Proc. Int. Symposium on Advances in Neutron Scattering Research, Tokai, 2000
J. Phys. Soc. Jpn. **70** (2001) Suppl. A pp. 411–413

Small Angle Neutron Scattering Study on Short and Long Chain Phosphatidylcholine Mixture in Trehalose Solution

Hiroshi TAKAHASHI

Department of Physics, Gunma University, Maebashi, 371-8510, Japan

Trehalose protects cells and proteins against various stresses due to low temperatures or dryness. In order to clarify the molecular mechanism of cryoprotective function of trehalose, we have studied the interaction between trehalose and phosphatidylcholine (PC) which is a main lipid component of cell membranes. In this study, the structural change of a binary PC mixture by the presence of trehalose was investigated by means of small angle neutron scattering. The PC binary mixture studied contains dihexanoyl-PC (diC_6PC) and dihexadecy-PC (DHPC). The former has short hydrocarbon chains and the latter has long hydrocarbon chains. The scattering profiles from the DHPC/diC_6PC mixture were changed, depending on trehalose concentrations. This change can be interpreted as suggesting that the presence of trehalose reduces the interfacial area between water and PCs.

KEYWORDS: trehalose, cryoprotectant, phosphatidylcholine, neutron scattering, interfacial area

§1. Introduction

Trehalose is a disaccharide of glucose and phosphatidylcholines (PCs) are one of the major phospholipid components of cell membranes. During the past two decades, interactions between trehalose and PCs have been considerably studied. This is because the interactions are believed to underlie the mechanisms whereby trehalose protects cell membranes and proteins from the damage due to low temperatures and dryness.[1,2]

Koynova *et al.*[3] have proposed a following hypothesis: Trehalose tends to reduce the interfacial area between aqueous and phospholipid phases. No clear experimental evidence, however, has been reported for this hypothesis. In order to examine the hypothesis by Kyonova *et al.*,[3] we have investigated dihexadecylphosphatidylcholine (DHPC) multibilayers in trehalose solutions.[4] The feature of DHPC is to form an interdigitated gel phase at room temperatures.[5] In the phase, the interfacial area per lipid molecule is about twice as compared with that of a normal gel phase bilayer (Fig.1). We have found that trehalose destabilizes the interdigitated gel phase of DHPC and induces to form a normal gel phase bilayer. In other words, the presence of trehalose reduces the interfacial area by about half.

Yoon *et al.*[6] have pointed out that dioleoly-PC bilayers are poorly permeable to trehalose and that trehalose may not always distribute homogeneously in multibilayer phospholipid vesicles. There is a possibility that osmotic pressure is given to a multibilayer phospholipid vesicle because of the inhomogeneous distribution of trehalose. In this connection, recently Hatanaka *et al.*[7] have revealed that the osmotic pressure by poly(ethylene glycol) induces a phase transformation from the interdigitated gel phase to normal bilayer gel phase in the multibilayer DHPC vesicles. Therefore it is necessary to reconsider whether our previous result for DHPC[4] is due to osmotic pressure by inhomogeneous distribution of trehalose molecules or not.

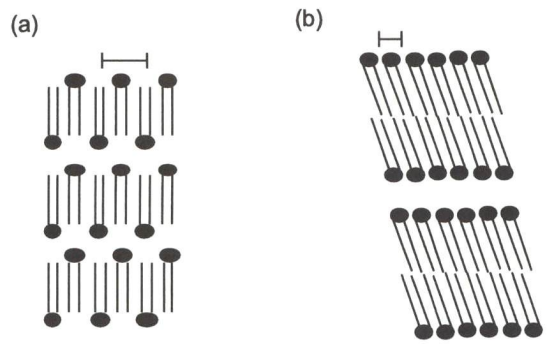

Fig. 1. Schematic representations of (a) interdigitated gel phase multibilayers and (b) normal gel phase multibilayers. Black circles corresponds to polar head groups of PC molecules and lines correspond to hydrocarbon chains.

In this study, we examined the effect of trehalose on the structure of the mixtures of dihexanoylphsohatidylcholine (diC_6PC) and DHPC, using small angle neutron scattering technique. DiC_6PC is one of short-chain PCs, having two fatty acyl chains with six carbons. The following is the reasons why we studied the PC binary system: In contrast to multibilayer vesicles, for micelle systems, there is no osmotic pressure due to the inhomogeneous distribution of solutes. Short-chain PCs with the chain lengths of four to eight carbons form micelles in water,[8] while long-chain PCs form multibilayer vesicles when they are dispersed in water. In addition, it has been reported that the binary mixture of diheptanoyl-PC (short-chain PC) and dipalmitoyl-PC (long-chain PC) forms disklike micelles, depending on the fraction of the short-chain PC.[9] It would be expected that, therefore, the mixture of the short-chain PC and DHPC also forms a micellar structure in trehalose solution, and that, as a result, the effect of trehalose on PCs can be examined under the condition of no osmotic pressure.

§2. Experimental

Powders of DHPC and diC_6PC were obtained from Fluka (Buchs, Switzerland) and Avanti Polar Lipids Inc. (Alabaster, AL, USA), respectively. These lipids gave a single spot on a silica gel thin-layer plate developed with $CHCl_3/CH_3OH/H_2O$ (65:35:4, by volume) and they were therefore used without further purification. Trehalose was obtained from Sigma (St. Louis, MO, USA) and it was purified by means of the recrystallization.[10)]

DHPC and diC_6PC were dissolved in chloroform and mixed to achieve the molar fraction of DHPC : diC_6PC = 4:1. The solvent was evaporated under a stream of oxygen free dry nitrogen. Residual solvents were removed by storage of the sample for 16 h in vacuum. The dried lipid samples were hydrated in 100 % D_2O containing various concentrations of trehalose by shaking on a Vortex mixer. The total lipid concentration was 25 mM.

Small angle neutron scattering measurements were performed with the SANS-U spectrometer at JRR-3M reactor at the Japan Atomic Energy Research Institute (Tokai, Japan). The detail of the spectrometer has been reported elsewhere.[11)] A mechanical velocity selector was used to get a monochromatic neutron beam from a polychromatic cold neutron beam from the reactor. The wavelength of the neutron beam was 7 Å. The samples were contained in a rectangular quartz cell with 1 mm optical path length. The data corrections were carried out at about 25°C. The exposure times were 0.5 ~ 2.5 h. The observed scattering intensity data were corrected by taking account of an instrumental background, backgrounds from buffers, a detector efficiency, transmissions, exposure times, and the thickness of samples.

§3. Results and Discussion

Figure 2 shows small angle neutron scattering spectra form the mixtures of 20 mM DHPC and 5 mM diC_6PC in trehalose solutions with various concentrations. Because the neutron scattering length density of trehalose is more nearly value of PCs than that of D_2O, the contrast of neutron scattering length densities between the lipids and trehalose solutions becomes smaller with increasing trehalose concentrations. Consequently, the scattering intensity decreases with increasing trehalose concentrations. It can be seen from Fig. 2 that, in addition to the decreasing of scattering intensity, the shape of the scattering curves changes with elevating trehalose concentrations. This indicates that structural changes of the DHPC/diC_6PC mixtures take place, depending on the trehalose concentrations.

For the mixture in pure D_2O, a sharp Bragg diffraction peak is observed at $Q = 0.12$ Å^{-1}. Here Q is the magnitude of the scattering vector. Judging from the spacing (52 Å), we can assign this peak to be originated from the periodic lamellar stacking of DHPC interdigitated gel bilayers.[5)] This result suggests that a phase separation between multilamellar vesicles of DHPC interdigitated gel bilayers and micellar structures occurs in the mixture in pure D_2O (Fig. 4 (a)). A very small Bragg diffraction peak is observed for the mixture in 0.4 M trehalose (Fig. 2 (Inset)).

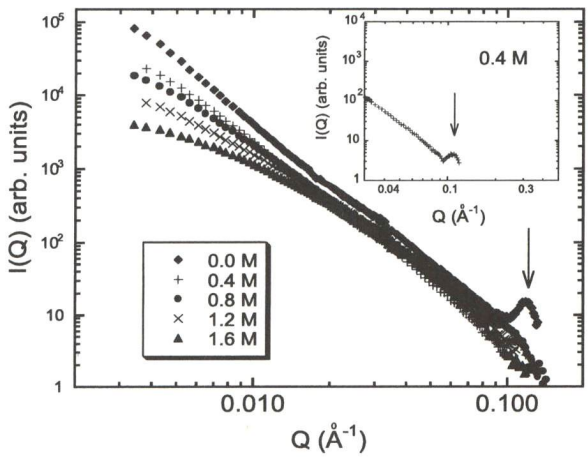

Fig. 2. Small angle neutron scattering curves form the mixtures of 20 mM DHPC and 5 mM diC_6PC in trehalose solution with various concentrations. Inset: Expanded plot of the scattering curve from the mixture in 0.4 M trehalose solution. The arrows indicate Bragg diffraction peaks (see text).

Next, we consider the structure of the mixtures in high trehalose concentrations. No Bragg diffraction peak is observed for the mixtures in more than 0.8 M trehalose. This indicates that no multibilayer structure exists in high concentration trehalose solutions. If lipid aggregates form a disklike two dimensional structure with radius R much larger than the thickness T and the disklike aggregates are randomly oriented, the neutron scattering intensity in the middle Q region can be approximated by[12)]

$$I(Q) \simeq n_p \Delta\rho^2 \frac{2\pi^2 R^2 T^2}{Q^2} \exp(-R_t^2 Q^2), \qquad (3.1)$$

where n_p is the number density of the particles, $\Delta\rho$ is the average contrast of neutron scattering length densities between the paticles and solvents, and R_t is the radius of gyration across the thickness of the disklike two dimensional structure. The R_t is equal to $T/\sqrt{12}$ for homogeneous disks. Therefore the scattering intensi-

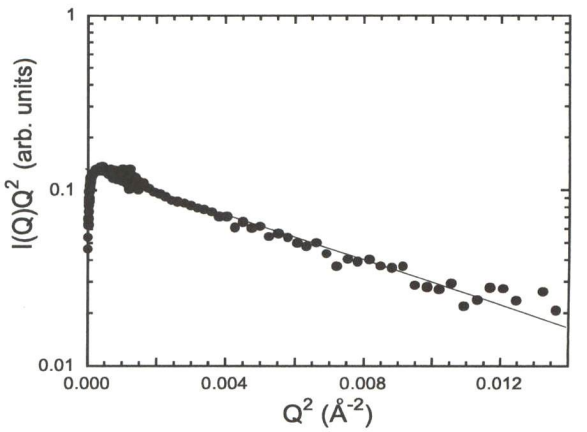

Fig. 3. Kratky-Porod plot of the measured small angle neutron scattering curve form the mixtures of 20 mM DHPC and 5 mM diC_6PC in 1.6 M trehalose solution.

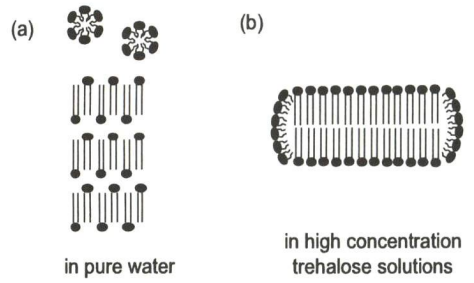

Fig. 4. Models for the structures of the DHPC/diC$_6$PC mixtures in (a) pure water and in (b) high concentration trehalose solutions.

ties of disklike particles in the middle Q region will fall on a straight line in the Kartky-Porod plot, which is by plotting $\ln(I(Q)Q^2)$ versus Q^2. Figure 3 represents the Kartky-Porod plot of the DHPC/diC$_6$PC mixture in 1.6 M trehalose solution. The data at $Q^2 > 0.001$ Å^{-2} fall on a straight line. For the mixture in 1.2 M trehalose, the similar result was obtained (data not shown). This strongly suggests that the mixture of DHPC/diC$_6$PC in high concentration trehalose solutions forms a disklike two dimensional structure.

From the slope of the fitted straight line in Fig. 3, R_t is found to be 12.2 Å. Because D_2O was used as water in this study, it can be assumed that the difference of neutron scattering density lengths between the solvents and the phosphocholine head groups is almost the same as that between the solvents and the hydrocarbon chain regions. Namely, one can regard the DHPC/diC$_6$PC mixtures as homogeneous. Hence, the thickness of the DHPC/diC$_6$PC mixtures in 1.6 M trehalose solutions is estimated to be about 42 Å from the R_t. Because the thickness of the interdigitated gel phase bilayer is about 30 Å,[5] the value (42 Å) is interpreted as suggesting that, in the DHPC/diC$_6$PC mixture, DHPC does not form the interdigitated structure but a normal bilayer structure. Judging from the chemical structures of DHPC and diC$_6$PC, we assume that the structure of the DHPC/diC$_6$PC mixtures in high concentration trehalose solutions is a bilayer structure with edge of the disk and that the diC6PC molecules cover the edge of the DHPC bilayer disk (Fig. 4 (b)).

In the gel phase bilayer of dipalmitoyl-PC, the hydrocarbon chains are tilted from the bilayer normal.[13] From the present available data, however, we can not determine whether the hydrocarbon chains in the DHPC/diC$_6$PC disklike aggregates are tilted or not.

The present study shows that DHPC form the normal gel phase bilayer in the micellar structures in the presence of more than 1.2 M trehalose. In other words, under the condition of no osmotic pressure, high concentration trehalose solutions destabilize the interdigitated gel phase bilayers of DHPC and induce the normal gel phase bilayers. It is concluded that what tehalose reduces the interfacial area between aqueous and phospholipid phases is not mainly due to the osmotic pressure by inhomogeneous distribution of trehalose molecules. The origin of the reduction effect of trehalose has been unclear, although several explanations have been proposed.[3,4]

Finally, we consider briefly the mechanism of cryoprotective function of trehalose. As discussed in our previous papars,[4,14] on the basis of various results of studies on the trehalose-phospholipid interaction, we can assume that the origin of the effect of trehalose is not due to direct interaction between trehalose and phospholipids. It would be expected, therefore that the effect of trehalose is common for amphiphilic macromolecules or assemblies of amphiphilic molecules and that trehalose also reduces the interfacial area of proteins in aqueous phases. Proteins undergo a cold-induced denaturation[15] and the denatured proteins do not fulfill their function. In the denatured state, the interfacial area contacting with water increases in comparison with the native state. Taking accounts of this fact, trehalose would be assumed to stabilize the native state of proteins at subzero temperatures by reducing the interfacial area. This may be correlated with the low temperature resistance by producing trehalose in some organisms

Acknowledgements

We would like to thank Dr. M. Nagao (ISSP, University of Tokyo) for his technical advice in the SANS-U spectrometer.

1) J. H. Crowe, J. F. Carpenter, and L. M. Crowe : Annu. Rev. Plant Physiol. **60** (1998) 73.
2) J. Wolfe and G. Bryant : Cryobiologly **39** (1999) 103.
3) R. D. Koynova, B. G. Tenchov and P. J. Quinn: Biochim. Biophys. Acta **980** (1989) 377.
4) H. Takahashi, H. Ohmae and I. Hatta: Biophys. J. **73** (1997) 3030.
5) J. T. Kim, J. Mattai and G. G. Shipley: Biochemistry **26** (1987) 6592.
6) Y. H. Yoon, J. M. Pope and J. Wolfe: Biophys. J. **74** (1998) 1949.
7) Y. Hatanaka, K. Kinoshita and M. Yamazaki: Biophys. Chem. **65** (1997) 229.
8) R. J. M. Taush, J. Karmiggelt, C. Oudshoorm and J. Th. G. Overbeek: Biophys. Chem. **1** (1974) 175.
9) T.-L. Lin, C.-C. Liu, M. F. Roberts and S.-H. Chen: J. Phys. Chem. **95** (1991) 6020.
10) E. A. Arnett, N. Harvey, E. A. Johnson, D. S. Johnston and D. Chapman: Biochemistry **25** (1986) 5239.
11) Y. Ito, M. Imai and S. Takahashi: Physica B **213-214** (1995) 889.
12) O. Glatter and O. Kratky: *Small angle X-ray scattering*, (Acadenic Press, New York, 1982).
13) A. Tardieu, V. Luzzati and F. C. Reman: J. Mol. Biol. **75** (1973) 711.
14) H. Takahashi, M. Imai, Y. Matsushita and I. Hatta: Progr. Colloid Polym. Sci. **106** (1997) 223.
15) P. Privalov and S. J. Gill: Adv. Protein Chem. **39** (1988) 191.