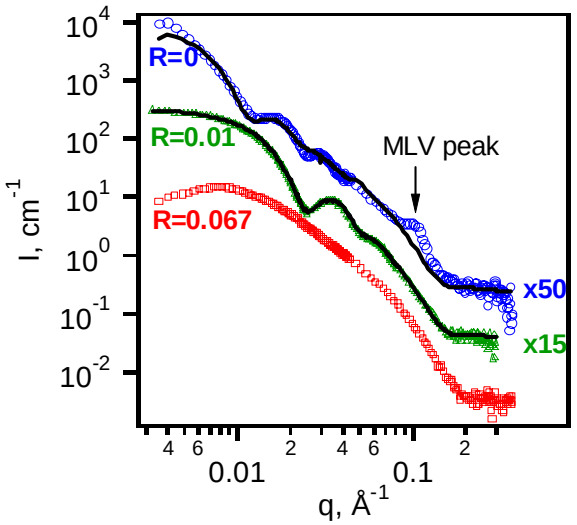

Figure 1: SANS data of DMPC/DHPC/DMPG-D_2O mixtures. R=0 (circles), 0.01 (triangles) and 0.067 (squares) with c_{lp} = 0.25, 0.1 and 0.25 wt.%, respectively.

Fig. 1 shows SANS results obtained from DMPC/DHPC samples doped with varying degrees of DMPG molar ratios (R = [DMPG]/[DMPC]) at T = 45 °C (heated from 10 °C). A smeared spherical core-shell (SCS) model with a constant shell scattering length density (SLD) and thickness as well as, a polydisperse diameter described by a Schulz distribution function was used to fit the data (Fig. 2). For the sample without DMPG (i.e., R=0), most of the data can be fit using the SCS model, except for the data in the vicinity of q = 0.1 $Å^{-1}$ where a quasi-Bragg peak is present, a measure of the d-spacing of a population of MLV. Presumably, the SANS data indicate coexistence of MLV and ULV (radius of 280 Å and polydispersity of 0.15). As for the sample with moderate charge density (R=0.01), the SCS model fits the SANS data perfectly, indicating ULV with a radius of 100 Å and polydispersity of 0.15. Finally, SANS data of the highly charged system (R=0.067) shows a scattering behavior characteristic of discoidal micelles (also known as "bicelles") [10].

Apparently, charge density plays an important role in the spontaneous formation of ULV. Electrostatic repulsion is needed for complete disruption of the lamellar stacks making-up MLV; however, bilayer membranes are not be able to fold into vesicles, if the electrostatic force is too strong for coalescence to take place (see the section of "Effect of c_{lp}"). At 45 °C, ULV are found to be stable over prolonged periods of time (>~ 2 weeks) [6].

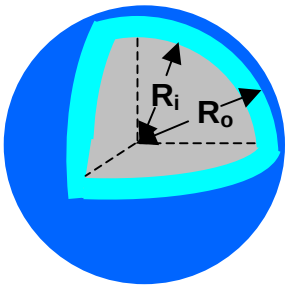

Figure 2: The SCS model for fitting SANS data

Effect of Q

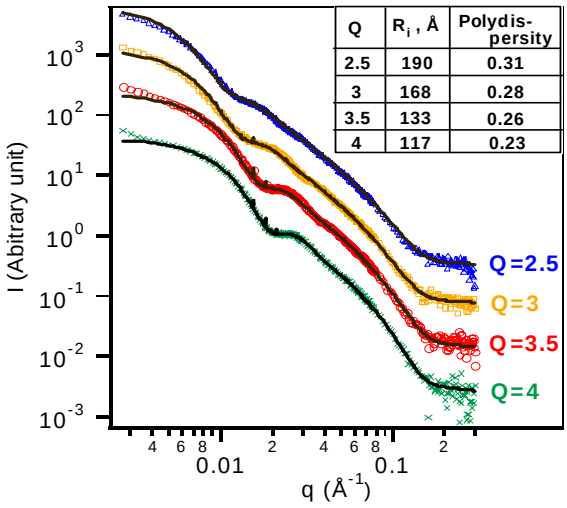

Figure 3: SANS data of DMPC/DHPC/DMPG-D2O solutions with various Q values (R=100 and c_{lp} = 0.1 wt.%). The size and polydispersity of the ULV are in the inset table.

The molar ratio of long- to short-chain lipid, Q, modulates the rigidity and spontaneous curvature of the membrane as well as the chemical composition of the outer and inner leaflets of the bilayer. We have investigated the size of ULV in lipid mixtures with the same charge density (i.e., R = 0.01) but various Q values (from 2.5 to 4) as shown in Fig 3. The SANS data indicate that all samples yield low polydispersity ULV. Moreover, ULV radii (R_i)

and polydispersities decrease with increasing Q values. These can be explained in terms of the transformation mechanism, the precursor bicelles coalescing into larger bicelles, then ULV [4]. Samples with larger Q values have less DHPC to stabilize the line tension at the rim of the bicelles, thus the bicelles fold more readily into ULV than those with greater amounts of DHPC. Therefore, the size of spontaneous ULV can effectively, be controlled.

Effect of c_{lp}

Spontaneously formed ULV are usually obtained via a phase transition from gel phase DMPC (< 22 °C) to L_α phase (> 30 °C) at low c_{lp}. In one such protocol for spontaneous ULV formation, the mixture was initially diluted from high c_{lp} (> 5 wt.%) to c_{lp} < 2 wt.% at low T, where bicelles were observed, and then heated up to 45 °C where bicelles coalesced and folded into ULV [4,11]. This protocol of preparation produces, as a function of c_{lp}, high polydispersity ULV. Fig 4 illustrates SANS data of samples (Q=3.2, R=0.01) with three c_{lp} (1.25, 0.5 and 0.1 wt.%). The best-fit radii for the three cases are 235, 134 and 104 Å, respectively, indicating the size of ULV decreases with decreased c_{lp}. This can be explained in terms of the bicelle precursors growing in size by coalescing with one another. Since bicelles at high c_{lp} have a greater likelihood to coalesce , they can grow larger than those at low-c_{lp} before they fold into ULV.

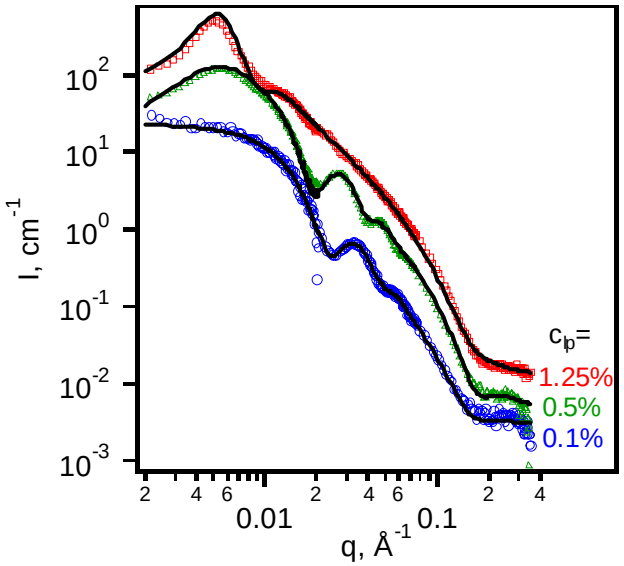

Figure 4: SANS result of DMPC/DHPC/DMPG-D_2O at three different c_{lp} = 1.25 (squares), 0.5 (triangles) and 0.1 (circles) wt.%

It should be noted that the high-c_{lp} (> 2 wt.%) mixture at 45 °C forms lamellar stacks. Diluting the system at such T induces complete unbinding of the lamellae, yielding larger polydisperse ULV, since there is no preferred length scale (or spontaneous curvature) for ULV formed in this manner. An example of a 1.25 wt% sample obtained from high-T dilution is shown in Fig 5 (top). The SANS intensity has a monotonic decay with a slope of q^{-2}, indicative of polydisperse ULV.

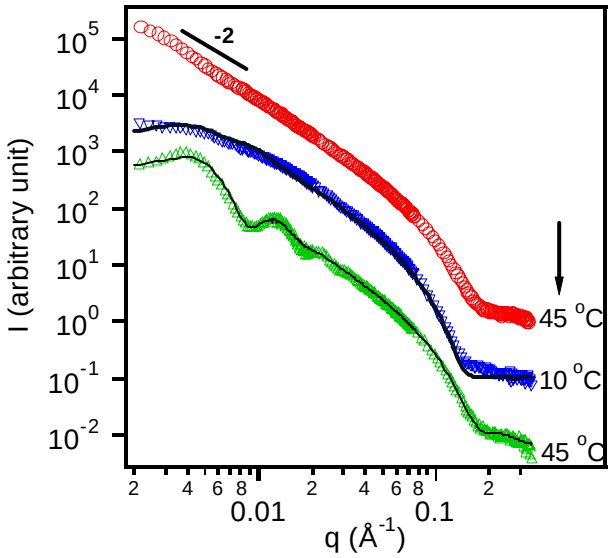

Figure 5: SANS data of 1.25 wt% DMPC/DHPC/DMPG-D_2O diluted at high T (45 °C; circles) and then cycling between 10 °C (tip-down triangles) and 45 °C (tip-up triangles). The arrow represents the thermo path.

Effect of T

It has been reported that by varying T between the gel and L_α phases of DMPC, the system can undergo two possible kinds of phase transitions. At low-c_{lp}, bicelles transform into ULV, while at high-c_{lp}, bicelles transform into lamellae [10]. These transitions at elevated T are presumably related to the increased solubility of DHPC in solution and intermixing with the long-chain lipid, DMPC [5]. SANS data of the 1.25 wt.% sample (Fig 5) shows that polydisperse ULV (top curve as described in the previous section) transform into bicelles (middle curve) as T drops to 10 °C, eventually transforming into low polydispersity ULV (bottom curve, where many peaks are observed) upon reheating to 45 °C. This result indicates that the precursor bicelles are necessary for the system to form spontaneous forming, low polydispersity ULV. Ideally, as low-c_{lp} mixtures undergo T cycling between DMPC the gel and L_α phases, the final product at high T can be low-polydispersity ULV. However, in the case of extremely low c_{lp}, it is possible for ULV to get "locked-in" the ULV morphology, even at low T.

Fig 6 shows two examples of these locked-in ULV. For 0.5 wt.% polydisperse ULV (the top two curves), T-cycling does not change the characteristic SANS data except for slight differences in the range 0.08 $Å^{-1}$ < q < 0.15 $Å^{-1}$, accounting for a thicker DMPC gel phase bilayer. However, bicelles are not found at low T. In the case of low-polydispersity ULV at c_{lp} = 0.1 wt.% (bottom two curves in

Fig 6), reduction of T also does not recover the bicelle morphology. Instead, oblate vesicles, ellipsoidal shells with two identical long axes and a short axis, can be best-fit to the low-T data. Their shell has the same approximate volume as that of the high-T spherical ULV after correction for the bilayer thickening effect in the gel phase. This implies that there is little or no exchange of lipids between ULV and solution, and that spherical ULV are simply deformed into an oblate shape at low T, presumably due to the phase separation of DMPC and DHPC within individual vesicles. The flat regions of the ellipsoids may be composed of DMPC disks which are joined at their edges by DHPC.

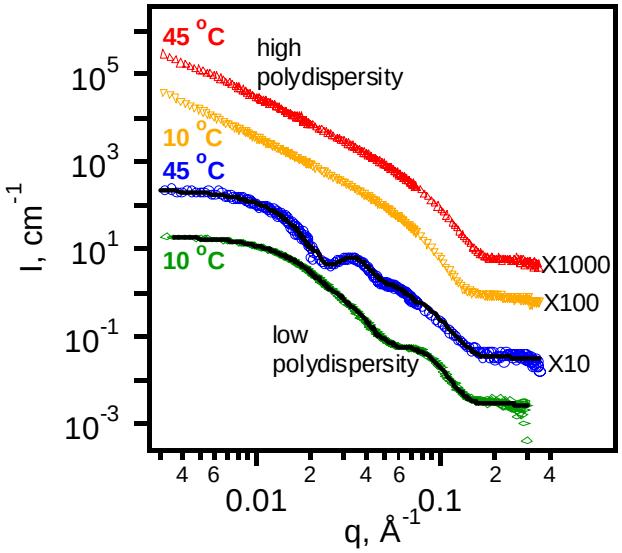

Figure 6: SANS data of 0.5 wt.% high-polydispersity ULV (top two data sets) and 0.1 wt.% low-polydispersity ULV(bottom two data sets). Both show "locked-in" ULV morphology. The 45 and 10 °C 0.1 wt.% data are fitted with spherical- and oblate- core-shell models (in solid curves), respectively.

Compared to low-polydispersity ULV, we found that high-polydispersity ULV are more stable. This may be due to the fact that high-polydispersity ULV have a greater "locked-in" c_{lp}. Since the curvature energy of vesicles is independent of their size (assuming zero spontaneous curvature), the number of ULV in solution thus determines their stability. The fewer the number of particles in solution at a given c_{lp} the more stable are those particles (i.e., large ULV more stable than small ULV).

Stability of the ULV

It has also been reported that ULV composed of DMPC/DHPC/DMPG mixtures are stable at 45 °C for 2 weeks [3]. However, by replacing DMPG with Ca^{2+}, in order to maintain the same charge density, yields unstable ULV. The size of Ca^{2+}-doped ULV continues to increase and becomes polydisperse after a period of two weeks, possibly due to the fusion of ULV induced or facilitated by Ca^{2+}. We have also found the size of ULV to be independent of the solution's salinity (using NaCl) at low salt concentrations (≤ 0.33 wt.%). However, higher salt concentrations (> 0.5 wt.%) can induce a change in the ULV size or the formation MLV [11].

CONCLUSION

We are able to create stable, spontaneously forming ULV in lipid mixtures. Some important parameters have been studied in order to understand the formation mechanism and to control the size and polydispersity of ULV. These ULV have great potential to serve as drug delivery carriers. In future, we will investigate the encapsulation efficiency of ULV and load them with useful peptides or proteins for *in-vitro* and *in-vivo* studies.

ACKNOWLEDGMENT

J. K., J. P., T. H. and M.-P. N. would like to thank the Advanced Foods and Materials Network (Networks of Centres of Excellence, Canada) and Canadian Neutron Beam Centre for financial assistance. This work utilized facilities supported in part by the National Science Foundation under Agreement No. DMR-9986442.

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