CHINESE JOURNAL OF PHYSICS VOL. 50, NO. 2 April 2012

Small-Angle X-ray Scattering Studies on the Structure of Mixed DPPC/diC$_7$PC Micelles in Aqueous Solutions

Po-Wei Yang,[1] Tsang-Lang Lin,[1,*] Yuan Hu,[1] and U-Ser Jeng[2]

[1]*Department of Engineering and System Science, National Tsing-Hua University, Hsinchu, Taiwan, R.O.C.*

[2]*National Synchrotron Radiation Research Center (NSRRC), Hsinchu, Taiwan, R.O.C.*

(Received March 9, 2012)

The phase behavior of mixed DPPC/diC$_7$PC micelles in aqueous solutions was investigated using small-angle x-ray scattering (SAXS) and Transmission electron microscopy (TEM). Phase transitions from multilamellar vesicle (MLV) to disc bicelle, ribbon-like, and rod-like aggregates are observed as the percentage of the short-chain diC$_7$PC is increased. At DPPC/diC$_7$PC molar ratio 3/1, the multilamellar liposomes are all transformed into disc bicelles. The long-chain DPPC lipid and short-chain diC$_7$PC lipid, respectively, form the planar and rim part of the bicelle with a radius and thickness of 98.4 and 55.3 Å, respectively, for the DPPC/diC$_7$PC mixing ratio of 3/1. Temperature-induced structure transformation is also observed for such a mixed system. The structural transformation is induced by the solubilization of diC$_7$PC into the planar long-chain lipid bilayer. An intermediate ribbon phase was found and coexisted with DPPC/diC$_7$PC multilamellar liposome phase at 40°C; while only multilamellar liposome phase was found at 45°C which is higher above the chain melting temperature of DPPC.

PACS numbers: 61.05.cf, 68.18.H-, 64.70.pv

I. INTRODUCTION

In aqueous solution, small self-assembled aggregates could form spontaneously by amphiphilic molecules in a variety of shape and size due to the differences in their hydrophilic head groups and hydrophobic acyl chain [1–3]. In addition to the factors of molecular structure and property, concentration, temperature and environment, such as salt concentration, are also important in determining the structure and size of the aggregates. Such aggregates have wide application, such as used in fabricating nanoparticle, templating nanostructural materials, mimic biological systems, drug delivery, gene therapy, *etc.* For bio-related applications, the zwitterionic 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) or 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) is often used in forming vesicular bilayer structures as a model system to mimic the cell membrane [4, 5]. Unlike the long chain lipids, the short-chain lipids, such as 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine (diC$_7$PC) or 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (diC$_6$PC), with 6-8 carbons in their acyl hydrocarbon chains, typically form globular to rod like micelles in aqueous solutions DPPC, a common phospholipid found in biological membranes, forms stable liposomes which are

*Electronic address: tllin@mx.nthu.edu.tw

promising vectors for formulating drug or gene delivery systems [6]. In the presence of short-chain lipids, such as diC_7PC or diC_6PC, at suitable ratios could turn the vesicles into disc-shaped bicelles [7, 8].

Over the past decade, there were lots of biophysical and biochemical studies of lipid bilayers to mimic the system of biological membranes [1, 9–12]. The bicelles have been widely and successfully used in studying the interactions of lipid bilayer with biomolecules [1, 10, 11]. The bicelles have disc-shapes with the long-chain lipids form the planar core and the short-chain lipids form a belt on the edge of the planar core to protect the open edge of the lipid bilayer from contacting with the water. [13, 14]. As we know, the most interesting property of the bicelles could be spontaneously aligned in presence of an external magnetic field [2, 15], which makes the bicelles very attractive for nuclear magnetic resonance (NMR) studies, especially applied to small membrane protein/peptide system [1, 16, 17]. Moreover, such kind of micelle can also be used as a template for synthesizing inorganic nanoparticle by using two oppositely charged surfactants [18].

A series of researches on characterizing the short-chain/long-chain unilamellar vesicles [19, 20] had been done by means of negative staining electron microscopy, NMR and Fourier transform infrared (FTIR) spectroscopy [3], and these results indicated the bicelle could be self assembled in aqueous suspension of long-chain lecithin with small amount of short-chain lecithin in a wide range of conditions [19]. So far, the bicelles consist of DMPC or anionic DMPG with diC_6PC have been widely studied by small angle scattering (SAS) [14] and NMR [8, 15, 20] to map out the complete phase diagram of these binary lipid/water system and to reveal the factors [14] affecting their structures, such as temperature [2, 21–23], lipid species, buffer pH [23]. In addition to the DMPC/diC_6PC mixed system, the DPPC/diC_7PC system is still not well studied in terms of their structures as the composition varies [13, 19]. In this report, we present a systematic study on the structure of mixed DPPC/diC_7PC system as a function of the mixing ratio by means of SAS and TEM. The temperature-induced structural changes of the DPPC/diC_7PC bicelles at a long-chin to short-chain ratio of 3/1 were also investigated.

II. METHODS AND MATERIALS

II-1. Small-Angle X-Ray Scattering (SAXS) and Transmission Electron Microscopy (TEM)

SAS is most suitable for investigating the size and shape of nanostructural materials suspended in aqueous solution, while TEM could provide direct observation of the structure as complementary tool to scattering methods.

SAXS measurements were carried out at the SAXS/WAXS beamline 23A of the National Synchrotron Radiation Research Center (NSRRC), Hsinchu, Taiwan. The photon energy and sample-to-detector distance are set to be 14 keV ($\Delta E/E = 1/7000$) and 2700 mm, respectively, in order to cover a scattering vector, $Q = (4\pi/\lambda)\sin(\theta/2)$, from 0.008 to 0.35 Å^{-1}. Here, θ is the scattering angle and λ is the wavelength of the incident X-rays. Liquid samples were loaded in cell with thickness of 5 mm with Kapton-walled windows

and the sample temperature was kept at 25°C. Scattered X-rays were collected by means of 2D MarCCD detector. The collected 2D data were circular averaged to give the 1D scattering intensity distribution as a function of scattering vector and treated with background subtraction, transmission correction and normalized to absolute scattering intensity. The TEM images were taken with 100 kV by the JEOL JEM-1230 in Chang Gung University.

II-2. Materials and Sample Preparation

The zwitterionic phospholipids of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine (diC_7PC), were purchased from Avanti Polar Lipids and used without further purification. Deionized water (resistivity > 18 MΩ/m) was purified using MilliQ system (Millipore). The appropriate amounts of lipids were first dissolved in chloroform with desired molar ratio. The solvent was evaporated and all the trace was then removed entirely in vacuum oven at least 8 hours. Subsequently, the dry residues were rehydrated by adding deionized water and treated by bath sonication at temperature of 30°C for 5 minutes in order to homogenize the lipids suspension in H_2O. The total lipid concentration is keep at 10 mM for all mixed DPPC/diC_7PC samples. The specimens for TEM measurement were prepared on a cupper grid with a supported polymer film. The solution used in SAXS measurement were diluted appropriately (1/20) and then applied by negative-staining with uranyl acetate (1 wt%) for 30 seconds in order to enhance the contrast [3].

III. RESULTS AND DISCUSSIONS

The formation of binary mixed long-chain lipid with short-chain lipids, DMPC/diC_6PC in various concentrations and temperature was well-established [14, 24, 25]. We, here, demonstrate a similar self-assembly structure by applying longer zwitterionic lipids which have acyl chains with 16 and 7 carbons for the long-chain and short-chain lipids, respectively. We use DPPC and diC_7PC to form mixed systems at different ratios. The effect of temperature on the bicelles was investigated with the mixed DPPC/diC_7PC system at a ratio of 3/1.

Fig. 1 shows the measured SAXS profiles for mixtures of the DPPC with diC_7PC at different mixing ratios in aqueous phase at 25°C. It was found that a structure transition occurs when the percentage of diC_7PC is gradually increased by turning the DPPC multilamellar vesicles gradually into disc bicelles. The first order diffraction peak of the DPPC multilamellar vesicles occurs at $Q = 0.099$ Å^{-1}, which corresponds to a spacing of $d = 63.5$ Å. As the percentage of the diC_7PC is increased, the intensity of the diffraction peaks gradually decreases and the full width at half maximum (FWHM) of the Bragg peaks also get broadened. These indicate that the number of the stacking layers of the DPPC liposomes are gradually reduced. However, the lamellar peaks do not disappear completely until DPPC/diC_7PC molar ratio reaches 3/1. For DPPC/diC_7PC $> 3/1$, DPPC multilamellar liposomes coexist with the disc bicelles. It needs sufficient amounts of the short-chain diC_7PC to solubilize all the DPPC bilayer membrane. The d-spacing of the DPPC multil-

amellar phase does not change when diC_7PC is added. This means that short-chain diC_7PC lipids solubilize DPPC membrane to form small pieces of disc-like aggregates rather than being solubilized in the long-chain lipid bilayer. The sample of DPPC/diC_7PC at 3/1 molar ratio appears to be highly transparent while DPPC multilamellar liposome sample is opaque due to the large size of the liposomes that scatters the visible light strongly. The transparency of the bicelle solution is a good indication of the existence of small aggregates.

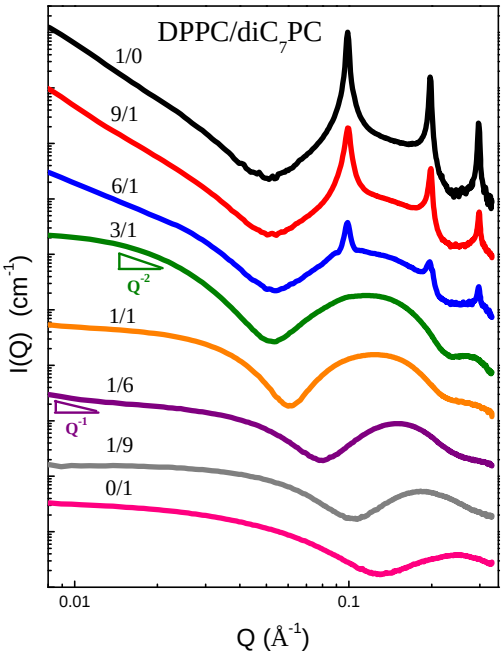

FIG. 1: The measured SAXS profiles of DPPC/diC_7PC system at different mixing molar ratios at 25°C. The scattering curves are shifted for clarity.

The measured SAXS data of the mixed bicelle at DPPC/diC_7PC = 3/1 were fitted with the "PolyCoreBicelle" model that had been developed by NCNR using the software IGOR-Pro [26]. It is assumed that the bicelle disc model has short-chain diC_7PC in the rim region and the long-chain DPPC form the center core region. The hydrophobic core and the hydrophilic shell in this simplified model consist of acyl chain and head group, respectively. The total lipid concentration in this study is only around 0.1 wt% so that the SAXS data can be fitted by the form factor only at such a dilute condition. Fig. 2 shows the fitted scattering curve and the schematic structural model of the disc bicelle according to the structural parameters obtained from the fitting. The disc bicelles have an averaged radius of 98.4 Å and a thickness of 55.3 Å. This radius includes the rim thickness of 14.9 Å and a mean core radius of 83.5 Å with 24% polydispersity in the bicelle radius. The total thickness of the bicelle includes the hydrophobic core thickness 25.5 Å and the thickness of the two hydrophilic head group layers, 14.9 Å each. Although the thickness of the rim (head group region of the short-chain lipids) and the planar shell (head group region of the

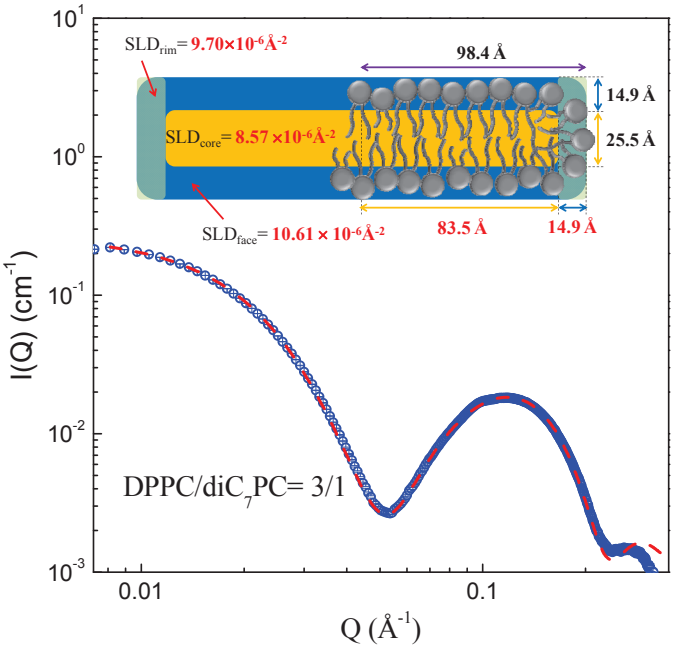

FIG. 2: The SAXS profile in together with the fitted curve, and the schematic model of the mixed bicelles of DPPC/diC$_7$PC at 3/1 molar ratio at 25°C.

long-chain lipids) can be set differently in the model fitting, it could still be reasonable and simpler to set equal shell thickness in the calculations. The X-ray scattering length density of the rim (9.7×10^{-6} Å^{-2}) is slightly lower than that of the planar region (10.6×10^{-6} Å^{-2}). This is reasonable since the packing of the head groups of the short-chain lipids in the rim region is less tight as compared with the more ordered structure of the planar region. The structural characteristics of the bicelle structure of DPPC/diC$_7$PC system at a molar ratio of 3/1 is comparable with that of DMPC/diC$_6$PC at 3/1 ratio [14]. The size of the bicelle can also be estimated theoretically by considering the packing constraints [11]. It is assumed that the head groups occupy different area sizes at the interface for DPPC and diC$_7$PC since the surface curvature in the rim and the planar regions are different. The long-chain lipid and short-chain lipid can only stay at planar and rim region, respectively. Therefore, the core radius, R, of the disc-shaped bicelles can be calculated by a given mixture molar ratio, q, and rim radius, r, by $R = 0.5krq[\pi + (\pi^2 + 8k/q)^{0.5}]$. Here, k is the surface area ratio of the short-chain lipid to long-chain lipid. With $q = 3$, $k = 0.6$ (as that set for the DHPC/diC$_6$PC bicelle), $r = 27.65$ Å (the radius of the rim set equal to half of the bicelle thickness according to ideal bicelle model of a hemispherical shape rim), it would predict a bicelle core radius of 162 Å, which is significantly larger than that obtained by SXAS analysis. For the DHPC/diC$_6$PC bicelles, it was found that using a much smaller value of $r = 20$ Å would give a better fit to the measured bicelle size. It is obvious that the shape of the rim could not be a hemispherical shape as described by this ideal biclle model

and the rim surface area covered by the short-chain lipids should be much smaller than that described by the ideal bicelle model. Although such a simple geometrical model could not predict the size of the bicelles accurately, it is still useful to illustrate the importance of packing constraints and the idea of phase separation in such a mixed system with very asymmetric components. For a thin cylindrical disc bicelle model as used in the SAXS analysis of this study, the radius of the bicelle can be roughly estimated by $R_{bicelle} = khq$ by assuming that the long-chain lipids cover all the top and the bottom surfaces and the the short-chain lipids cover the flat rim with a height h. According to this simple model, with $q = 3$, $k = 0.6$, and $h = 55.3$ Å, the bicelle radius is predicted to be 99.5 Å, which is very close to the value of 98.4 Å determined by the SAXS analysis.

Furthermore, it was found that the size of the mixed aggregates decreases as the percentage of short-chain lipid is increased and the shape/structure would also change as the ratio of DPPC to diC_7PC is further decreased. At DPPC/diC_7PC molar ratio 1/6, the scattering intensity at the low-Q region increase significantly with decreasing Q, which indicates a structural transition into very long aggregates, probably ribbon-like as that observed for the DMPC/diC_6PC system. Eventually the scattering curves exhibit the feature of rod-like micelles for pure diC_7PC suspension. As shown in Fig. 3, the TEM image shows the bicelles at top view with circular shape and side view with oblong shape. The size is estimated to be around is $20 \sim 30$ nm in diameter which close to the size obtained from the SAXS analysis.

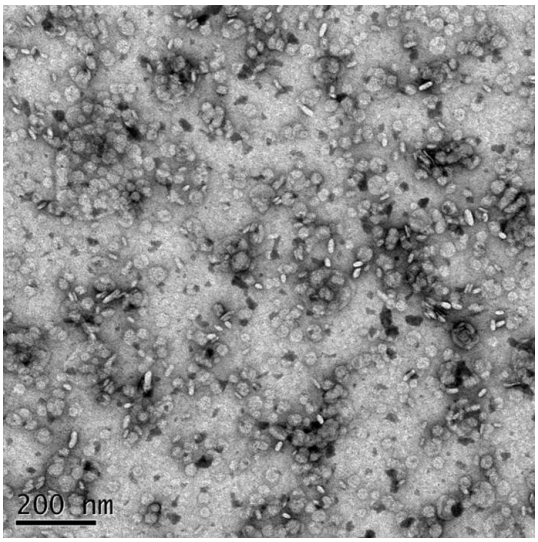

FIG. 3: The TEM image of DPPC/diC_7PC at molar ratio 3/1. The bicelle image is obtained at a 20-fold dilution from 10 mM SAXS solution sample. The solution sample was transferred to the grid with negative-staining and the picture was taken at $\times 100,000$ magnification.

The structural stability against temperature changes is also investigated for the DPPC/diC_7PC bicelles formed with a mixing ratio of 3/1. The transparent bicelle sample was heated from 25°C to 45°C, which is above the chain melting temperature of DPPC

($T_m = 41°C$), at 5°C step. The measured SAXS profiles are shown in Fig. 4. Below 35°C, at gel phase of DPPC, the disc shaped bicelles dominated the suspension and the bicelles size got larger as the temperature is increased as indicated by the change of the slope in low-Q region. As the temperature is increased, some of the short-chain lipids get solubilized into the planar long-chain lipid bilayer. The shortage of short- chain lipids to form the protecting rim would induce the bicelles to fuse into larger bicelles to reduce the total edge surface need to be protected by the short-chain lipids. It is also likely that the presence of large amounts of diC$_7$PC in the planar DPPC bilayer region could result in an increased curvature so as to form ribbon-like structures as indicated by the $1/Q$ dependence of the low-Q scattering data [25]. At 40°C, the ribbon phase is mostly replaced by a multilamellar phase with intense diffraction peaks at $Q = 0.096\text{Å}^{-1}$ which has a spacing of $d = 65.4$ Å. This d-spacing value is 1.9 Å larger than the spacing for pure DPPC multilamellar phase at room temperature. When the temperature is raised above the T_m of DPPC, at 45°C, the suspension entirely transforms into multilamellar liposomes and the solution turns opaque. The formation of DPPC/diC$_7$PC mixed multilamellar liposomes can be indicated by Q^{-4} dependence of scattered intensity in the low-Q region.

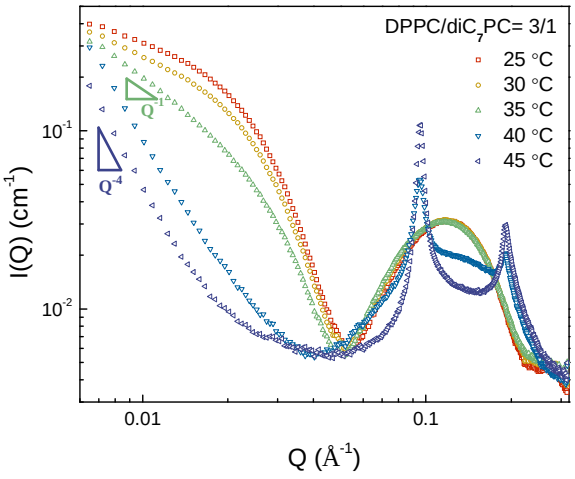

FIG. 4: The temperature dependent SAXS profiles of the mixed DPPC/diC$_7$PC at 3/1 molar ratio.

IV. SUMMARY

Using SAXS and TEM, we demonstrate that the structural changes and the temperature dependent stability of the mixed long-chain/short-chain lipid aggregates in aqueous solution can be well studied by using SAXS and TEM. As the percentage of the short-chain lipid, diC$_7$PC, is gradually increased, the DPPC multilamellar liposomes are gradually

transformed into disc bicelles. Well defined bicelles formed at DPPC/diC_7PC molar ratio 3/1. The bicelle structure is stabilized by the short-chain lipids that form the rim of the planar bilayer. However, the bicelle structure is destabilized when the temperature is raised that induce the lost of the short-chain lipids from the rim to be solubilized in the planar region. Both the geometrical constraint and the interaction among these two highly asymmetric lipids are important in determining the equilibrium mixed structure.

Acknowledgements

The authors would like to thank the NSRRC, Hsinchu, Taiwan, for providing the SAXS beam time at the BL23A beam line and the assistance in carrying out the measurements. This research is supported by the National Science Council, project No. NSC 99-2113-M-007-014-MY3 (T.-L. Lin) and also partly supported by the Frontier Research Center on Fundamental and Applied Sciences of Matters of National Tsing Hua University.

References

[1] J. Katsaras, T. A. Harroun, J. Pencer, *et al.*, Naturwissenschaften **92**, 355 (2005).
[2] M. P. Nieh, V. A. Raghunathan, C. J. Glinka, *et al.*, Langmuir **20**, 7893 (2004).
[3] N. E. Gabriel and M. F. Roberts, Biochemistry **25**, 2812 (1986).
[4] G. Pabst, N. Kucerka, M. P. Nieh, *et al.*, Chemistry and Physics of Lipids **163**, 460 (2010).
[5] T. A. Harroun, N. Kucerka, M. P. Nieh, *et al.*, Soft Matter **5**, 2694 (2009).
[6] A. Manosroi, K. Thathang, R. G. Werner, *et al.*, Arzneimittelforschung **58**, 485 (2008).
[7] N. Skar-Gislinge and L. Arleth, Physical Chemistry Chemical Physics **13**, 3161 (2011).
[8] L. van Dam, G. Karlsson, and K. Edwards, Biochim. Biophys. Acta-Biomembr. **1664**, 241 (2004).
[9] R. S. Prosser, F. Evanics, J. L. Kitevski, *et al.*, Biochemistry **45**, 8453 (2006).
[10] C. R. Sanders and R. S. Prosser, Structure **6**, 1227 (1998).
[11] K. J. Glover, J. A. Whiles, G. Wu, *et al.*, Biophy. J. **81**, 2163 (2001).
[12] R. R. Vold, R. S. Prosser, and A. J. Deese, J. Biomol. NMR, **9**, 329 (1997).
[13] T. L. Lin, C. C. Liu, M. F. Roberts, *et al.*, J. of Phys. Chem., **95**, 6020 (1991).
[14] M. P. Nieh, C. J. Glinka, S. Krueger, *et al.*, Langmuir **17**, 2629 (2001).
[15] C. R. Sanders and J. P. Schwonek, Biochemistry **31**, 8898 (1992).
[16] J. A. Whiles, R. Deems, R. R. Vold, *et al.*, Bioorganic Chem. **30**, 431 (2002).
[17] J. A. Whiles, K. J. Glover, R. R. Vold, *et al.*, J. Magn. Reson. **158**, 149 (2002).
[18] Y. J. Song, R. M. Dorin, R. M. Garcia, *et al.*, J. Am. Chem. Soc. **130**, 12602 (2008).
[19] N. E. Gabriel and M. F. Roberts, Biochemistry **23**, 4011 (1984).
[20] N. E. Gabriel and M. F. Roberts, Biochemistry **26**, 2432 (1987).
[21] G. Raffard, S. Steinbruckner, A. Arnold, *et al.*, Langmuir **16**, 7655 (2000).
[22] E. Sternin, D. Nizza, and K. Gawrisch, Langmuir **17**, 2610 (2001).
[23] J. Bolze, T. Fujisawa, T. Nagao, *et al.*, Chemical Physics Letters, **329**, 215 (2000).
[24] M. P. Nieh, V. A. Raghunathan, C. J. Glinka, *et al.*, Macromol. Symp. **219**, 135 (2004).
[25] M. P. Nieh, P. Dolinar, N. Kucerka, *et al.*, Langmuir **27**, 14308 (2001).
[26] S. R. Kline, J. Appl. Crystallogr. **39**, 895 (2006).