PHYSICAL REVIEW E, VOLUME 64, 040902(R)

Sugar-induced stabilization of the monoolein $Pn3m$ bicontinuous cubic phase during dehydration

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(Received 23 April 2001; revised manuscript received 16 July 2001; published 21 September 2001)

To explore the molecular mechanism of the protective function of sugars on cubic lipidic systems, the mesomorphic properties of the monoolein-water system, dehydrated in the presence of a series of sugars, have been studied by osmotic stress experiments. Two bicontinuous inverse cubic structures ($Pn3m$ and $Ia3d$) and a lamellar L_α phase form under dehydration in pure water. In sugar solutions, the $Pn3m$ phase shows an extraordinary stability: as a function of sugar concentration, the lattice parameter decreases to very low values, but no phase transitions occur. Instead, the $Pn3m$ to $Ia3d$ phase transition is obtained by equilibrating the lipid phase with aqueous polymer solutions of increasing osmotic pressure. As a result, the pressure at which the phase transition occurs strongly depends on sugar concentration. The free-energy curves obtained from the osmotic-pressure unit-cell data show that the sugar exerts an additional stabilization on both the cubic phases. The analysis of the structural parameters indicates that sugars alter the interface geometry. We suggest that a consequent release of stretching contributions in the chain packing or a reduction of the inhomogeneity in molecular splay mainly stabilize the $Pn3m$ phase and prevent the transition to the $Ia3d$ phase on dehydration.

DOI: 10.1103/PhysRevE.64.040902 PACS number(s): 87.15.−v, 61.30.St, 87.14.Cc, 61.30.Cz

Cubic bicontinuous lipidic mesophases have been recently proved to be useful media for growing crystals of membrane proteins (*in cubo* crystallization) [1,2] and for the rational design of biocompatible materials for encapsulation, controlled release and uptake, and delivery of drugs and proteins [3]. Being strongly related to the integrity of the medium, both applications point out the special interest in controlling and eventually extending the range of stability in lipidic cubic phases.

It is well known that sugars exert a stabilizing effect on lipid systems [4–9]. In fully hydrated conditions, a reduction of the surface-*per*-polar head at the lipid-water interface has been detected. As a consequence, structures with low surface areas (like inverted hexagonal H_{II} phases) are stabilized, and transitions from structures with larger to structures with lower interfacial area (for example, from lamellar L_β to $L_{\beta'}$ phases) are induced. These changes in structural properties have been analyzed, suggesting that sugar molecules act as substitutes for water molecules, forming hydrogen bonds with the hydrophilic surfaces [4] or as a manifestation of the Hofmeister effect, in which the sugar acts as a kosmotropic reagent, stabilizing the structure of water [6].

Concerning bicontinuous cubic phases, we recently reported about the protective effects that trehalose (a disaccharide) exerts on the monoolein-water system [10], just the lipid on which the *in cubo* method has been based [1,2]. Monoolein (MO) in water forms as a function of concentration several mesophases characterized by a highly disordered conformation of the hydrocarbon chains [11–14]. Among them, two inverted (type II) bicontinuous cubic phases have been identified, namely the $Pn3m$, which extends in a very limited range of hydration, from excess water conditions (water volume concentration $\phi \approx 0.39$) to $\phi = 0.34$ (at 25 °C), and the $Ia3d$, which at the same temperature exists in the range $0.21 < \phi < 0.34$. Both cubic phases are described in terms of infinite periodic minimal surfaces (IPMS), i.e., infinite arrays of connected saddle surfaces with zero mean curvature in every point [15]. In these structures, lipid monolayers are draped across each side of the IPMS, touching it with their terminal methyl groups; it results in a three-dimensional periodic bicontinuous structure, formed by distinct water and lipid volumes. The lattice parameter of both the cubic phases depends on composition: at 25 °C, the unit cell of the $Pn3m$ decreases from 104 Å ($\phi \approx 0.39$) to about 97 Å ($\phi = 0.34$), while in the $Ia3d$ phase the cell reduces from 146 to 119 Å when ϕ decreases from 0.33 to 0.21. In excess water solution, we have found that trehalose leads to a reduction of the cell dimension to 90 Å, largely below the threshold value observed in pure water at the phase transition [10]. We also found that the $Pn3m$ phase exists even in trehalose glasses in extremely dry conditions and even if the lattice parameter reduces to very small values (as low as 70 Å, observed in trehalose platelets dehydrated at 80 °C for more than one day) [10].

No doubt that both the stabilization of $Pn3m$ induced by sugars and the possibility to modulate the cell dimensions in excess water are of crucial importance for technological applications. In order to get at the molecular mechanism responsible for such effects, we decided to analyze by the osmotic stress method the $Pn3m$ dehydration process in the presence of different sugars over a range of concentrations. Osmotic stress is the controlled removal of water from the system under investigation [16]: the system is let to come in equilibrium with a polymer solution of known osmotic pressure and the structure of the phase is determined by x-ray diffraction experiments following the usual procedure [13]. Under a condition in which the polymer is excluded from the macroaggregate lattice, the osmotic pressure of the polymer solution is the osmotic stress dehydrating the sample. Briefly, MO samples (1-monoolein, from Sigma Chemical Company, >99% purity) were equilibrated with an excess solution of polyethylene glycol (PEG 15000-20000 MW, Sigma Chemical Company, 99% purity), whose osmotic pressure have been directly measured [16]. PEG solutions

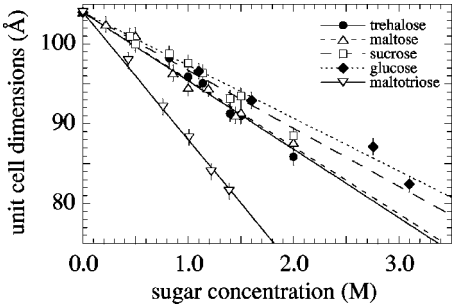

FIG. 1. $Pn3m$ unit-cell dependence on sugar concentration in excess water conditions. The lines are the best fits used to calculate the deydration rates $dn_w/d(M)$ (see Table I).

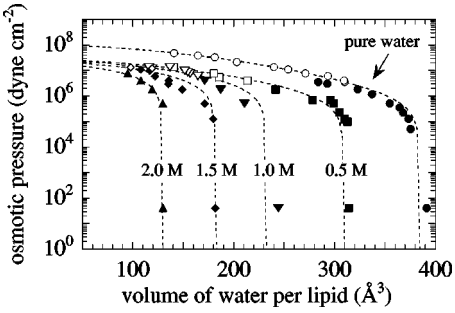

FIG. 2. Osmotic pressure dependence on the volume of water *per* MO molecule measured at different trehalose concentrations on the $Pn3m$ (filled symbols) and $Ia3d$ (open symbols) cubic phases. Lines, obtained by fitting data using stretched exponentials, are visual guides to show the general trend.

were prepared over a range of sugar concentrations: D-glucose (a monosaccharide), D-maltose monohydrate, D-trehalose dihydrate, sucrose (disaccharides), and maltotriose (a trisaccharide), were used (Sigma Chemical Company, 95% purity). X-ray diffraction of these samples (a Philips PW1830 X-ray generator equipped with a Guinier-type focusing camera operating in vacuum with a bent quartz crystal monochromator was used; diffraction patterns were recorded on a stack of four Kodak DEF-392 films) yields their structure, unit cell dimension, *a*, and, by reference to gravimetrically prepared samples [10], their composition (that we will alternatively indicate in terms of number of water molecules *per* lipid, n_w, or volume of water *per* lipid, V_w [17]). Errors in extrapolated concentrations have been estimated by gravimetric analysis to be lower than 5%.

In Fig. 1, the $Pn3m$ lattice parameter is plotted as a function of sugar concentration in absence of external stresses, clearly proving the ability of the different sugars in affecting the cubic structure. The corresponding dehydration rates, $dn_w/d[M]$, calculated plotting the number of water molecule *per* lipid versus the sugar molar concentration, are reported in Table I. Noticeable is the fact the $dn_w/d[M]$ show a linear dependence on the number of monosaccharide rings present on the sugar molecule: the averaged dehydration rate *per* unit monosaccharide ring is -2.44 ± 0.20 mol/M. Another point to be stressed is that MO in excess water still forms the $Pn3m$ phase, even if the increased sugar concentration leads to a strong reduction of the cell dimensions (82 Å, detected in glucose and in maltotriose), largely below the threshold cell dimension observed in pure water at the $Pn3m$-$Ia3d$ phase transition ($a=97$ Å). Related to the reduction of the unit cell is the expected decreasing of the surface-*per*-polar head at the lipid-water interface. However, since no phase transitions are detected, a simple osmotic mechanism can be excluded.

The unit cell variations observed during osmotic stress are better discussed providing the dependence of osmotic pressure, π, on the volume of water *per* lipid, V_w, as reported by Caffrey and co-workers [17]. $Pn3m$ data obtained in trehalose solutions are reported in Fig. 2: at low pressures, the lattice parameter (and then V_w) is clearly dependent on sugar concentration, while in strong dehydrating conditions (i.e., high osmotic pressure) data seem to converge to a common curve. Moreover, the $Pn3m$ to $Ia3d$ phase transition is detected on dehydration. The osmotic pressure at which the transition occurs depends on molarity and chemical structure of the sugar (see Table I), even if similar pressure lattice-parameter dependencies have been detected for all the investigated sugars.

TABLE I. Dehydration rates, $dn_w/d[M]$ (mol/M), determined in the absence of osmotic stress, and osmotic pressure, π_{trans} (10^6 dyne cm^{-2}), at which the $Pn3m$ to $Ia3d$ phase transition occurs at the different sugar concentrations (M) (at 25 °C).

$dn_w/d(M)$	Pure water	Glucose -2.75 ± 0.21	Trehalose -4.73 ± 0.09	Maltose -4.69 ± 0.13	Sucrose -4.48 ± 0.09	Maltotriose -7.56 ± 0.19
concentration		0.23	0.5	0.5	0.5	0.43
π_{trans}	1.8	>4.1	2.3	1.9	1.8	4.1
concentration		0.80	1.0	1.0	1.0	0.76
π_{trans}		>4.2	5.0	5.7	4.1	>4.2
concentration		1.38	1.5	1.5	1.5	1.02
π_{trans}		>4.2	10.5	8.7	5.7	>4.2
concentration		1.55	2.0	2.0	2.0	1.22
π_{trans}		>4.2	11.6	>10.4	>10.4	3.1 [a]
concentration						1.39
π_{trans}						0.4 [a]

[a]The occurrence of the $Pn3m$ to H_{II} phase transition.

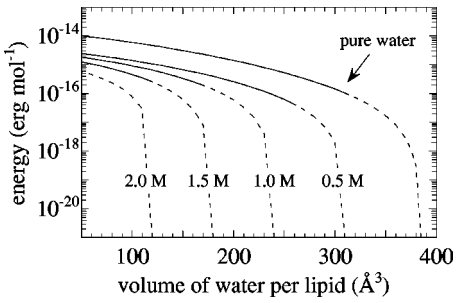

FIG. 3. Concentration (in terms of V_w) dependence of the overall free energy as determined in different trehalose solutions on both $Pn3m$ (dotted line) and $Ia3d$ (continuous line) cubic phases.

By numerically integrating the osmotic pressure over the volume of water *per* lipid, the overall free energy of the system can be obtained [17]. This energy is the work required to dehydrate the hydrophilic surfaces and includes among others, the cost of bending the lipid layer (i.e., the curvature energy, which is believed to be a critical term governing the stability of the nonlamellar phase and the ability of lipid and cellular membrane to bend), and the cost of lateral packing the lipid molecules (i.e., the stretching energy) [18,19]. In Fig. 3, the overall free energy calculated at different trehalose concentrations is reported as a function of V_w. It appears that the presence of sugar reduces the free energy of the system: however, stabilization occurs in similar way for both the cubic phases and then no explanation for the singular $Pn3m$ stabilization can be extracted.

An indication about energy contributions during dehydration can be derived from the analysis of the very existence of the pivotal surface, i.e., that molecular location at which the molecular cross-sectional area is invariant upon isothermal bending [17–22]. It has been in fact observed that by measuring the bending energy with respect to this surface, one excludes, to first order, any energy due to lateral monolayer stretching. In the case of inverse bicontinuous cubic phases, the pivotal surface is assumed to be parallel to the underlying IPMS [17–22]. According to this model, the pivotal surface geometry, i.e., the molecular area at the pivotal plane, A_n, and the molecular volume between this plane and the end of the chains, V_n (both surface averaged due to the inhomogeneity of the curvature of the underlying minimal surface), can be obtained fitting through the cubic equation derived by Templer and co-workers [22] the unit cells as a function of the water volume fraction:

$$\left(\frac{\langle A_n\rangle}{\langle v\rangle}\right)^3 a^3 + 6A_0\left(\frac{\langle A_n\rangle}{\langle v\rangle}\right)^2 \frac{a^2}{(1-\phi)} - \left[\frac{36\pi\chi}{(1-\phi)}\left(\frac{\langle V_n\rangle}{\langle v\rangle}\right)^2 + \frac{32A_0^3}{(1-\phi)^3}\right] = 0. \quad (1)$$

v is the lipid molecular volume, χ is a constant for the minimal surface, A_0 is the area of the minimal surface, and $\langle\ \rangle$ denotes the surface average. From the fitting parameters, the distance of the pivotal plane from the IPMS, ξ_n can be derived [22]:

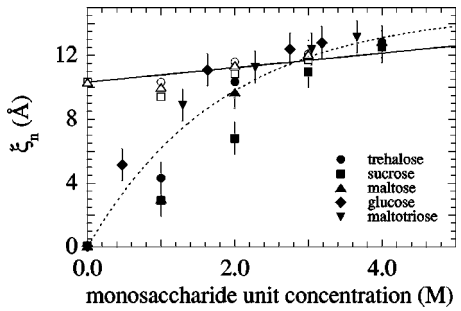

FIG. 4. Dependence on sugar concentration of the pivotal surface location, ξ_n, averaged from an osmotic stress series. To show the general trend, sugar concentrations are reported as molarity of monosaccharide units. Filled and open symbols refer to the $Pn3m$ and to the $Ia3d$ cubic phases, respectively. Lines are guides to the eyes (dotted line, $Pn3m$; continuous line, $Ia3d$).

$$\frac{\langle A_n\rangle}{\langle V_n\rangle} = \frac{1}{\xi_n}\left(\frac{1+\langle K\rangle\xi_n^2}{1+1/3\langle K\rangle\xi_n^2}\right), \quad (2)$$

where $\langle K\rangle$ is the surface averaged Gaussian curvature at the minimal surface. Using this model, reasonable estimates of the location of the pivotal plane in several aqueous lipidic structures [17,19,21,22], except in the $Pn3m$ phase [18,20], have been obtained. Remarkably, in MO, in which both $Pn3m$ and $Ia3d$ phases exist, a realistic location for the pivotal surface has been found only in the latter [20].

Present results confirm that the pivotal plane in the $Pn3m$ phase, at least in pure water and in dilute sugar solutions (lower than 1 M), practically corresponds to the minimal surface. This position is unphysical and could indicate an idiosyncrasy of the $Pn3m$ symmetry to the used interfacial geometrical model. However, as shown in Fig. 4, on increasing sugar concentration, the pivotal surface moves from $\xi_n \approx 0$ to a distance of about 12 Å from the IPMS, similar to the one observed in the $Ia3d$ phase (where the pivotal surface position is only slightly dependent on sugar concentration). During dehydration, sugars remain within the aqueous channels and alter the interface geometry. As a result, the polar groups are forced into such a close proximity by the decreasing of the area-*per*-polar head, that steric headgroup-headgroup interactions become strongly repulsive. As observed in swelling experiments [21,22], the final effect is a displacement of the pivotal surface toward the polar-apolar interface. Therefore, these results could indicate that on dehydration the $Pn3m$ interface is bending and stretching simultaneously in pure water and at low sugar [20], while only bending at high sugar, as well as in the $Ia3d$ phase.

This sugar effect could be the key to understand the singular stabilization observed on the $Pn3m$ phase. By modifying the interface properties, sugars can change the chain packing, as demonstrated by the appearance of the pivotal surface. As a consequence, the packing stresses in the $Pn3m$ could be reduced to levels where this phase is at lower free energy than the $Ia3d$, so that no phase transition occurs on dehydration. Note that the packing stress relieving should be quite large to counterbalance the increase of the bending energy due to the small dimensions of the $Pn3m$ unit cell. It

might equally be that the sugars enable the system to reduce the inhomogeneity in molecular splay and thereby stabilize the $Pn3m$ phase.

Packing frustrations in the H_{II} phase are usually larger than those observed in the $Pn3m$, so that in the MO-water system this phase forms only at high temperature [13,14]. It has been recently observed that appropriate amounts of hydrophobic molecules (like tricosane) added to MO induce the transition into the H_{II} phase because of the packing stress relief [19]. If packing frustrations were relevant also in the present case, we would expect that sugar addition can also get MO in the hexagonal phase. Indeed, at very high sugar concentrations (but this is possible only in the case of maltotriose due to solubility limits), the $Pn3m$-H_{II} phase transition is detected (see Table I). Confirming the relevant contribution of the stretching to drive the structural changes and phase behavior of lipidic mesophases, the increase of sugar concentration in the excess solution can relieve the packing stress both in the $Pn3m$ phase, which results stabilized with respect to the $Ia3d$, and in the H_{II} phase, which probably forms when almost all of the packing stress is relieved.

This work has been cofinanced by European Community funds for regional development.

[1] E.M. Landau and J.P. Rosenbusch, Proc. Natl. Acad. Sci. U.S.A. **93**, 14532 (1996).
[2] M. Caffrey, Curr. Opin. Struct. Biol. **10**, 486 (2000).
[3] J.C. Shah, Y. Sadhale, and D.M. Chilukuri, Adv. Drug Deliv. Rev. **47**, 229 (2001).
[4] L.M. Crowe and J.H. Crowe, Biochim. Biophys. Acta **946**, 193 (1988).
[5] R.D. Koynova, B.G. Tenchov, and P.J. Quinn, Biochim. Biophys. Acta **980**, 377 (1989).
[6] P.W. Sanderson, L.J. Lis, P.J. Quinn, and W.P. Williams, Biochim. Biophys. Acta **1067**, 43 (1991).
[7] L.I. Tsonev, M.G. Tihova, A.P.R. Brain, Z.-W. Yu, and P.J. Quinn, Liq. Cryst. **17**, 717 (1994).
[8] R. Koynova and M. Caffrey, Chem. Phys. Lipids **62**, 253 (1994).
[9] H. Takahashi, H. Ohmae, and I. Hatta, Biophys. J. **73**, 3030 (1997).
[10] P. Mariani, F. Rustichelli, L. Saturni, and L. Cordone, Eur. Biophys. J. **28**, 294 (1999).
[11] W. Longley and T.J. McIntosh, Nature (London) **303**, 612 (1983).
[12] S.T. Hyde, S. Andersson, B. Ericsson, and K. Larsson, Z. Kristallogr. **168**, 213 (1984).
[13] P. Mariani, V. Luzzati, and H. Delacroix, J. Mol. Biol. **204**, 165 (1988).
[14] J. Briggs, H. Chung, and M. Caffrey, J. Phys. II (Paris) **6**, 723 (1996).
[15] L.E. Scriven, Nature (London) **263**, 123 (1976).
[16] V.A. Parsegian, R.P. Rand, N.L. Fuller, and D.C. Rau, Methods Enzymol. **127**, 400 (1986).
[17] H. Chung, and M. Caffrey, Nature (London) **368**, 224 (1994).
[18] R.H. Templer, D.C. Turner, P. Harper, and J.M. Seddon, J. Phys. II (Paris) **5**, 1053 (1995).
[19] H. Vacklin, B.J. Khoo, K.H. Madan, J.M. Seddon, and R.H. Templer, Langmuir **16**, 4741 (2000).
[20] M. Pisani, S. Bernstorff, C. Ferrero, and P. Mariani, J. Phys. Chem. **105**, 3109 (2001).
[21] P.R. Rand, N. Fuller, S.M. Gruner, and A. Parsegian, Biochemistry **29**, 76 (1990).
[22] R.H. Templer, J.M. Seddon, N.A. Warrender, A. Syrykh, and Z. Huang, J. Phys. Chem. **102**, 7251 (1998).