Structural Phase Behavior of High-Concentration, Alignable Biomimetic Bicelle Mixtures

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Summary: The structures of bicelle mixtures composed of dimyristoyl and dihexanoyl phosphatidylcholines (DMPC and DHPC) with DMPC/DHPC molar ratios of 3.2 and 5 are characterized using polarized optical microscopy (POM) and small angle neutron scattering (SANS). Three phases, isotropic (I), chiral nematic (N^*) and smectic (S) are observed as temperature (T) varies from 10 to 70 °C. The structure of the magnetically alignable N^* phase, which was previously considered to be made up of discoidal micelles, is found to be composed of "ribbons". Doping with the charged lipid, dimyristoyl phosphatidylglycerol (DMPG), which has the same 14:0 hydrocarbon chains as DMPC, results in a structural change of the aggregates where only the isotropic and smectic phases are observed. The smectic phase for the mixtures doped with DMPG is shear-alignable and follows one-dimensional swelling. However, at high-T zwitterionic DMPC/DHPC mixtures form multi-lamellar vesicles (MLV) with a relatively constant lamellar spacing of 66 Å, independent of water content.

Keywords: bicelle; DHPC; DMPC; DMPG; lamellae; multilamellar vesicle (MLV); neutron diffraction; optical microscopy; phospholipids; ribbons; small angle neutron scattering (SANS)

Introduction

Bicelle mixtures have been extensively used as magnetically alignable membrane substrates for the study of membrane-associated proteins/peptides.[1-3] Their chemical compositions include at least one long-chain and one short-chain lipid/surfactant molecule.[4-6] From nuclear magnetic resonance (NMR) studies a discoidal structure has been inferred with the short-chain lipid sequestered at the rim of the bilayered micelle, or so-called "bicelle", and the long-chain lipid forming the planar bilayer. Because the edge energy at the disk's rim can be reduced by the above-mentioned lipid arrangement, the proposed structure has been widely accepted. Recently, another possible structure for the

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magnetically alignable phase, namely perforated lamellae, was proposed based on SANS data. From the data, it was inferred that the short-chain lipids coat the rims of the perforations, minimizing the pore curvature energy.[7,8] In order to understand the structures and their self-assembly mechanisms, we have investigated a series of high-concentration bicelle mixtures at two different long- to short-chain lipid molar ratios, Q (=3.2 and 5), using POM and SANS. At least three phases (isotropic, chiral nematic and smectic) were observed with increasing temperature (e.g., 10 to 70 °C).

In general, the lipid bilayer normal (N) tends to align perpendicular to the applied magnetic field (B) due to the negative diamagnetic susceptibility of the lipids. When doped with certain lanthanide ions (Tm^{3+}, Yb^{3+}, etc.),[9-11] the bilayers can align with N || B, eliminating the degree of freedom for the rotation of N. These lanthanide ion-doped bicelles mixtures are popular because they can serve as a "biological goniometer" for membrane-associated proteins/peptides, making it possible to obtain their in-plane or out-of-plane structure. However, the non-biologically relevant lanthanide ions may bind with the protein/peptide of interest, altering its native conformation. Moreover, a strong magnetic field is required to align the bicelle mixture resulting, with the exception of NMR, in a nontrivial experimental setup.

Here we report a bicelle formulation that is alignable under a weak shear flow in absence of lanthanide ions and strong magnetic fields. The "alignability" is comparable to those systems oriented in the presence of strong magnetic fields.[12]

Experimental Section

DMPC, DHPC and dimyristoyl phosphatidylglycerol (DMPG) were purchased from Avanti Polar Lipids† and used without further purification. The mixtures were dissolved in D_2O (99.9% in purity, Chalk River Lab.) at a total lipid concentration = 25 wt.% and two Q values, 3.2 and 5. DMPG-doped mixtures with Q = 5 were prepared at different molar ratios of DMPG to DMPC, [DMPG]/[DMPC] = 0.02 and 1, yielding different charge densities.

The POM studies were performed on an Olympus BX52 microscope and the images were captured with a CCD camera. Samples loaded in sealed rectangular capillaries (2mm X 0.1mm, VitroCom Inc.), were placed in a temperature-controlled stage (Linkam

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TMHS600) to obtain the desired T with an accuracy of ± 0.05 °C. Each sample was equilibrated at the desired T for at least 0.5 hours before the image was recorded.

SANS experiments were performed at the 30-m SANS instrument (NG7) located at the National Institute of Standards and Technology (NIST, Gaithersburg, Maryland, USA). Two sample-to-detector distances (1.25 and 15.3 m) were selected to cover a range of scattering vectors, q, (= $4\pi/\lambda \sin\frac{\theta}{2}$, where λ and θ are the wavelength of the neutron and the scattering angle, respectively) from 0.002 to 0.35 $Å^{-1}$. Samples were loaded in cells with a path length of 2 mm and placed in a temperature controlled sample holder capable of handling 10 samples. Temperature was controlled by a circulating water bath. Each measurement was taken after the sample was equilibrated at the desired T for 0.5 hours. Two-dimentional SANS data were corrected for background (blocked beam), and reduced to an absolute intensity scale based on the incident neutron flux. The data were finally circularly averaged to yield one-dimentsional SANS data.[13]

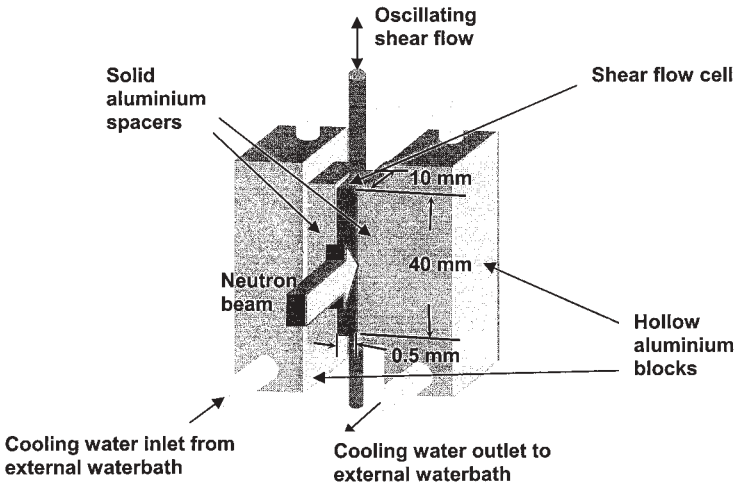

Figure 1. Neutron diffraction experimental setup for shear-aligned samples.

Neutron diffraction experiments were conducted at the N5 diffractometer located at the National Research Universal (NRU) reactor (Chalk River Laboratories, Ontario, Canada). 2.37 Å neutrons were selected using the (002) reflection of a pyrolytic–graphite

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monochromator, while a graphite filter was used for the scattered beam to reduce diffraction arising from $\lambda/2$ and $\lambda/3$ incident neutrons. Samples were aligned using an oscillating shear flow along the long axis of a rectangular cell with openings at both ends (0.5mm X 10mm X 40mm, Starna Cells, Inc.). After shear, the normal of the alignable bilayers remains perpendicular to the 10mm X 40mm surfaces for extended periods of time (at least several days). Sample cells were sandwiched between two aluminium blocks, whose temperature was controlled using a circulating waterbath (Figure 1). The incident beam was parallel to the 10mm dimension of the cell. To determine the degree of sample alignment, rocking curves were obtained using the first-order Bragg peak position.

Results and Discussion

Non-doped DMPC/DHPC mixtures. Q = 3.2 and 5 bicelle mixtures have a similar temperature dependence for appearance and viscosity. At low T (< 20 °C), the samples are more or less transparent with water-like viscosity (Figure 2A). When T is increased slightly above 23 °C (phase transition temperature, T_M, of DMPC), the sample's appearance is either transparent (Q = 3.2) or translucent (Q=5), and both become highly viscous (Figure 2B). On further elevation of T, the samples turn opaque and experience a drop in viscosity (Figure 2C). Our observation is consistent with those in the literature, where the viscosity of the systems is reported to have a maximum at T ~ 30 °C.[14,15] The POM results of the Q = 3.2 mixture identify three distinct textures, namely smectic, chiral nematic and isotropic phases on decreasing T. In the high-T smectic phase, the bright four-fold brushes of a "Maltese cross" are often observed (Figure 3A). This texture is characteristic of multi-lamellar vesicles coexisting with the isotropic phase, a phase that has no birefringence (dark regions). With decreasing T, a S → N^* phase transition is observed where POM data show a "fingerprint" texture (Figure 3B).[16] Moreover, the high viscosity N^* phase (Figure 2B) indicates the presence of entangled aggregates, difficult to justify with a discoidal structure. The S → N^* transition temperature, $T_{S\rightarrow N^*}$, is also strongly Q-dependent ($T_{S\rightarrow N^*}$ = 30 and 52 °C for Q = 5 and 3.2, respectively), indicating the importance of DHPC. Decreasing T below the T_M of DMPC induces a N^* → I transition, where POM shows no birefringence intensity. Unlike the $T_{S\rightarrow N^*}$, the N^* → I transition temperature, $T_{N^*\rightarrow I}$, is independent of Q and coincides with the T_M of DMPC (23 °C).

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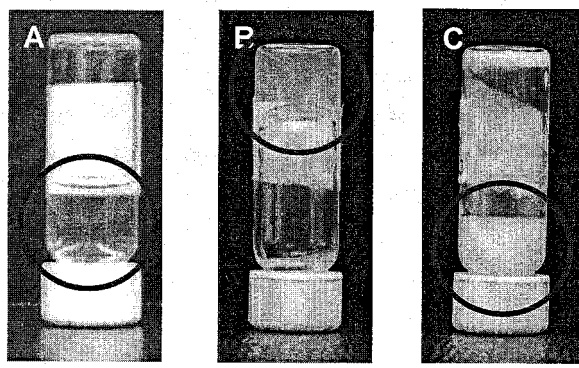

Figure 2. Appearance and viscosity of the 25 wt.% Q = 5 sample at (A) T = 10 °C, where the low viscosity sample is transparent, (B) at T = 25 °C , where the highly viscous sample is translucent and finally, (C) at 50 °C, where the sample experiences a drop in viscosity and turns opaque. Blue circles indicate the sample's location with respect to the glass vial.

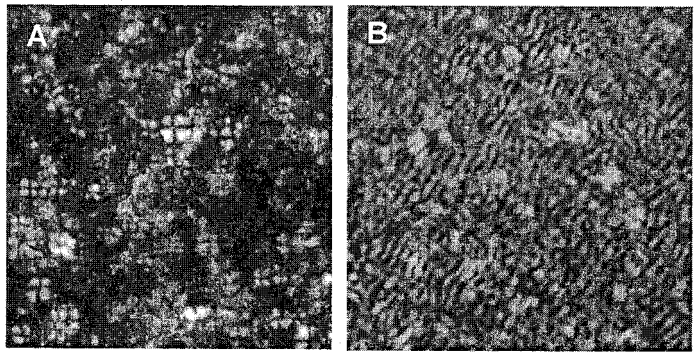

Figure 3. POM textures of a 25 wt.% Q = 3.2 sample (A) at T = 70 °C showing a "Maltese cross" texture, and representative of MLV. (B) "Fingerprint" texture at T = 45 °C characteristic of an N^* phase.

The microscopic structures of these three phases were characterized using SANS. Figure 4 shows SANS data of a Q = 3.2 sample at 3 different temperatures. It should be noted that the Q = 5 sample exhibits a similar behaviour as the Q = 3.2 sample (data not shown). At high T (70 °C), the sharp peak at q = 0.095 $Å^{-1}$ is indicative of a smectic phase with a lamellar spacing of ~ 66 Å, consistent with fully hydrated DMPC bilayers and the POM data showing MLV (Figure 3A). In the low-q regime, the intensity follows a q^{-4} decay,

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characteristic of large MLV of the order of hundreds of nm (Porod law). This result is also consistent with the opaqueness seen in these samples, and DHPC phase separating from from DMPC, as reported in several NMR studies.[17,18]

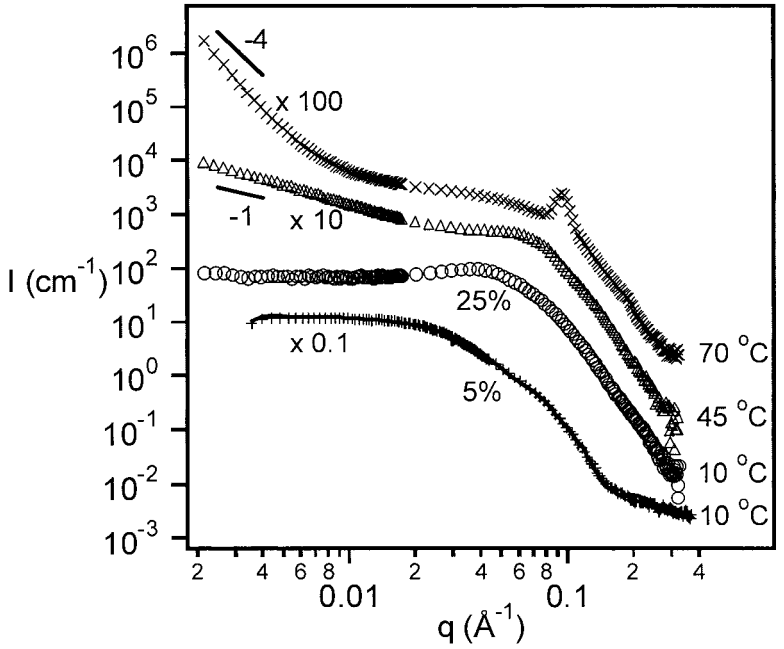

Figure 4. SANS results for the 25 wt.% Q = 3.2 sample at three different temperatures (70, 45 and 10 °C represented by x, Δ and o, respectively). SANS data for a 5 wt.% dilute solution (+) and its best-fit using a discoidal model (solid curve), are also shown. Structure factor was not taken into account when fitting the data.

At T = 45 °C, a less well-defined peak is found at q = 0.05 - 0.06 $Å^{-1}$, indicative of a poorly ordered structure. At this temperature the POM data shows the presence of N^* phase (Figure 3B). This phase was previously found to be magnetically alignable with the bilayer normal ⊥ to the applied magnetic field, and the proposed morphology was purported to be a discoidal bilayered micelle, or commonly referred-to bicelle.[2,4-6] The low-q SANS intensity shows a q^{-1} decay over a decade of q (from 0.002 to 0.02 $Å^{-1}$), representative of long objects (e.g., cylinders or ribbons). This holds true assuming that the contribution from the structure factor does not alter the slope over this q range.[16] Judging from numerous NMR experiments carried-out in this phase, the consensus is that some of

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the DHPC phase separates from DMPC.[1-6] Combining the SANS and NMR evidence along with the high viscosity of the solution, it therefore seems likely that the morphology is one of entangled ribbon-like objects with DMPC found on the flat surface of the ribbons and DHPC coating the ribbon's rim, thus minimizing the structure's edge energy. From the SANS data the average minimal persistence length and the ribbon width can be estimated to be > 3000 and 300Å, respectively. Most recently, cryo-transmitted electron microscopy of a similar system also shows enlongated aggregates over this T range.[19]

As mentioned previously, $T_{S\rightarrow N*}$ is strongly Q-dependent: the larger the Q (i.e., lower short-chain composition) the narrower the range of the N^* phase. Decreasing T, presumably results in more DHPC molecules at the edge, either due to lower solubility of DHPC in water, or to the two lipids phase separating. The extra DHPC molecules break up the MLV structure while stabilizing the ribbons by coating the rim of the ribbons and minimizing the edge energy. Therefore, the $T_{S\rightarrow N*}$ is lower for higher Q value samples (i.e., less DHPC). The reason for this is that a sufficient amount of DHPC needs to be released either from bilayers or solution in order stabilize the ribbons.

At T = 10 °C, the SANS result shows a weak interparticle interaction peak at q ~ 0.04 $Å^{-1}$ and a plateau region at low q values (Figure 4). SANS data from a lower lipid concentration (5 wt.%) sample, where no such peak is observed, can be best-fitted using a disk model confirming that the isotropic phase consists of discoidal aggregates, similar to bicelles. The best-fit diameter and thickness of the resultant bicelles are ~ 160 and 50 Å, respectively. Since $T_{N*\rightarrow I}$ always occurs around the T_M of DMPC for both Q = 3.2 and Q = 5 samples, the implication is that the formation of bicelles requires an ordered DMPC gel phase. It is expected that more DHPC is needed to coat the rim of a bicelle than the rim of a ribbon because the bilayer area of an individual ribbon is much larger than that of an individual disk. A possible interpretation is that L_α-phase DHPC is highly immiscible with gel-phase DMPC, resulting in almost all of the DHPC being driven out from the bilayer region. As a result, ribbons break up into the smaller bicelles in order to accommodate the extra DHPC. However, more experiments are needed to further elucidate this proposal.

In summary, three structures (bicelles, ribbons and MLV) are found in DMPC/DHPC zwitterionic mixtures as a function of T. The location and number density of DHPC molecules in the structure presumably play an important role in determining the structural phase of the mixtures.

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DMPG-doped DMPC/DHPC mixtures. Neutron diffraction experiments[12] and NMR spectroscopic studies[10] have shown that doping the zwitterionic DMPC/DHPC mixtures with paramagnetic ions (e.g., Tm^{3+}) results in a nematic → smectic phase transition. In our experiments, instead of using lanthanide ions, we doped the mixtures with a small amount of biologically relevant DMPG to yield a membrane with good alignment properties after the application of shear. A previous study has shown that doping DMPC/DHPC mixtures with DMPG results in the alignment of lamellar domains in two distinct orientations.[20] Presently, this phenomenon is not well-understood, and the study of charged systems may provide us with better insight of the influence of charge density on the morphology of bicelle mixtures.

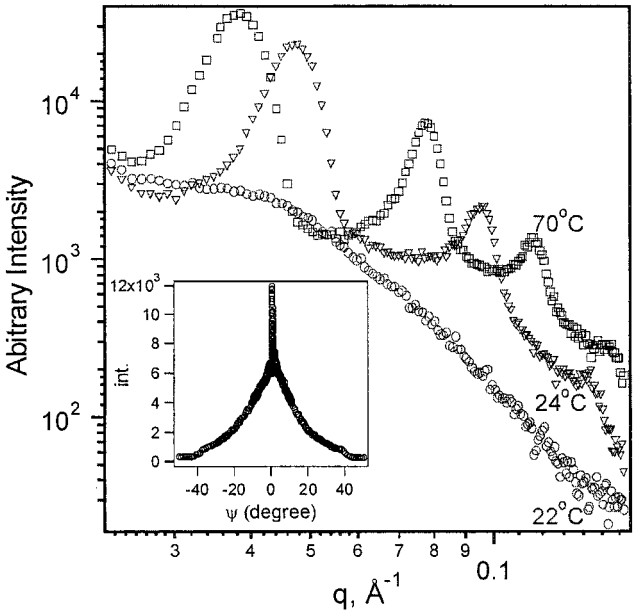

Figure 5. Neutron diffraction patterns of a 20 wt.% Q = 5 and [DMPG]/[DMPC] = 0.02 sample at three temperatures (22, 24 and 70 °C represented by o, Δ and □, respectively). A bicelle → lamellar transition is observed at T ~ 23 °C. The inset is the "rocking curve" at the first order Bragg position, q_{max} for T = 70 °C.

Figure 5 shows the neutron diffraction data for a 20 wt.% Q = 5 and [DMPG]/[DMPC] = 0.02 sample using the experimental setup shown in Figure 1. The data indicate a bicelle

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(isotropic) → lamellar (smectic) transition taking place at ~ 23 °C (~ T_M of DMPC). This lamellar phase persists until 70 °C (Figure 5). From previous studies we know that diluting the system to 5 wt.% results in one-dimensional swelling, characteristic of a lamellar phase without excess water.[8] This behaviour is very different from that of zwitterionic MLV, whose lamellar repeat spacing is insensitive to dilution. Apparently, even this small amount of DMPG is more than enough to result in "complete unbinding", as recently reported by Demé et al. on dioleoylphosphatidylserine solutions.[21,22] The fact that the spacing of these lamellae increases with T from 133 Å (24 °C) to 165 Å (70 °C), is presumably the result of perforations, present in the low T phase, annealing at high T.[23]

The degree of sample alignment can be obtained from the "rocking curve", which shows the scattered intensity at specific q_o (usually at a peak position, q_{max}) as a function of sample angle, ψ, with respect to the incident neutron beam. The intensity corresponds to the population of lamellae with a spacing $2\pi/q_o$ oriented at $(\psi - \phi_o/2)$ with the confining surface, where ϕ_o is the scattering angle of the first order Bragg reflection. The inset to Figure 5 shows a spike at $\psi_o = \phi_o/2$ with a full width at half maximum (FWHM) of less than 1° and broad wings spanning over ± 40°. The spike represents a portion of lamellae that align with the confining surfaces, while the broad wings are the result of a population of misaligned lamellae which deviate from ψ_o. The alignment of DMPG-doped lamellae is found to be dependant on the membrane charge density in a manner that the peak intensity of the rocking curve for a sample with a higher membrane charge density is higher, indicative of more lamellae aligning with the confining surfaces.

We have also found that the alignability of DMPG-doped samples ([DMPG]/[DMPC] = 1) after a weak shear is comparable with, if not better than, that of Tm^{3+}-doped membranes aligned in a strong magnetic field (2.6 Tesla),[12] as shown in Figure 6. The DMPG-doped mixture has a larger lamellar spacing than the previously studied Tm^{3+}-doped system (~ 23 wt.%),[12] due to a slightly lower lipid content. Up to six Bragg reflections from the shear aligned sample are observed.

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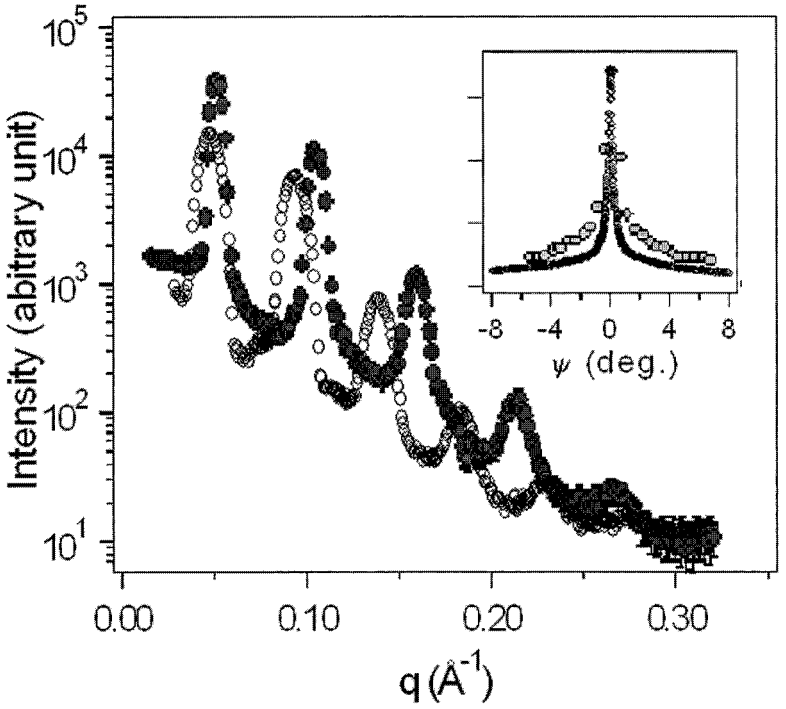

Figure 6. Neutron diffraction patterns of Tm^{3+}-doped mixtures (23 wt.%) in magnetic field ~ 2.6 Tesla (filled symbols)[12] and DMPG-doped mixtures (20 wt.%, [DMPG]/[DMPG] = 1) after experiencing a weak shear (open symbols). The inset to the figure shows the rocking curves of the two aligned samples.

Conclusion

We have shown that DMPC/DHPC mixtures (Q = 3.2 and 5) undergo isotropic (bicelle) → nematic (ribbon) → smectic (MLV) transitions with increasing T. $T_{I\rightarrow N^*}$ is tied to the T_M of DMPC, whereas $T_{N^*\rightarrow S}$ is strongly Q-dependant. The magnetically alignable nematic phase seems to be made up of ribbons, instead of bicelles as thought previously. Bicelles, however, form the low-T isotropic phase, while MLV are the resultant structure at high T. For DMPG-doped systems no N^* (ribbon) phase is found; only the I → S transition is observed with $T_{I\rightarrow S}$ taking place around the T_M of DMPC. Also, unlike excess water zwitterionic MLV, the smectic phase for DMPG-doped mixtures can swell up to several hundred Å. DMPG-doped systems can yield a comparable degree of alignment, after the application of a weak oscillating shear, as the previously studied Tm^{3+}-doped systems in a

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strong magnetic field. The DMPG-doped mixtures can potentially be used as a "biological goniometer" to study the in-plane and out-of-plane structure of proteins and peptides associated with biomembranes without the use of lanthanide ions and strong magnetic fields.

Acknowledgement

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