Spontaneously Forming Unilamellar Phospholipid Vesicles

Baohua Yue,[1] *Chien-Yueh Huang*,*[1] *Mu-Ping Nieh*,[2,3] *Charles J. Glinka*,[4] *John Katsaras*[2]

[1]York Department of Chemical Engineering, Department of Chemistry and Environmental Science, New Jersey Institute of Technology, Newark, NJ 07102, USA
E-mail: mhuang@adm.njit.edu
[2]National Research Council, Steacie Institute for Molecular Sciences, Chalk River, Ontario K0J 1J0, Canada
[3]Department of Physics, University of Guelph, Guelph, Ontario N1G-2W1, Canada
[4]Center for Neutron Research, National Institute of Standards and Technology, Gaithersburg, MD 20899, USA

Summary: Unilamellar vesicles (ULV) consisting of a single lipid bilayer are of special interest as drug delivery vehicles. Here, we report on a spontaneously forming ULV system composed of the short- and long-chain phospholipids, dihexanoyl (DHPC) and dimyristoyl (DMPC) phosphorylcholine, respectively, doped with the negatively charged lipid, dimyristoyl phosphorylglycerol (DMPG). Small-angle neutron scattering (SANS) and dynamic light scattering (DLS) were employed to systematically investigate the effects of lipid concentration, salinity, and time on vesicle stability. It is found that ULV size is practically constant over a range of lipid concentration and temperature. The spontaneously formed ULV are stable for periods of four months, or greater, without the use of stabilizers.

Keywords: dynamic light scattering; membranes; neutron scattering; phospholipids; vesicles

Abbreviations: DLS – dynamic light scattering; DHPC – dihexanoyl phosphorylcholine; DMPC – dimyristoyl phosphorylcholine; DMPG – dimyristoyl phosphorylglycerol; MLV – multilamellar vesicle; NG7 – neutron beamline; NIST – National Institute of Standards and Technology; P-B theory – Poisson-Boltzmann theory; PEG – polyethylene glycol; SANS – small angel neutron scattering; ULV – unilamellar vesicle

Introduction

Phospholipid vesicles are considered promising candidates for encapsulation and delivery devices of drugs and biomaterials. Stable, spontaneously forming ULV are of particular interest as there is no need for stabilizers. Nevertheless, spontaneously formed ULV have

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predominantly been observed in surfactant systems;[1-13] stable bicompatible phospholipid ULV rarely form spontaneously.

Conventional methods of preparing lipid ULV often involve tedious procedures such as, multi-stage extrusion and sonication, necessary in breaking-up multilamellar vesicles (MLV). However, these ULV are unstable and eventually revert back to MLV.

Some vesicles can be stabilized by polymers, e.g. PEG (polyethylene glycol)-grafting, but the release of the encapsulated materials may be hindered due to low membrane permeability.[14,15] In comparison, spontaneously formed ULV have the possible advantage of high stability, easy preparation, and fast release under appropriate conditions. For drug delivery, of most interest are monodisperse ULV with diameters between 50 and 150 nm, a compromise between loading efficiency, stability and ability to extravasate after administration.[16-17]

Previously, lipid ULV were prepared using long and short-chain lipid mixtures.[18-26] The average vesicle radius $<R_o>$ was found to change with lipid concentration, C_{lp}. A recent study has shown that the structures and sizes of DMPG or Ca^{2+} doped spontaneously formed ULV were practically C_{lp} indepemdent.[27-28] However, when the systems were doped with both DMPG and Ca^{2+}, the ULV radius became C_{lp} dependent. It is known that Ca^{2+} binds tenaciously with the membrane, simultaneously affecting bilayer surface charge and local screening.

In the present study, we replaced Ca^{2+} with Na^{+} in order to alleviate the complexity from surface binding, and to study the effect of ionic strength on spontaneous vesiculation. The self-assembled structures from NaCl-doped DMPC/DMPG/DHPC mixtures were studied using SANS and DLS. The vesicle size was monitored as a function of C_{lp}, solution salinity, C_s, and time t. The surface charge was introduced via the bilayer embedded negatively charged lipid, DMPG.

Under various experimental conditions, it is found that ULV size is insensitive to both C_{lp} and C_s. The formation of vesicles can be explained by the Poisson-Boltzmann theory (P-B theory) of charged fluid membranes, where a negative Gaussian modulus can be obtained[29-31] and spontaneous vesiculation can lead to stable ULV. As salinity increases, the total elastic energy increases and eventually leads to a ULV-MLV transition. The reason for nearly constant vesicle sizes under various lipid concentrations and salinity is presently not very clear. It may result from the dominant role of enthalpic interactions at low lipid concentration or from the kinetics of the system. Despite this, DLS data show most samples remaining stable for weeks and even months.

Experimental Section

Materials: DMPC, DHPC and DMPG were purchased from Avanti Polar Lipids* (Alabaster AL); sodium chloride (NaCl) was obtained from Sigma-Aldrich (St. Louis, MO). All chemicals were used as received. Prior to use, deuterium oxide (99.8 %, Fisher Scientific) solvent was filtered through a 0.1 μm Millipore Millex-VV filter.

Sample Preparation: Solutions of DMPC/DMPG/DHPC molar ratio of 60/1/15 were prepared to a C_{lp} of 1.00 wt% in D_2O (diluting from higher C_{lp} of 5wt.%) by dissolving dry lipid powders. Sodium chloride was then added to yield a C_s in the range of 0.10 % to 1.00 %. After a freezing and thawing cycle for homogenization, samples were diluted with appropriate amounts of D_2O to their final C_{lp} and C_s. Samples were stored at 4 °C for four months, while selected samples were further incubated at 30 °C for an additional month. Most samples were tested with DLS multiple times over a five-month period, while SANS experiments were conducted one month after the sample preparation.

Small Angle Neutron Scattering: SANS experiments were conducted on the 30m NG7 beamline at the NIST Center for Neutron Research, Gaithersburg, MD. Two sample-to-detector distances (1.50 and 15.30 m) were selected, covering an effective Q range between 0.002 and 0.3 A^{-1}.

Dynamic Light Scattering: DLS experiments were performed on a Beckman N4 Plus Photon Correlation Spectrometer equipped with a laser source of wavelength 632.8 nm at a scattering angle of 90°.

Results

All final ULV solutions were transparently with a bluish tinge, a characteristic of solutions containing nanometer sized vesicles.[6] Solutions of MLV, on the other hand, appear opaque and undergo phase separation with time.

Effects of C_{lp} vesicle stability and size: To study the effect of C_{lp} on the self-assembled structures, ULV size for a series of C_{lp} concentrations (e.g., 0.033, 0.10, 0.33, 0.50 and 0.75 wt%, and C_s = 0.1 wt%), were measured.

SANS data and the corresponding best-fit curves of a spherical shell model are shown in

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Figure 1. Data of the C_{lp} = 0.75 wt% sample are not fitted because of large particle size and high polydispersity. The fitted results are in good agreement with the experimental data for all samples with $C_{lp} \leq 0.5$ wt%. The results also indicate that all the vesicular structures have a constant bilayer thickness of ~ 32 Å as a function of C_{lp}, consistent with previously reported data.[25-28] Strikingly, vesicle radii remain practically unaltered (24 ± 1 nm) and are C_{lp} independent for $C_{lp} \leq 0.33$ wt%. Larger monodisperse ULV (~ 31 nm) are observed in the C_{lp} = 0.5 wt% sample. The monotonic decay for the 0.75% sample implies the existence of polydisperse large aggregates.

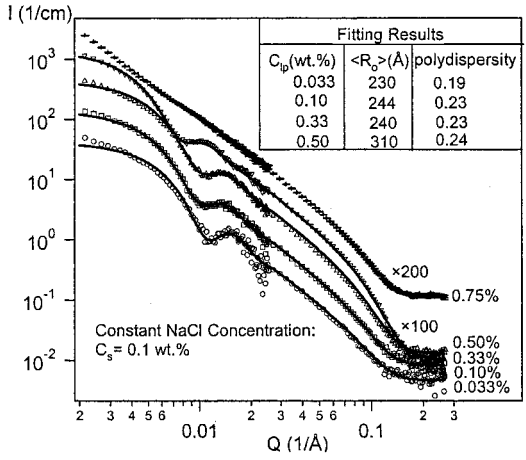

Figure 1. SANS data, fitted curves (solid lines) and results for samples with varying C_{lp} and a constant NaCl concentration of C_s = 0.10 wt%. The C_{lp} = 0.50 and 0.75 wt % samples were scaled for better viewing.

DLS measurements of the same systems (Figure 2) show results consistent with SANS data such as, unimodal size distribution with low polydispersity and a hydrodynamic radius, R_H, ~ 40 nm. As shown in Figure 2, that although particle size remains reasonably constant for three C_{lp} samples (i.e., 0.033, 0.10 and 0.33 wt%), the size distribution for the 0.50 wt% sample is much larger. In the case of the 0.75 wt% sample a bimodal distribution is observed immediately after preparation, with the sample turning turbid. Note that the best-fit vesicle radius from SANS experiments is always smaller than the hydrodynamic radius (R_H) obtained from DLS data. This difference is a result of the

different size-averaging algorithms. Moreover, R_H also includes the contribution from associated water molecules, and which is not the case for the radii determined from SANS data. Finally, large aggregates (R_H > 50 nm) have been observed in DLS experiments, and may contribute to the misfit of the SANS data at very low Q (< 0.003 $Å^{-1}$) values (Figure 1).

Both SANS and DLS results show that the ULV size is, within a certain C_{lp} range (≤ 0.33 wt%), practically invariant. As C_{lp} increases above 0.5 wt%, the ULV size becomed C_{lp} dependent. Although highly stable ULV were found when doped either with a charged lipid (DMPG) or a salt ($CaCl_2$),[27,28] this is the first time that a highly stable ULV system doped with both charged a lipid (DMPG) and a salt (NaCl), has been reported.

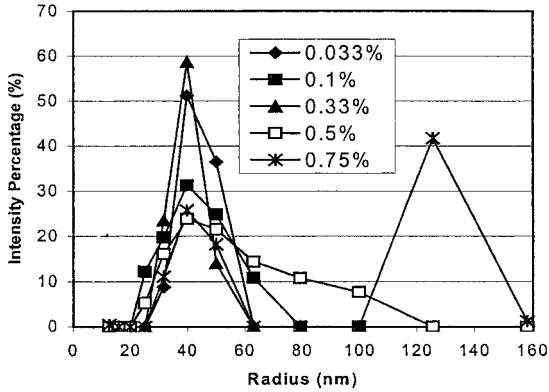

Figure 2. Size distribution functions obtained from DLS data for samples with C_{lp} ranging between 0.033 and 0.75 wt%, at a fixed C_s of 0.10 wt%.

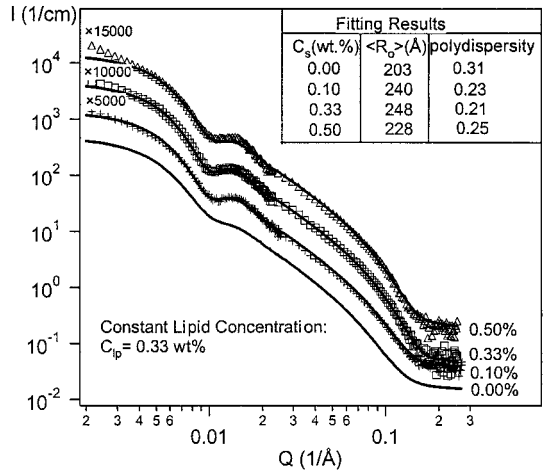

Figure 3. SANS data, fitted curves (solid lines) and results for varying C_s samples and C_{lp} = 0.33 wt%. Data was scaled by an appropriate factor (shown) for better visualization.

Effects of C_s on vesicle stability and size: To study the effect of ionic strength, NaCl was added to vesicle solutions. For a C_{lp} = 0.33 wt% sample, five salt concentrations (C_s = 0, 0.10, 0.33, 0.50 and 1.00 wt%) were examined (Figure 3, except for the C_s = 1.0 wt% sample). Conspicuously, the best fits to the SANS data show the existence of ULV with invariant radii (24 ± 1 nm), and polydispersity ~ 20%, for all samples except those with C_s = 0 and 1 wt%. The sample C_s = 0 wt% sample (non-NaCl doped) has a slightly smaller radius (~ 20 nm) and a slightly higher polydispersity (~ 30%); the C_s = 1.00 wt% sample turned opaque only one day after being prepared, presumably forming MLV.

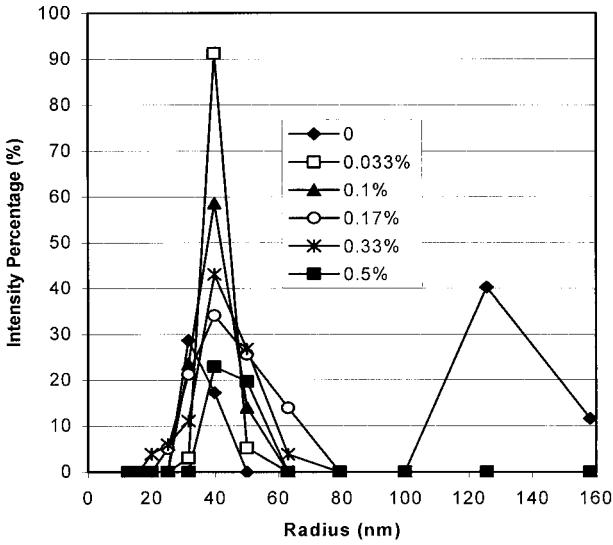

Figure 4. Size distribution functions from DLS for samples with C_s varying from 0.0 to 0.50%, and C_{lp} = 0.33 wt %.

The finding of forming nearly constant vesicle size as a function of C_s is further supported by DLS data (Figure 4), which show ULV radii remainin virtually unaltered at ~ 40 nm. Poydispersity, on the other hand, does seem to be variable. In some cases such as, C_s = 0, another peak with a much larger hydrodynamic radius (R_H >100nm) is found. This coexistence of ULV and the large particles may also help in explaining the discrepancy between the SANS data and the fit to the data in very low Q regime (< 0.004 $Å^{-1}$).

Time Evolution: The stability of ULV size versus time was monitored by DLS for four different C_{lp} (0.033, 0.1, 0.33 and 0.5 wt%) samples. All samples were kept at 4 ^oC for the first four months and at 30 ^oC during the fifth month. Figure 5 depicts the size changes of the samples: for ULV formed in mixtures with $C_{lp} \leq 0.1$ wt%, R_H were almost invariant over the five month period; for ULV formed with C_{lp}/C_s = 0.33 wt%/0.33 wt%, R_H showed a slight increase in the first four months, followed by a significant increase, as judged from the appearance of a second peak in DLS data, after the fifth month. In the case of C_{lp}/C_s = 0.50 wt%/0.1 wt%, the sample became turbid after two months, indicative of MLV formation.

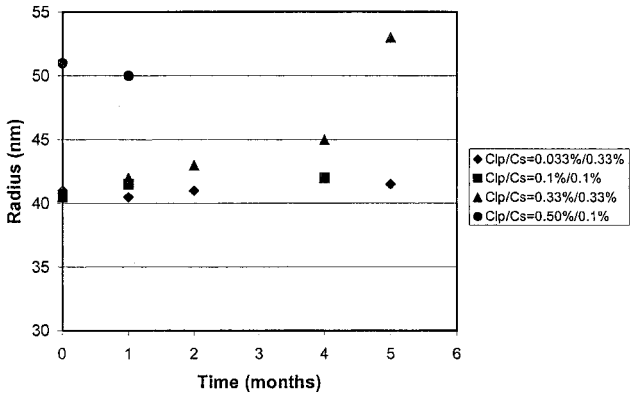

Figure 5. Hydrodynamic radius (R_H) of ULV as a function of time after sample preparation; C_{lp}/C_s = 0.033 wt%/0.33 wt%, 0.10 wt%/0.10 wt%, 0.33 wt%/0.33 wt% and 0.50 wt%/0.10 wt% .

Discussion

Effect of Lipid concentration, C_{lp}: Our observations of variable size ULV for $C_{lp} > 0.33$ wt% samples are consistent with previous studies.[27-28] For $C_{lp} \leq 0.33$ wt% samples, vesicle size is C_{lp}-independent and consistent with what has previously been observed in systems doped with either DMPG or Ca^{2+}, but not with both.[27-28] A previous study of non-doped ([DMPC]/[DMPG] = ∞) and strongly DMPG-doped ([DMPC]/[DMPG] = 15) DMPC/DHPC systems at temperatures and lipid concentrations similar to those studied here, showed the presence of MLV and bicelles, respectively.[26] When an appropriate amount of charged is introduced into DMPC/DHPC mixtures, the mixtures exhibit spontaneous, monodisperse ULV formation . This implies that surface charge may induce a reasonably deep local free energy minimum rendering the ULV insensitive to changes in C_{lp} and C_s. The fixed ratio between charged (DMPG) and non-charged lipids used in this study provides a condition for constant surface charge density assuming that, under the conditions investigated, the amount of lipids in solution does not affect the chemical potential. The insensitivity of ULV size to changes in C_{lp} also contradicts the predictions of Oberdisse et al.,[32-34] whose results show a ULV size increase with increasing surfactant concentration in a system of high surface charge density.

Recently, Egelhaaf et al.[35-36] suggested a kinetic model explaining the formation

mechanism of ULV in lecithin/bile salt systems. The model states that ULV could be kinetically trapped as the detergent (i.e., charged short-chain lipid) molecules are removed from the rim of disk-like micelles (i.e., bicelles) upon dilution. Once vesicles are formed, the size may be insensitive to a certain degree of change in either C_s or C_{lp} since only a small amount of bile salt is left residing in lipid membranes. The formation of ULV in our study is different from the above scenario. Although bicelles were found at C_{lp} = 1 wt% and low temperatures[28], the short chain DHPC lipid does not carry any charge and probably distributes itself at the high curvature bicelle rim. This, of course, does not preclude the formation of ULV via another kinetically controlled mechanism. However, more studies on the stability and size of ULV formed *via* various pathways are needed to further clarify this issue.

Effect of Ionic strength: The screening effect of coulombic interactions on the self-assembled charged structures can be studied by altering the ionic strength, e.g., salt concentrations, and thus the Debye length χ_D. Our experimental results show a slightly smaller vesicle radius in NaCl-free mixtures (~ 20 nm, SANS) compared to those ULV formed from lipid mixtures with NaCl (~ 24 nm, SANS). Assuming the complete dissociation of DMPG in a NaCl-free mixture, the calculated χ_D is about 37 nm, larger than the radius of curvature, and the surface charge density of the bilayer membrane is about 3.5×10^{-3} C/m^2 (neglecting the charge difference between the inner and outer layers and taking vesicle outer radius R_o = 24 nm and inner radius R_i = 21 nm). Compared to the calculations from the simplified P-B theory by Winterhalter and Helfrich, [31] the above experimentally obtained charge density and the Debye length predict a small positive bending modulus and a negative Gaussian modulus, leading to a negative total elastic energy which favors spontaneous vesiculation. Reducing the screen lengths (by increasing salt concentration) renders a reduction in bending modulus, however, under the given charge density, the negative Gaussian modulus *increases* with the ionic strength and eventually leads to a positive total energy at certain threshold $C_{s,MLV}$, where a ULV to MLV transition takes place. For samples doped with NaCl, χ_D varies from 4 nm (C_s = 0.033 wt%, 5.6 mM) to 1.3 nm (C_s = 0.33 wt%, 56 mM), ULV size remains unaltered. Though there is no direct comparison between our data and calculations from more detailed treatments such as, P-B theory with translational entropy, asymmetric charge density, etc., the insensitivity of ULV size may be attributed to a low entropic contribution resulting from low lipid concentration and the dominant role of enthalpic interactions in

forming self-assembled structures. As lipid concentration is increased, other degrees of freedom should be considered, particularly their contributions to entropy. The entropic contribution should increase with the total lipid concentration that eventually destabilizes ULV at a threshold salinity $C_{s,MLV}$, which decreases with the total lipid concentration.

Aging Effect: Although the size of ULV, over a five month period, is found to be invariant (Figure 5), the issue of whether these ULV are kinetically trapped or thermodynamically stable is not yet understood. Note that the samples were stored at 4 ^{o}C, lower than the T_M of DMPC, except during the measurements. Most of the time, the DMPC was in the gel phase, a more rigid molecular structure than the L_α phase, and hence the exchange rate of DMPC from ULV to solution, or vice versa, was slow. The invariant ULV size at low temperature (4 ^{o}C) may suggest that the vesicular structures are kinetically trapped.

Conclusions

Spontaneously formed ULV with low polydispersity are found in DMPC/DMPG/DHPC phospholipids mixtures by dilution from high concentration lipid solution at low temperature. SANS and DLS results show that the ULV are reasonably stable over prolonged periods of time and variations of lipid concentration ($C_{lp} < 0.33\%$) and ionic strength ($C_s < 0.5$ wt%), both below and above the phase transition temperature, T_M, of DMPC. These results are different from some theoretical predictions and experimental reports. A possible explanation is that the enthalpic contribution from a negative Gaussian modulus dominates over the effects from other degrees of freedom at low lipid concentrations. Another possible explanation may be the energetically favorable packing of DMPC and DMPG that renders a slow response to any change in chemical potential at low concentrations. Further studies of the resultant structures *via* various pathways in preparation with the same chemical composition are needed to better understand the formation mechanism of ULV. The stability of ULV and the ability to control their size may facilitate the development of pharmaceutical or biomedical applications.

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