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Effect of urea, dimethylurea, and tetramethylurea on the phase behavior of dioleoylphosphatidylethanolamine

Ying Feng [a], Zhi-Wu Yu [a,*], Peter J. Quinn [b]

[a] *Department of Chemistry, Bioorganic Phosphorous Chemistry Laboratory, Tsinghua University, Beijing 100084, PR China*
[b] *Division of Life Sciences, King's College London, 150 Stamford Street, London SE1 8WA, UK*

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Abstract

The phase behavior of dioleoylphosphatidylethanolamine in aqueous solutions of urea, *N*,*N*′-dimethylurea (DMU), and *N*,*N*,*N*′,*N*′-tetramethylurea (TMU) has been characterized by synchrotron X-ray diffraction and differential scanning calorimetry. All three solutes stabilize the lamellar liquid-crystalline phase at the expense of lamellar-gel phase and inverted hexagonal phase of the phospholipid when present in concentrations up to 3 M. X-ray diffraction data demonstrated that the repeat spacing of DOPE increased with increasing urea concentration, but decreased as the DMU and TMU concentrations increased. The repeat spacing of DOPE in the liquid-crystal phase dispersed in the three solutes is $d(\text{urea}) > d(\text{DMU}) > d(\text{TMU})$. The molecular mechanisms underlying these observations are discussed in terms of either membrane Hofmeister effect, where urea acts as a water structure breaker, or a direct insertion effect of the amphiphilic DMU and TMU molecules into the lipid head groups in the interfacial region of the phospholipid bilayer.

Keywords: Phospholipid; Phase transition; Hofmeister effect; Urea derivative; X-ray diffraction; Differential scanning calorimetry

1. Introduction

Phase separation of non-bilayer-forming lipids such as phosphatidylethanolamines (PEs) from membrane lipid mixtures has been suggested to play a major role in freezing or chilling injuries of tissues, organs, and bioorganisms exposed to low temperatures (Seddon et al., 1983; Yeagle and Sen, 1986; Sanderson et al., 1991; Williams et al., 1991; Yang and Huang, 1996). Among the many PEs, dioleoylphosphatidylethanolamine (DOPE) is one of the most studied molecules with unsaturated hydrocarbon chains. It has been used as a model system to study the influence of different solutes, both inorganic and organic, on lipid phase transitions and freeze-induced destabilization of biological membranes (Zhang and Liu, 1987; Koynova and Caffrey, 1994; Yu et al., 1996; Shalaev and Steponkus, 1999). Some of these additives such as thiocyanate and guanidine hydrochloride tend to increase the lamellar liquid-crystal (L_α) to inverted hexagonal (H_{II}) transition temperature and to decrease the lamellar-gel (L_β)

* Corresponding author. Tel.: +86-10-6277-2767; fax: +86-10-6277-1149.
E-mail address: yuzhw@tsinghua.edu.cn (Z.-W. Yu).

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to L_α transition temperature so as to stabilize the L_α phase (Sanderson et al., 1991). Other cosolutes, such as dimethyl sulphoxide, tend to destabilize the L_α phase in favor of the L_β and H_{II} phases (Yu and Quinn, 1998). These differences have been explained in terms of the so-called Hofmeister effect (Collins and Washabaugh, 1985; Cacace et al., 1997; Koynova et al., 1997), which considers only an indirect interaction between host and guest molecules mediated by water. Cosolutes are categorized as chaotropes if their presence tends to disrupt the hydrogen-bonded water structure and kosmotropes if they act to promote or stabilize water structure. Accordingly, thiocyanate is an example of a chaotropic agent and dimethyl sulphoxide is a kosmotrope. The significance of direct interactions between cosolutes and phospholipid molecules independent of their effect on water structure has yet to be assessed.

As a biologically important solute found in living organisms, urea has been chosen by many to study its effect on the stability of proteins and biomembranes (Brennan et al., 1994; Surrey and Jaehnig, 1995; Carlson and Goldstein, 1997; Patra et al., 1998). In a study by Sanderson et al. (1991), urea was found to increase the $L_\alpha \rightarrow H_{II}$ temperature and to decrease the $L_\beta \rightarrow L_\alpha$ temperature of palmitoleoylphosphatidylethanolamine, thus to stabilize the L_α phase. Urea was therefore regarded as a chaotrope because it tends to enlarge the interfacial area of lipid, bearing in mind that the L_α phase has the largest surface area among the three phases formed by the hydrated phospholipid. This is in line with the position of urea in the Hofmeister series (Collins and Washabaugh, 1985).

It has been reported, however, that urea might interact directly with proteins or lipids. The decrease in thermal stability of protein by urea has been explained as the hydrophobic interaction between urea and the non-polar groups of protein, while the interaction with membranes may be due to the combination of the effect of urea on the hydrophobic core of the lipid assembly and its effect on the hydrophilic head groups (Barton et al., 1999; Poklar et al., 1999). Urea can also be used as the penetration enhancer to influence the flux across membranes and the retention of the drugs in cells (Bentley et al., 1997). In addition, urea may form hydrogen bonds directly with the head groups of lipid molecules in competition with hydrating water molecules.

To understand more precisely the molecular mechanism of these possible effects of urea, we have compared the effect of urea with its two derivatives, *N*,*N*′-dimethylurea (DMU) and *N*,*N*,*N*′,*N*′-tetramethylurea (TMU), on the phase behavior of DOPE, using differential scanning calorimetry and synchrotron X-ray diffraction methods. Collins and Washabaugh (1985) once categorized both DMU and TMU as kosmotropes. The experimental results reported in the present work, do not support such claim. Rather, direct insertion of these two molecules into the head group region of DOPE can better explain the experimental observations.

2. Materials and methods

1,2-Dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE) of 99% purity was purchased from Sigma (USA) and was used without further purification. AR-grade urea was purchased from Beijing Liulidian Chemical Factory, and GC-grade DMU and TMU were from Sigma-Aldrich Co. Ltd. Solutions of urea and its derivatives were prepared using deionized distilled water.

2.1. Differential scanning calorimetry (DSC)

Calorimetry measurements were made using a Metller Toledo DSC 820^e calorimeter. Lipid samples were prepared directly in aluminum pans by adding solutions of urea, DMU or TMU to dry lipid powder with a weight ratio of 2:1. To ensure proper mixing, they were cycled between -40 and 40 °C several times prior to data collection until identical heating thermograms were recorded. For each sample, a minimum of two identical heating thermograms were recorded at a scan rate of 1°/min. Transition temperature was taken as the onset temperature of each endothermogram upon heating.

Usually two transitions of the lipid dispersions could be identified in the temperature range of −20 to 40 °C in addition to ice–water transition. In order to avoid dehydration effect on phase transition due to ice-formation, heating/cooling protocols were designed so that samples were super-cooled until the presence of the exotherm of the main phase transition was observed and kept at that temperature for 2 min. Phase transition temperatures were then determined during the subsequent heating scans.

2.2. X-ray diffraction (XRD)

Real-time X-ray diffraction was conducted at Station 8.2 of the Synchrotron Radiation Source of the SERC Daresbury Laboratory, using a method described previously (Yu and Quinn, 2000). Briefly, lipid samples were prepared in small centrifuge tubes by adding aqueous solutions to lipid dry powder with a weight ratio of 2:1. Then they were thermally cycled using water baths to provide an identical thermal history to the samples used in DSC studies. Each of the lipid samples was then mounted in a cell of 1 mm in thickness and sealed with thin mica windows. Measurements were performed at heating/cooling rates of 3°/min. The small angle (SAXS) and wide-angle X-ray scattering (WAXS) patterns were recorded simultaneously.

3. Results

3.1. DSC measurement

To assess the effect of cosolutes on enthalpic phase transitions of aqueous dispersions of DOPE, differential scanning calorimetric studies were undertaken. The results presented in Fig. 1 are thermograms of DOPE dispersions in excess water and aqueous urea solutions. Two transitions can be observed in each heating scan. The low-temperature transition, or main transition, T_m, is designated as transformation from L_β to L_α, and the endotherm above 0 °C is due to a transition from L_α to H_{II} (confirmed below by X-ray results). The transition temperatures in the absence of urea were determined to be −8.7 and 11.0 °C, respectively. These are in good agreement with published values, i.e. −8.6 and 11.5 °C (Wistrom et al., 1989; Fenske and Cullis, 1992).

The thermograms recorded from DOPE dispersed in urea, DMU and TMU solutions follow similar patterns to that of fully hydrated DOPE. The concentration dependence of the main transition ($L_\beta \rightarrow L_\alpha$) temperature T_m and the temperature of the phase transition ($L_\alpha \rightarrow H_{II}$) temperature T_h is summarized in Fig. 2. It can be seen from the data presented in Fig. 2 that T_m decreases slightly with increasing concentration of all these solutes, whilst an increase in T_h with increasing concentration is much pronounced. The greater sensitivity of the second transition to the addition of solutes can be explained by a model proposed by Koynova et al. (1997), where the transition with lower latent heat is more sensitive than that with larger latent heat. Replacement of water by 3 M aqueous urea solution caused a decrease of the main transition temperature from −8.7 to −10.9 °C, while the temperature of L_α to H_{II} transition increased from 11.0 to 20.4 °C. In the case of 2.4 M aqueous DMU solution, the

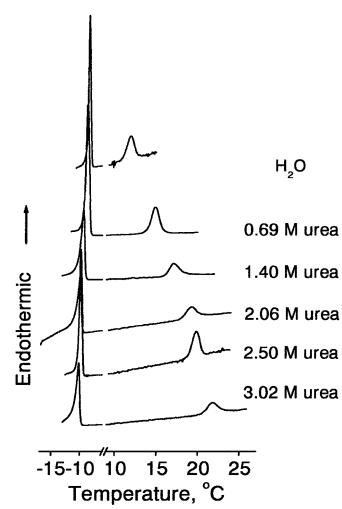

Fig. 1. DSC heating thermograms showing phase transitions from lamellar-gel to lamellar liquid-crystal and then to inverted hexagonal phases of DOPE dispersions in a series of aqueous urea solutions. (The peaks on the right are enlarged by five times.)

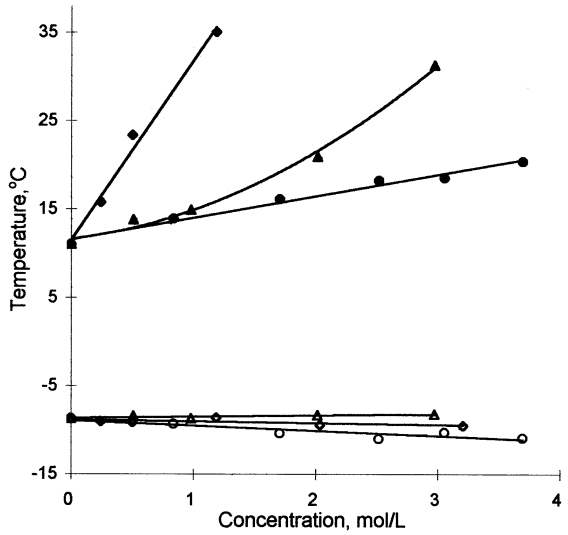

Fig. 2. Plots showing the dependence of transition temperatures of T_m (open symbols) and T_h (solid symbols) of DOPE dispersed in solution of urea (●, ○), DMU (□, △), and TMU (□, ◇) on the solute concentration.

increase in T_h is 21.3 °C, more significant than that of urea. TMU has the greatest effect of the three solutes in modulating the transition temperatures towards the non-bilayer phase. An increase of 23.5 °C in T_h was observed even with a concentration of just 1.2 M aqueous TMU solution.

It can be clearly seen from the data presented in Figs. 1 and 2 that increasing the concentration of urea, DMU, and TMU in the aqueous phase expands the temperature range of lamellar liquid-crystal phase, i.e. stabilization of L_α phase at the expense of L_β and H_{II} phases.

3.2. X-ray diffraction

X-ray diffraction patterns were recorded during heating and cooling of dispersions of DOPE in differing concentrations of either urea, DMU, or TMU to correlate structural changes with the thermal transitions observed by calorimetry. The data presented in Fig. 3 is an example of the small-angle (SAXS) and wide-angle (WAXS) X-ray scattering patterns recorded from DOPE dispersions in 3 M urea during heating from −11 to 36 °C at 3°/min. The WAXS pattern at −11 °C is characterized by a sharp diffraction peak centered at about 0.43 nm, evidencing a lamellar-gel phase. The three additional peaks in the wide-range pattern centered at 0.392, 0.367 and 0.356 nm correspond to the reflections from hexagonal ice (Yu et al., 1996). The replacement of the sharp diffraction peak by a broad scattering band around 0.45 nm at about −9 °C indicates that this is a transition from L_β to a liquid-crystal phase. SAXS pattern with a spacing ratio of 1:1/2:1/3 for different diffraction orders demonstrates the new phase is an L_α phase (only the first order is shown). The $L_\beta \rightarrow L_\alpha$ transition is also characterized by an abrupt decrease in the lamellar repeat spacing from 6.07 to 5.56 nm.

A second transition at about 21 °C is characterized by the appearance of the first three diffraction orders in the SAXS region with d-spacing ratio of $1:1/\sqrt{3}:1/\sqrt{4}$, indicating the formation of the H_{II} phase. The repeat spacing increases from 5.32 to 6.43 nm at the transition temperature. In comparison with the X-ray diffraction pattern of aqueous dispersions of DOPE reported previously (Yu et al., 1996), the presence of urea, DMU, and TMU has little effect on the characteristic X-ray diffraction patterns other than to shift the phase transition temperature. Upon cooling the diffraction patterns were almost reversed except the slight difference in transition temperature of DOPE (data not shown).

Another interesting observation is the different effect of urea and its derivatives on the repeat

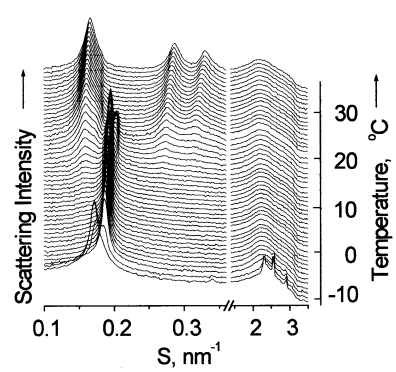

Fig. 3. Real-time synchrotron X-ray scattering intensity profiles recorded at small-angle (left) and wide-angle (right) during a heating scan at 3°/min, showing phase transitions of DOPE dispersed in 3 M aqueous urea solution.

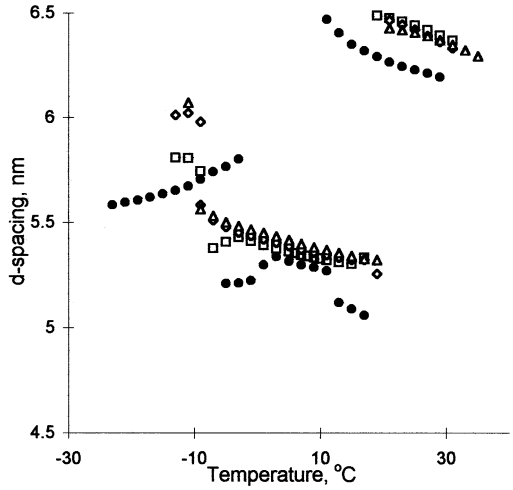

Fig. 4. Plots of temperature-dependence of the repeat spacing (d) of DOPE dispersed in H_2O (●), 0.5 M urea (□), 2 M urea (◇), 3 M urea (△).

spacing of DOPE. The repeat spacing of DOPE dispersed in the aqueous solutions of urea, DMU, and TMU at different concentrations and temperatures are summarized in Figs. 4–6, respectively. These show that with all three solutes the transition of DOPE in heating scans proceeds with a decrease of repeat spacing in the $L_\beta \rightarrow L_\alpha$ phase transition and an increase of repeat spacing in the $L_\alpha \rightarrow H_{II}$ phase transition.

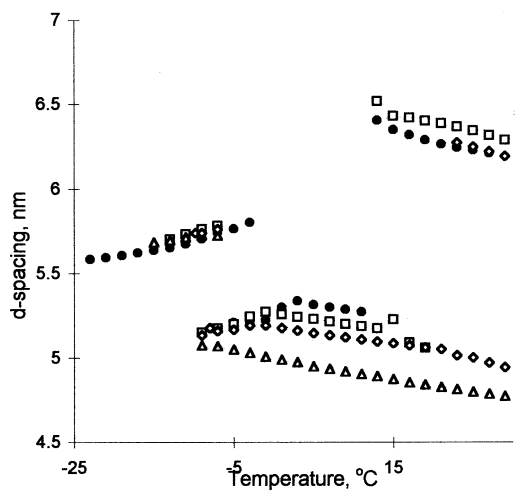

Fig. 5. Plots of temperature-dependence of the repeat spacing (d) of DOPE dispersed in H_2O (●), 0.5 M DMU (□), 1 M DMU (◇), 3 M DMU (△).

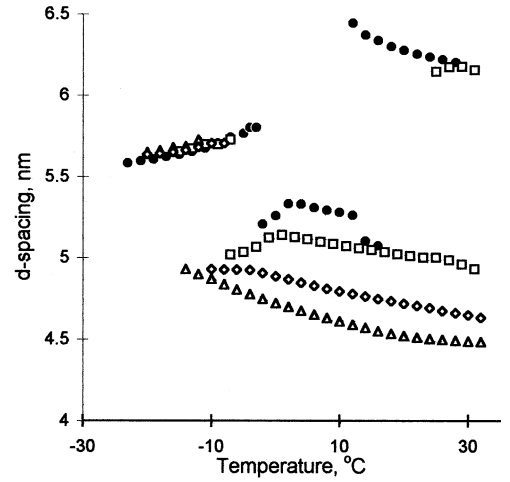

Fig. 6. Plots of temperature-dependence of the repeat spacing (d) of DOPE dispersed in H_2O (●), 0.49 M TMU (□), 0.91 M TMU (◇), 3.21 M TMU (△).

The most obvious difference between data presented in the three figures is the effect of urea, DMU, and TMU solutes on the repeat spacing of DOPE in L_α phase. Plots of d-spacings as a function of solute concentration at 7 °C, at which all the dispersions are in L_α phase, is presented in Fig. 7. It can be seen that the spacing of the L_α phase increases with increasing urea concentration, but decreases with increasing in DMU and

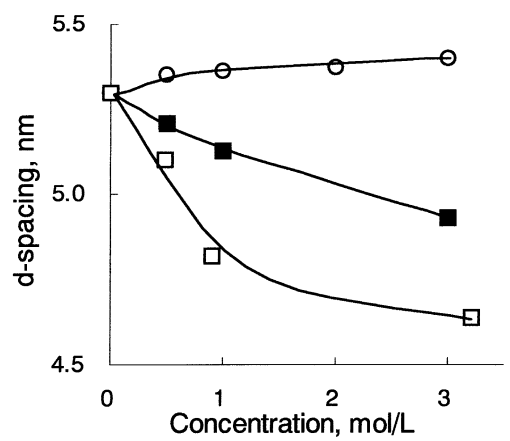

Fig. 7. The dependence of repeat spacing of DOPE dispersions on the concentration of solutes at 7 °C: urea (○), DMU (■), and TMU (□).

TMU concentration. For a fixed concentration of these solutes, the repeat spacing of DOPE dispersions at L_α state follows the sequence of $d(\text{urea}) > d(\text{DMU}) > d(\text{TMU})$.

Because TMU is a liquid at ambient conditions, it is possible to examine the phase behavior of DOPE in pure TMU. Both DSC and X-ray diffraction studies over the temperature range -15 to 30 °C recorded only one phase transition at about 28 °C. WAXS patterns of both phases showed broad scattering bands centered at 0.47 nm indicating both phases are liquid-crystalline. The repeat spacings of both phases were almost unchanged upon heating, but decreasing from 4.13 to 3.32 nm at the phase transition.

4. Discussion

4.1. The stability of liquid-crystal phase

We have demonstrated in the present results that both urea and its two derivatives, dimethyl urea, tetramethyl urea, can increase the temperature of the $L_\alpha \rightarrow H_{II}$ phase transition and decrease the temperature of the $L_\beta \rightarrow L_\alpha$ phase transition. This suggests that these small cosolutes stabilize the lamellar liquid-crystal phase of the phospholipid. This conclusion is consistent with previous studies of the modulation effect of urea on two kinds of non-bilayer-forming lipids, namely, soy phosphatidylethanolamine (Yeagle and Sen, 1986) and palmitoyloleoylphosphatidylethanolamine (Sanderson et al., 1991). The dimethyl- and tetramethyl-derivatives of urea were found for the first time to stabilize the lamellar liquid-crystal phase more dramatically.

The effect of urea on thermotropic lipid phase transitions has been discussed in terms of Hofmeister effect in the two earlier publications. Considered as a chaotrope, or water structure breaker, urea should be able to induce an enlargement of interfacial area of an assembly composed of amphiphilic molecules or segments (Ruiz, 1995). This will result in denaturation or destabilization of water-soluble proteins upon addition of urea. Similarly, urea can stabilize the L_α phase of lipids because it has a larger surface area than that of L_β and H_{II} phases (Seddon et al., 1984; Koynova et al., 1997)

A recent paper by Tovchigrechko et al. (1999) has investigated the underlying molecular mechanism of the interaction of urea and TMU using molecular dynamics simulations. They concluded that only three of nine water molecules comprising the first hydration shell of urea could be, on average, in strong interaction (H-bonding) with the urea molecules. Furthermore, an enhanced mobility of water has been found in this region corresponding to the hydration of the carbonyl oxygen beyond the first hydration shell. These results indicate that urea interacts weakly with water molecules, in comparison with interactions between water molecules. This could be the reason that the hydrophilic urea molecule, with six H-bonding positions, breaks water structures after dissolving in water.

Interestingly, both DMU and TMU were regarded as kosmotropes, or water-structure makers in a review article by Collins and Washabaugh (1985). The cited studies supporting the classification include the temperature-dependence of relative viscosity, heat capacity of transfer from H_2O to D_2O and structural temperature of solution as measured by infrared difference spectra of dilute H_2O to D_2O. This appears to contrast with the present results showing that DMU and TMU both stabilize the L_α phase of DOPE.

To resolve this apparent discrepancy, we consider possible direct interactions between the two urea-derivatives with lipid assemblies. By replacing amino hydrogens with methyl groups, the urea-derivatives tend to become less hydrophilic. This shift in amphipathicity allows DMU or TMU molecules to enrich in the interfacial region and, possibly, to insert between the lipid head groups. As a result, the intrinsic curvature tension in the interfacial part of the bilayer structure will be released (Seddon, 1990), making it more difficult to form non-bilayer structures. Of the two derivatives, TMU is more hydrophobic and bulky and is thus more effective in stabilizing L_α phase at the expense of the H_{II} phase.

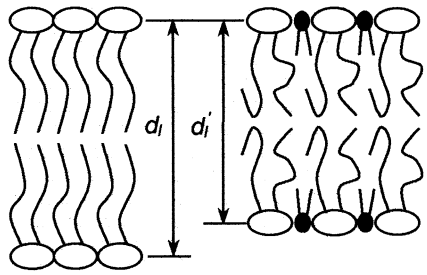

Fig. 8. Schematic presentation to show the shortening of lipid bilayer thickness from d_l to d'_l due to insertion of amphipathic molecules into the lipid head groups. Open symbols with tails represent phospholipid molecules, solid ones with tails are amphipathic molecules such as dimethyl urea.

4.2. The d-spacings of DOPE at different aqueous solutions

The results presented in Fig. 7 show that the d-spacing of DOPE in L_α phase increases with increasing urea concentration but decreases with increasing in DMU and TMU concentrations. The change in the repeat spacing of the lamellar phases could be due to either a change of the thickness of the bilayer, a change of the solvent space separating lipid bilayers, or a combination of both. As discussed above, the amphiphilic properties of DMU and TMU may permit the molecules insert into the lipid head groups, separating them, so as to result in a decrease of the lipid bilayer thickness. This is shown schematically in Fig. 8. This could explain why the addition of DMU and TMU results in a decrease in the repeat spacing of the multiplayer structures.

To consider any change in the solvent layer thickness separating the lipid bilayers, we need to analyze both attractive and repulsive forces between the opposing bilayers. For the non-net-charged DOPE, electrostatic force is taken to be negligible (Vierl et al., 1994), leaving attractive van der Waals force and repulsive hydration and undulation forces as the more significant interacting forces. The van der Waals force per unit area between two planar surfaces, P_v, can be calculated from the relationship:

$$P_v = -H/6\pi D^3,$$

where H is the non-retarded Hamaker constant and D is the separation of the two planar surfaces (Israelachvili, 1992) The Hamaker constant can be expressed as:

$$H \approx 3kT/4((\varepsilon_l - \varepsilon_f)/(\varepsilon_l + \varepsilon_f))^2 + 3h\nu_e(n_l^2 - n_f^2)^2/16\sqrt{2}(n_l^2 + n_f^2)^{3/2},$$

where $h\nu_e$ is the ionization energy of the bilayer (approximately 2×10^{-18} J), $kT = 4.114 \times 10^{-21}$ J at 25 °C, ε_l, ε_f, n_l and n_f are the static dielectric constants and the refractive indexes of the lipid and fluid layer, respectively (Israelachvili, 1992). The dielectric constant and refractive index of lipid are taken as 2.0 and 1.45, respectively (McIntosh et al., 1989). Thus the Hamaker constants can be calculated for various solutions containing urea, DMU, and TMU. These values are presented in Table 1. It can be seen that the Hamaker constants of aqueous urea solutions decrease with increasing urea concentrations, which may result in the decrease of van der Waals attractive force between bilayer separations. This could be the primary reason that the d-spacing of the DOPE dispersion in aqueous urea solutions increases with increasing of urea concentrations.

Table 1 also shows that the Hamaker constants of the DMU and TMU solutions decrease with increasing cosolute solutions. Apparently, this could lead to increases of the d-spacings. However, the reduction of the lipid bilayer thickness transcends such effects and causes the decrease of the lamellar repeat distances.

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Table 1
Hamaker constants of a few aqueous solutions at 25 °C

System	Concentration (mol/l)	ε_f	n_f^e	H ($\times 10^{-21}$ J)
Water	0	80.10[a]	1.333	6.47
Urea	0.71	82.33[b]	1.338	6.17
	1.39	84.48[b]	1.344	5.82
	2.04	86.54[b]	1.350	5.47
	2.51	87.99[b]	1.356	5.20
	3.00	89.55[b]	1.359	5.03
DMU	0.40	81.31[c]	1.336	6.27
	0.78	82.43[c]	1.341	5.97
	1.60	84.89[c]	1.351	5.41
	2.36	87.17[c]	1.361	4.93
TMU	0.50	79.56[d]	1.340	6.05
	1.19	78.72[d]	1.351	5.43
	2.04	77.49[d]	1.365	4.71
	3.21	75.29[d]	1.386	3.87
	Pure TMU	23.10[e]	1.447	2.18

[a] Data from Lide (1999).
[b] Data evaluated from the equation $\varepsilon_f = 78.30 + \delta C$, where C is the concentration of urea and δ equals to 3.15 for urea (Hasted, 1973).
[c] Data evaluated from the equations as above with C as the concentration of DMU and $\delta = 3$ is the concentration increment (Hasted, 1973).
[d] Data calculated by assuming an ideal mixing between TMU and water.
[e] Obtained from this study.

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