Concentration-Independent Spontaneously Forming Biomimetric Vesicles

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In this Letter we present small-angle neutron scattering data from a biomimetic system composed of the phospholipids dimyristoyl and dihexanoyl phosphorylcholine (DMPC and DHPC, respectively). Doping DMPC-DHPC multilamellar vesicles with either the negatively charged lipid dimyristoyl phosphorylglycerol (DMPG, net charge -1) or the divalent cation, calcium (Ca^{2+}), leads to the spontaneous formation of energetically stabilized monodisperse unilamellar vesicles whose radii are concentration independent and in contrast with previous experimental observations.

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Unilamellar vesicles (ULVs) are single-bilayer shells with radii commonly between 10 and 100 nm, and are widely used as model membranes, drug delivery systems, microreactors and substrates for a variety of enzymes and proteins. A common method of making ULVs is the extrusion of multilamellar vesicles (MLVs) through synthetic membranes of known pore size [1]. These extruded ULVs are invariably unstable and in due time revert back to MLVs [2]. Over the years there have been many reports of the spontaneous formation of stable ULVs in surfactant [3–6], lipid [7–9], and lipid-detergent [10–13] mixtures, with their formation occurring over a range of surfactant-lipid concentrations, C_{lp}, the limits of which are set either by the critical micellar concentration (CMC) of the system, where either micelles, MLVs, or extended lamellae are formed, or by the appearance of crystalline precipitate in mixtures of opposite charged surfactants. These ULVs have sometimes been shown to be monodisperse and their radii found, with the exception of one study [14], to vary with C_{lp} [3,5,6,8–12,15].

Theoretical work on the origins of spontaneously forming ULVs has generally been based on minimizing the total free energy of the system, which can include electrostatic and entropic contributions, and the Helfrich free energy [5,16–22]. Generally speaking, spontaneously forming ULVs can either be entropically or energetically stabilized. Entropically stabilized ULVs are usually characterized by ''soft'' membranes (i.e., the bending modulus, $K_b \sim kT \sim 10^{-20}$ J) and a spontaneous curvature, $C_0 \sim 0$ [16,18]. In this case, ULVs exhibit a wider size distribution function [23] and their size increases with increasing lipid concentration [16]. In contrast, energetically stabilized ULVs usually exist in surfactant or lipid mixtures with a high K_b and $C_0 \neq 0$. The formation of such ULVs can be attributed to the following: (i) differing chemical composition found in the outer and inner leaflets of the ULV [22], (ii) strong attraction between the two surfactant species [19,20], (iii) electrostatic interactions [21]. Such ULV mixtures usually result in a narrower size distribution function since the system would experience a higher energy penalty if the ULVs deviated substantially from their preferred radius [23]. To the best of our knowledge, no explicit form for the concentration dependence of vesicular size is presently available.

Recently, it was shown by Nieh *et al.* [9] that in the absence of any dopants a 1.0 wt.% 3.2:1 mixture of dimyristoyl phosphatidylcholine (DMPC) and dihexanoyl phosphatidylcholine (DHPC) at 45 °C formed MLVs which were not monodisperse. However, diluting the system to 0.25 wt.% resulted in either smaller, but monodisperse MLVs, or mixtures of MLVs and monodisperse ULVs. At 45 °C, doping the 1.0 wt.% system (DMPC:DHPC, 3.2:1) with significant amounts of dimyristoyl phosphatidyglycerol (DMPG) [DMPG:DMPC = 0.067] led to the formation of so-called ''bicelles'' or bilayered micelles [8,9] in coexistence with extended bilayers (i.e., lamellar sheets). Diluting the doped system to a C_{lp} of 0.25 wt.% resulted in a single population of bilayered micelles.

Here we present small-angle neutron scattering (SANS) studies of biologically relevant materials composed of the ''long-chain'' phospholipid DMPC and the ''short-chain'' lipid DHPC. These two diacyl phosphatidylcholines (PCs) contain two saturated fatty acid chains of 14 and 6 carbons, respectively. Doping the MLV DMPC-DHPC mixtures with either the negatively charged lipid DMPG (net charge -1) or the divalent cation calcium (Ca^{2+}) led to the spontaneous formation of monodisperse ULVs, whose radii are concentration independent, and contrary to previous experimental observations.

DMPC and DHPC were obtained from Avanti Polar Lipids (Birmingham, AL) and used without further purification. [The identification of any commercial product or trade name does not imply endorsement or recommendation by the National Institute of Standards and Technology (NIST)]. Samples were prepared as follows: Three 20 wt.% solutions of molar ratio 3.2:1 DMPC to

DHPC were prepared. They included DMPG-doped (DMPC:DMPG = 5), Ca^{2+}-doped (DMPC:Ca^{2+} = 1), and nondoped solutions. Individual solutions were vortexed in D_2O solvent (99.9%, Cambridge Isotope Co.) and temperature cycled (10-60 °C) until the lipids were completely dissolved. Once this was accomplished, the 20 wt.% solutions were diluted, in steps, to 2 wt.% using D_2O. Subsequent to this, the 2 wt.% nondoped solution was mixed with the 2 wt.% DMPG-doped solution in a volume ratio of 25:1 yielding a 2 wt.% DMPG-doped solution with a final DMPC:DHPC:DMPG molar ratio of 3.2:1.0:0.021. Ca^{2+}-doped solutions with a final DMPC:DHPC:Ca^{2+} of 3.2:1.0:1.6 were obtained by mixing the 2 wt.% nondoped solution with 20 wt.% $CaCl_2$ solution in a volume ratio of 123:1. Samples doped with both DMPG and Ca^{2+} were prepared by mixing the 2 wt.% DMPG-doped solution with 20 wt.% $CaCl_2$ solution in a molar ratio of 149:1 resulting in a DMPC:DHPC:DMPG:Ca^{2+} molar ratio of 3.2:1:0.021:1.6. 1, 0.5, and 0.25 wt.% solutions were then obtained through further dilutions with D_2O solvent. Each dilution or mixing of solutions was followed by a minimum of 20 s of vigorous vortexing. Samples at their chosen wt.% were equilibrated at ~10 °C for approximately 5 weeks prior to being loaded into quartz sample cells suitable for SANS experimentation. At this temperature the samples are in a low viscous phase [8,9]. Some samples were equilibrated at 45 °C for up to 4 days.

SANS experiments were carried out at the NG7 Instrument (NIST, Gaithersburg, MD) using 8.0 Å neutrons having a wavelength spread ($\Delta\lambda/\lambda$) of 11% and covering a Q range of between 0.002 and 0.3 $Å^{-1}$. Samples were taken up in $1 \times 8 \times 13$ mm^3 quartz cells and their temperature controlled using a circulating water bath. The effective neutron path length was 1 mm. The data were corrected in the standard manner described by Nieh *et al.* [8,9].

A ULV can be described by an outer and inner radius, R_o and R_i, respectively, using a polydisperse core shell spherical model. $R_o - R_i$ is equal to the shell's thickness and is described by a single lipid bilayer. The model's form factor, $P_{\text{vesicle}}(Q)$, can be written as [8,24]

$$P_{\text{vesicle}}(Q) = \frac{1}{V_{\text{vesicle}}} \int_0^\infty f(r) A_0^2(Qr) dr, \qquad (1)$$

where V_{vesicle} is the total volume of a ULV, $f(r)$ is the Schultz particle size distribution, and A_0 is the amplitude of the form factor for vesicles with $R_o = r$. The instrumental resolution was folded into $P_{\text{vesicle}}(Q)$. For charged ULVs, at higher concentrations and low ionic strength (low salt concentration), a structure factor $S(Q)$ accounting for the interparticle interference had to be employed. Without $S(Q)$, fitting the data over its entire Q range, especially the low Q regime, proved to be impossible. Hayter and Penfold [25] have used the mean spherical approximation (MSA) to solve the Ornstein-Zernike equation and derived an analytical form of the structure factor, $S_{\text{MSA}}(Q)$, for charged particles. Combining the $S_{\text{MSA}}(Q)$ with the method described by Nieh *et al.* [8,9] the SANS data were fitted and the best fits are presented as solid curves in Figs. 1–3. The agreement of the fits with the experimental data supports the proposed ULV model. From the Schultz distribution one can obtain the polydispersity p of the ULV radius. p is defined as $\sigma/\langle R_o \rangle$, where σ^2 is the variance and $\langle R_o \rangle$ is the mean of the outer radius of the ULV.

Figure 1 shows SANS data for three lipid concentrations of the DMPG-doped system at 45 °C. Data for the 0.5 wt.% sample after a 4 day incubation at 45 °C are also shown [Fig. 1(*B*)]. One can clearly discern oscillations in the data which are indicative of a very monodisperse vesicular dispersion—dynamic light scattering data are also consistent with the existence of a single population of particles (to be published). Moreover, the fact that the oscillations occur at similar Q values, regardless of C_{lp}, shows that vesicular size is not affected by changes in lipid concentration.

It has previously been reported that in some surfactant mixtures ULV size distribution evolves over a period of a few months [4,15]. In order to check if such behavior is exhibited by the present system, we have studied the 0.5 wt.% sample after annealing for 4 days at 45 °C [Fig. 1(*B*)], and found that neither $\langle R_o \rangle$ (382 ± 15 Å) nor p (15%) had been altered (Fig. 1). Hence incubating the samples for a period of 5 weeks at 10 °C is clearly sufficient for equilibration. Furthermore, invariance of the average ULV radius as a function of C_{lp} was also observed at 10 °C over a twofold increase (0.25 and 0.5 wt.%) in C_{lp}. However, $\langle R_o \rangle$ for the ULVs at 10 °C was approximately 50 Å smaller than the same ULVs at 45 °C while the polydispersities of the ULVs at the two different temperatures remained comparable.

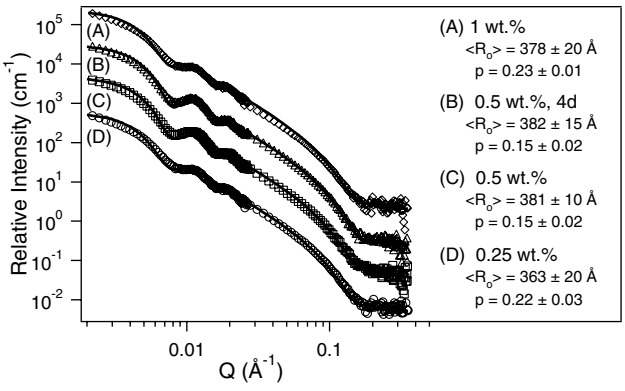

FIG. 1. SANS data for DMPG-doped samples at 45 °C with C_{lp} of 1.0 (*A*), 0.5 (*B* and *C*) and 0.25 (*D*) wt.%. Annealing the 0.5 wt.% sample for 4 days at 45 °C (*B*) did not result in any changes to $\langle R_o \rangle$.

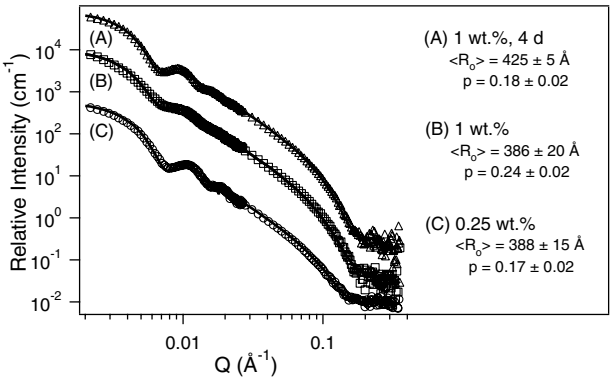

FIG. 2. SANS data of Ca^{2+}-doped samples at 45 °C and lipid concentrations C_{lp} of 1.0 (*A* and *B*) and 0.25 (*C*) wt.%. The 1.0 wt.% sample was annealed for 4 days at 45 °C (*A*) with a resultant increase to $\langle R_o \rangle$.

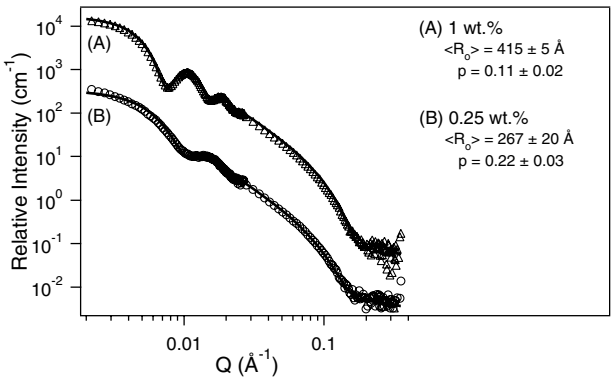

FIG. 3. SANS data of DMPG- and Ca^{2+}-doped ULVs at 45 °C and lipid concentrations C_{lp} of 1.0 (*A*) and 0.25 (*B*) wt.%. The changes in $\langle R_o \rangle$ are reflected by the shift in the oscillations along the scattering curves. More oscillations along the scattering curve are indicative of greater monodispersity.

DMPG incorporates itself into DMPC-DHPC MLVs and imparts a constant charge to the system. Doping the system with $CaCl_2$ is intrinsically different as the Ca^{2+} cations both bind to the phosphorylcholine headgroups and exist freely in solution. Figure 2 shows SANS data for two concentrations of the Ca^{2+}-doped ULVs at 45 °C. As in the DMPG-doped system, $\langle R_o \rangle$ was unaffected over a fourfold increase in C_{lp}. For the one Ca^{2+}-doped sample ($C_{lp} = 0.25$ wt.%) for which data were collected at 10 °C, the ULVs had an $\langle R_o \rangle$ and p similar to the DMPG-doped sample at the same temperature and consistent with dynamic light scattering data (not shown). However, the 1.0 wt.% sample, after incubation at 45 °C for 4 days [Fig. 2*(A)*], exhibited a large increase in $\langle R_o \rangle$ (425 ± 5 Å), with a concomitant improvement in polydispersity. This is unlike the DMPG-doped samples whose $\langle R_o \rangle$ remained unaltered over that same period of time.

Figure 3 depicts SANS data of the DMPC-DHPC system doped with both DMPG and $CaCl_2$ at 45 °C and lipid concentrations of 1.0 wt.% [Fig. 3*(A)*] and 0.25 wt.% [Fig. 3*(B)*]. It is clear from the data that in the presence of the two oppositely charged dopants the resultant ULVs, although monodisperse, are no longer insensitive to the total lipid concentration. In this case, a fourfold increase in C_{lp} leads to a 55% increase in ULV radius.

Since DMPC is generally considered as forming a "stiff" membrane at room temperature with a $K_b \sim 20kT$ [26–28], all of the present systems can be taken to be energetically stabilized. The Helfrich free energy for a closed vesicle can be expressed as $f_b = 1/2K_b(2C - C_0)^2 + K_G C^2$, where K_G is the bending modulus associated with the Gaussian curvature and C is the curvature of the vesicle ($1/R$). The addition of DHPC can effectively lower the K_b of the system as well as induce a compositional asymmetry between the inner and outer leaflets resulting in a nonzero C_0, thus a lower free energy of ULV formation. A previous study using noncharged DMPC-DHPC binary mixtures underwent a phase transition where DHPC phase separated from DMPC [9]. This result is consistent with a nonelectrostatic theory described by MacKintosh and Safran [29] which stated that vesicular structures are stabilized when mixtures are near or undergoing phase separation. Nevertheless, the reduction in the free energy achieved by the addition of DHPC is insufficient for monodisperse ULV formation, as DMPC-DHPC mixtures require that they be doped with charged species (e.g., DMPG or $CaCl_2$) to form ULVs. Noncharged DMPC-DHPC mixtures form MLVs [9].

In the Helfrich free energy the contribution of Gaussian curvature to the deformation energy is a topological invariant, and hence can be neglected where there is no change in topology. However, Helfrich, and several others, has attributed the appearance of vesicles to negative K_G values [30,31]. For $K_G < 0$, the formation of monodisperse ULVs is energetically favored as exemplified by our data.

In the present case, a low surface charge (DMPG-doped) or high salt (Ca^{2+}-doped) content induces tension in the bilayer, due to an asymmetric charge density resulting from a different radius of curvature between inner and outer part of the ULV, which in turn introduces a new electrical component to the bending moduli (e.g., $K_G \sim -K_b \sim 10^{-19}$ J) such that f_b is now small enough to induce ULV formation [30]. Assuming a symmetric distribution of DMPG molecules in each of the bilayer's two leaflets, the charge density for ULVs of radius 388 Å is 0.001 C/m^2. The only electrolytes in the DMPG-doped system are the dissociated Na^+ ions, meaning the Debye length is quite large, ~750 Å. For the Ca^{2+}-doped system, assuming a dissociation constant of 150 mM, the charge density is a factor of 5 greater than for the DMPG-doped system. The dissociated Ca^{2+} and Cl^- ions thus shorten the Debye length to tens of Å instead of hundreds of Å. Nevertheless, within these values, the qualitative magnitude of the electrical contribution to K_G is $\sim -2kT$, and to K_b this contribution is $\sim 1kT$.

It therefore seems that the spontaneous formation of ULVs in the doped systems can be explained by a large negative change in the modulus of Gaussian curvature, induced by electrical surface charge. K_b dominates the magnitude of the spontaneous curvature and determines the radius of the vesicle. With both dopants present in the system, the concentration dependence of $\langle R_o \rangle$ is not yet fully understood. A possible explanation is that the free energy of the electrical contribution to K_b decreases as a result of a shorter Debye length, due to a higher salt concentration, and, possibly, a lower surface charge depending on how Ca^{2+} ions bind to the various lipids in the mixture. This would allow the ULVs to adopt sizes other than those determined by the spontaneous curvature. Besides giving rise to ULVs the small surface charge associated with them, along with repulsive forces arising from thermal fluctuating bilayers [16], prevents the ULVs from forming MLVs. It thus seems that lipid chain asymmetry, which determines the ULVs spontaneous curvature, may be responsible for the insensitivity of vesicle radius to concentration. Note that the CMC for DHPC is $\sim$15 mM [32], much higher than the concentration of DHPC found in the present mixtures (< 4 mM) and indicating that no DHPC micelles exist in our samples. However, DHPC monomers may exist in solution and may possibly exchange with lipids in the ULVs.

In conclusion, we have reported on a spontaneously forming biomimetic ULV system possessing either a net negative (DMPG-doped) or net positive (Ca^{2+}-doped) charge and whose size (i.e., R_o) is insensitive to lipid concentration. This result is in contrast to previous experimental observations. We believe these monodisperse ULVs to be energetically, rather than entropically, stabilized for the following reasons: (i) the ULVs are highly monodisperse and (ii) R_o is insensitive to lipid concentration. Doping the system with both negative (e.g., DMPG) and positive (e.g., Ca^{2+}) charges also seems to result in energetically stabilized ULVs, whose size, although no longer independent of concentration, still exhibits a high degree of monodispersity. Unlike many ULV forming systems reported in the literature, the ULVs reported here are comprised of biocompatible molecules. It is known that enhanced uptake by tissues and organs, as a result of an extended circulation half-life, requires formulations that result in small and relatively monodisperse preparations [33]. The present system meets these requirements and therefore has the potential to be highly suitable for biomedical applications such as drug delivery.

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