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Effect of headgroup type on the miscibility of homologous phospholipids with different acyl chain lengths in hydrated bilayer

Yoshinori Nibu, Tohru Inoue *, Isamu Motoda

Department of Chemistry, Faculty of Science, Fukuoka University, Nanakuma, Jonan-ku, Fukuoka 814-01, Japan

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Abstract

The miscibility of homologous phosphatidylcholines with different acyl chain lengths in hydrated bilayer was examined through the binary phase diagram constructed by differential scanning calorimetry. By analyzing the phase diagram according to a thermodynamic model based on the Bragg–Williams approximation to evaluate the excess free energy of mixing, the non-ideality parameter of mixing, ρ_0, was estimated, which allows one to interpret the mixing behavior of the two lipid components in terms of the difference in the pair-interaction energies between like-pairs and mixed-pairs formed in the mixture. By summarizing the ρ_0 values obtained previously for other classes of phospholipids, it was found that ρ_0 increases in the order of phosphatidylglycerol (PG) ≈ phosphatidylcholine (PC) < phosphatidylethanolamine (PE) < phosphatidic acid (PA). Since the difference in the pair-interaction energies is considered to be determined by the relative contribution of inter-headgroup interaction to the overall intermolecular interaction, this sequence of ρ_0 value suggests that the headgroup interaction in hydrated bilayer increases in the order of PA < PE < PC ≈ PG.

Keywords: Phospholipid mixture; Phase diagram; Mixing behavior; Headgroup interaction; Differential scanning calorimetry

1. Introduction

Biomembranes are multicomponent systems constituted of various kinds of proteins and lipid species, the former being embedded in the bilayered lipid matrices [1]. Since it is believed that the physical and functional properties of biomembranes depend on the mixing state of different lipid species in the membranes [1], the elucidation of the mixing behavior of different lipids in hydrated bilayer becomes of fundamental importance from a biophysical and biotechnological point of view. In order to understand the lipid miscibility in biomembranes, an approach using model systems may be helpful. For this reason, many investigations have been reported concerning the mixing behavior of binary lipid mixtures [2], particularly stressing on phospholipids which are major constituents of biomembranes. Among the classes of phospholipids, the mixtures of phosphatidylcholines (PC) and phosphatidylethanolamines (PE) have been most frequently studied [3–15] because of their most abundant occurrence in living bodies as membrane-constituting lipids [16]. The mixtures of PC and

* Corresponding author.

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phosphatidylglycerols (PG) have also attracted considerable attention in connection with their function as lung surfactants [17–21].

The miscibility property of different components is reflected upon a phase diagram of the mixture, and hence, the phase diagram can become a useful tool to examine the mixing behavior of the components. The phase diagrams so far accumulated for various binary phospholipid mixtures have revealed quite diverse aspects for the miscibility in hydrated bilayers, from nearly ideal mixing to restricted miscibility or complete immiscibility, depending on the difference in the headgroup structure as well as that in the length and type of acyl chains between the two components. The miscibility also depends strongly on the state of the bilayer: gel or liquid–crystalline. The mixing behavior of different components would be determined, as a first approximation, by the difference in the pair-interaction energies among the like-pairs and the mixed-pairs being formed in the mixture as a result of mixing of the components. Thus, the diversity of the miscibility observed in phospholipid mixtures may be regarded as a reflection of the diverse nature of the intermolecular interaction among the different phospholipid species within the hydrated bilayers. In order to clarify the details concerning the intermolecular interactions responsible for the mixing behavior, and to extract the factors governing the miscibility of different phospholipid species, it may be desirable to investigate on systematically selected combinations of two phospholipids such as those with the same headgroup but different acyl chains, and also those with the same acyl chain but different headgroups.

From the above point of view, we have been studying the miscibility of homologous phospholipids with saturated acyl chains of different lengths based on the binary phase diagrams constructed by differential scanning calorimetry (DSC); the phospholipid species previously examined are phosphatidic acids (PA) [22,23], PE [24], and PG [25]. By analyzing the phase diagram according to the thermodynamic equations derived by evaluating the excess free energy of mixing within a framework of the Bragg–Williams approximation, the non-ideality parameter of mixing, ρ_0, has been estimated for these phospholipid mixtures. Furthermore, on the basis of the ρ_0 values, the mixing behavior of these homologous phospholipids has been interpreted in terms of the difference in the pair-interaction energies between like-pair and mixed-pair. As an extension of this series of study, the present paper deals with the miscibility of PC with different acyl chain lengths of $n = 14$ to 18 (n is the number of carbon atoms per chain) in hydrated bilayer. By comparing the results obtained for several phospholipid species, the effect of headgroup type on the miscibility property of homologous phospholipids will be revealed through the inter-headgroup interaction acting in the hydrated lipid bilayer.

2. Materials and methods

2.1. Materials and sample preparation

Synthetic dimyristoyl-($n = 14$), dipalmitoyl-($n = 16$), and distearoyl-($n = 18$) phosphatidylcholine (DMPC, DPPC, and DSPC, respectively) were obtained from Sigma, and were used without further purification. The purities of these lipids are $> 99\%$.

Samples of the lipid mixture for DSC measurements were prepared as follows. The appropriate amounts of two dry lipids were weighed into a glass cuvette to give a desired composition. Chloroform was added to it in an amount enough to just dissolve the lipid mixture completely. Then, the solvent was removed by gentle blowing of dry N_2 gas, followed by evaporation under reduced pressure.

2.2. DSC measurements

DSC measurements were performed using a Seiko Denshi Model SSC5000. The pure lipid or lipid mixture of about 5 mg was weighed in a sealable sample pan, to which 20 μl of water was added, and then the pan was sealed. The sample was held at 90°C for 2 h in the oven of the DSC apparatus in order to assure homogeneous mixing of the lipid and water. Then, a cooling/heating cycle was repeated for several times at the rate of 2°C/min in the temperature range of 5–90°C.

2.3. Construction of phase diagram from DSC curves

The binary phase diagrams of PC mixtures were constructed from the DSC thermograms by the fol-

lowing procedure. The onset and completion temperatures for the main phase transition of hydrated bilayer, i.e., gel-to-liquid-crystalline transition, were determined as the temperatures corresponding to the intersection between the tangent to the leading edge and the baseline of the DSC curves [22,24,26–28]. These temperatures were corrected for the finite widths of the transitions of pure components according to the method described by several authors [8,22,24,26–30]. The corrected temperatures were plotted against the mole fraction of one of the two components in the mixture to obtain the 'solidus' and 'liquidus' lines in a binary phase diagram [22,24,26,27].

3. Results

The DSC thermograms obtained for DPPC/DSPC mixtures and DMPC/DSPC mixtures at various compositions are shown in Fig. 1 and Fig. 2, respectively. As is well established, the large endothermic peaks appearing in the thermograms are ascribed to the bilayer phase transition from gel to liquid–crystalline, which is characteristic to the hydrated phospholipid systems. The transition temperatures (peak temperatures) and the transition enthalpy for pure components were 25.0°C and 5.2 kcal/mol (DMPC), 42.6°C and 7.3 kcal/mol (DPPC), and 56.0°C and 9.1 kcal/mol (DSPC). These values are in agreement with those reported in literature [31].

For a DPPC/DSPC mixture composed of two lipids differing in the acyl chain length by two methylene units, the transition temperature shifts toward higher temperature with the increase in DSPC content, simultaneously accompanied by a slight broadening of the transition peak up to the intermediate composition range. This thermotropic behavior associated with the variation of the composition indicates that the two lipids are mutually miscible in both liquid–crystalline and gel phases. On the other hand, the DMPC/DSPC mixture, the components in which differ in their chain length by four methylene units, provide extremely broad endothermic peaks. This indicates that the bilayer phase transition proceeds over a wide temperature range, or in other words, the gel and liquid–crystalline states are coexisting in a wide temperature range. In particular, the

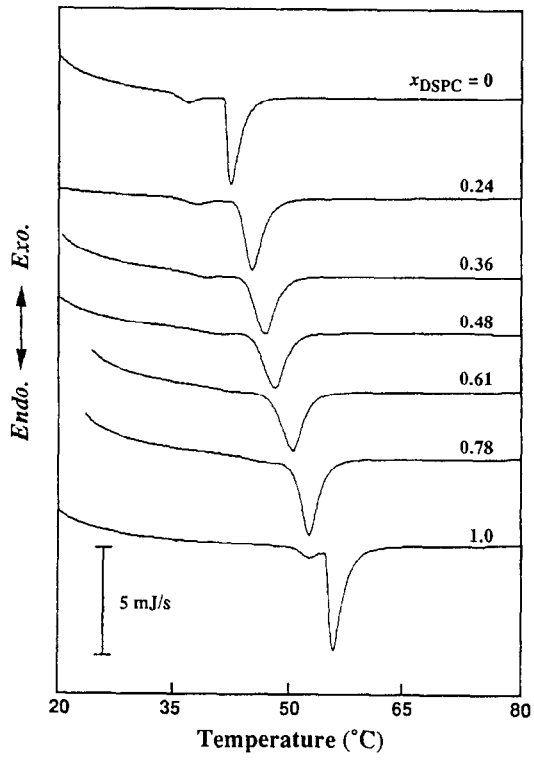

Fig. 1. DSC heating thermograms obtained for hydrated DPPC/DSPC mixtures at various compositions. The mole fractions of DSPC in the mixture, x_{DSPC}, are indicated in the figure.

onset temperature of the phase transition remains almost unaltered in the composition range from $x_{DSPC} = 0.23$ to $x_{DSPC} = 0.77$, where x_{DSPC} represents the mole fraction of DSPC. This suggests that the partial demixing occurs in the gel bilayer within this composition range. The reduced miscibility of DMPC/DSPC mixture may be attributed to the increased difference in the acyl chain lengths between the two components for this system.

The onset and the completion temperatures of the phase transition were determined from the DSC curves presented in Figs. 1 and 2, and after the correction for the finite transition widths of pure lipid components, they were plotted against the mole fraction of DSPC to construct the binary phase diagrams for the mixtures. Figs. 3 and 4 depict the phase diagrams thus obtained for DPPC/DSPC and DMPC/DSPC mixtures, respectively. Several authors have so far reported on the binary phase diagrams for DPPC/DSPC mixture [3], DMPC/DPPC

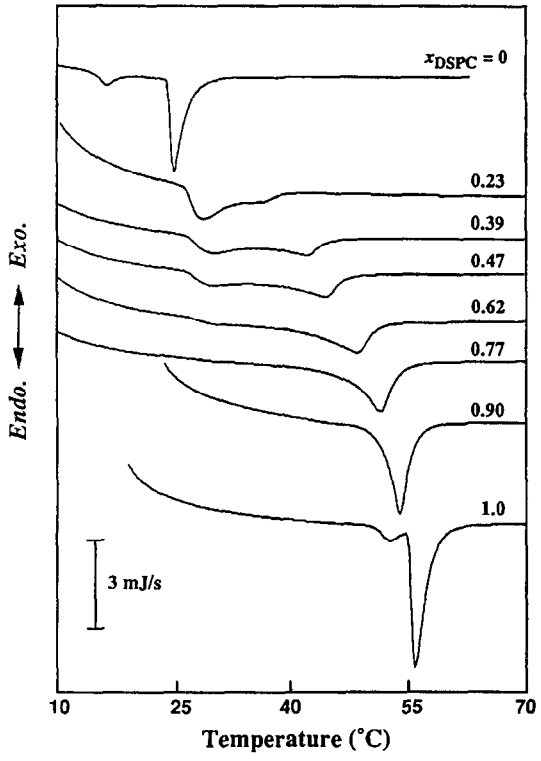

Fig. 2. DSC heating thermograms obtained for hydrated DMPC/DSPC mixtures at various compositions. The mole fractions of DSPC in the mixture, x_{DSPC}, are indicated in the figure.

mixture [3,5,6,8,10,32,33], and DMPC/DSPC mixture [3,8,10,32–34], which have been constructed by various methods such as spin-label [3,10], fluorescence probe [6,33], and DSC [5,8,32,34]. The phase diagrams obtained in the present study are in rather good agreement with those reported in literature for both DPPC/DSPC and DMPC/DSPC mixtures. It can be seen by surveying literature that the shape of phase boundaries for DPPC/DSPC mixture is quite similar to that for DMPC/DPPC. This demonstrates that the mixing behavior of homologous PC species is mostly determined by the chain length difference of the two components.

The cigar-shaped phase boundary for DPPC/DSPC mixture indicates that the two components are mutually miscible in both liquid-crystalline and gel phases of the bilayer. However, the mixing deviates considerably from ideal behavior, as demonstrated by the departure of the experimental phase boundary from dashed curves which are expected for ideal mixing of the two components. Several methods have so far been applied to simulate the binary phase diagrams for non-ideal lipid mixtures [3,11,32,34–38]. The most frequently used method utilizes the thermodynamic equations derived by considering the excess free energy of mixing. To evaluate the excess free energy of mixing, the Bragg–Williams approximation [39] may be valid for the lipid mixtures such as in the present case, because the components in the mixture have a similarity in both molecular structure and molecular volume. Thus, the phase diagrams obtained in the present study for PC mixtures were analyzed based on this model. According to this thermodynamic treatment, the phase boundaries corresponding to the

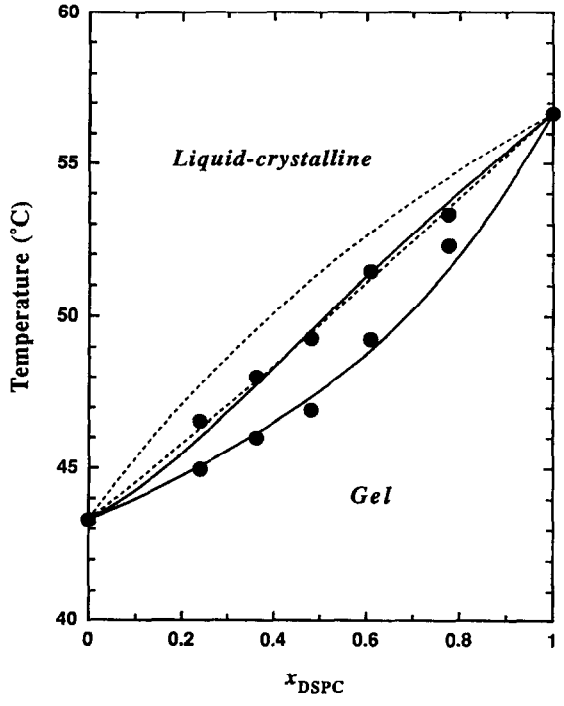

Fig. 3. Pseudo-binary phase diagram for hydrated DPPC/DSPC mixtures constructed from DSC curves presented in Fig. 1. Dashed lines are phase boundaries calculated by assuming the ideal mixing of the two components in both gel and liquid-crystalline bilayers. Solid lines are phase boundaries calculated according to Eqs. (1) and (2) (see text for details).

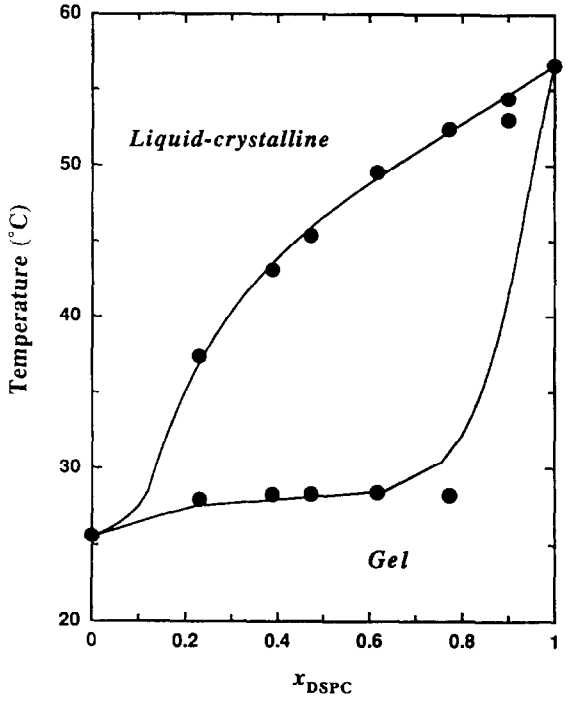

Fig. 4. Pseudo-binary phase diagram for hydrated DMPC/DSPC mixtures constructed from DSC curves presented in Fig. 2. Solid lines are phase boundaries calculated according to Eqs. (1) and (2) (see text for details).

'solidus' and 'liquidus' lines are given by solving the following simultaneous equations [22,36,37,40].

$$\ln\frac{x_A^{(L)}}{x_A^{(S)}} + \frac{\rho_0^{(L)}\left(1-x_A^{(L)}\right)^2 - \rho_0^{(S)}\left(1-x_A^{(S)}\right)^2}{RT} = -\frac{\Delta H_A}{R}\left(\frac{1}{T}-\frac{1}{T_A}\right) \quad (1)$$

$$\ln\frac{1-x_A^{(L)}}{1-x_A^{(S)}} + \frac{\rho_0^{(L)}\left(x_A^{(L)}\right)^2 - \rho_0^{(S)}\left(x_A^{(S)}\right)^2}{RT} = -\frac{\Delta H_B}{R}\left(\frac{1}{T}-\frac{1}{T_B}\right) \quad (2)$$

where $x_A^{(L)}$ and $x_A^{(S)}$ refer to the mole fractions of component A in liquid–crystalline and gel phase bilayers being in equilibrium with each other, T_A and T_B are the transition temperatures and ΔH_A and ΔH_B are the latent heats for pure species denoted by the subscripts, and $\rho_0^{(L)}$ and $\rho_0^{(S)}$ are parameters characterizing the non-ideality of mixing in liquid-crystalline and gel phases, being given by

$$\rho_0 = z\left(u_{AB} - \frac{u_{AA}+u_{BB}}{2}\right) \quad (3)$$

where z is the first coordination number and u_{AA}, u_{BB}, and u_{AB} are the molar energies of A–A, B–B, and A–B pair interactions.

In applying Eqs. (1) and (2) to the analysis of phase diagram, the non-ideality parameters $\rho_0^{(L)}$ and $\rho_0^{(S)}$ were determined so that the calculated phase boundaries reproduce the experimental phase boundaries most satisfactorily. This was done by searching their values to minimize the sum of squares of the difference between experimental and calculated temperatures; the procedure has been described elsewhere in some detail [24]. The values of T_A, T_B, ΔH_A, and ΔH_B required for the calculation are known, as described above. The ρ_0 values determined by this procedure are as follows; $\rho_0^{(L)} = 180$ cal/mol, $\rho_0^{(S)} = 380$ cal/mol for DPPC/DSPC mixtures, and $\rho_0^{(L)} = 500$ cal/mol, $\rho_0^{(S)} = 1210$ cal/mol for DMPC/DSPC mixtures. The solid lines in Figs. 3 and 4 were drawn according to Eqs. (1) and (2) using these parameter values. The agreement between the experimental and computed phase boundaries is satisfactory, although it becomes somewhat poor for DMPC/DSPC mixture at the composition range close to $x_{DSPC} = 1$.

4. Discussion

As demonstrated in the present work and also in previous studies [23–25], the phase diagrams of binary mixtures of homologous phospholipids with different acyl chain length can be well described by a thermodynamic model based on the Bragg–Williams approximation for the non-ideality of mixing. The non-ideality parameter, ρ_0, derived from this thermodynamic treatment characterizes the mixing behavior of different components in terms of the difference in the pair-interaction energies between like-pair and mixed-pair formed in the mixture. The ideal mixing corresponds to $\rho_0 = 0$ which is the case where intermolecular interaction for mixed-pair is just the same as that for like-pair. In other words, the

components in an ideal mixture do not differ from each other in interaction energy. The non-ideal behavior of mixing appears when ρ_0 deviates from zero; the positive ρ_0 means that the mixed-pair formation in the mixture is energetically less favorable than the like-pair formation. Thus, some aspects concerning the phospholipid miscibility may be revealed based on the intermolecular interaction by comparing the ρ_0 values estimated for various phospholipid mixtures.

For the mixtures of homologous phospholipids with different acyl chains, the difference in the pair-interaction energies between like-pair and mixed-pair may depend on a relative contribution of inter-headgroup interaction to the overall intermolecular interaction; the larger the headgroup contribution is, the smaller the difference in the pair-interaction energies becomes, because the difference in the intermolecular interaction between like-pair and mixed-pair arising from the chain length difference is hidden behind the large inter-headgroup interaction. Thus, the mixing behavior of homologous phospholipids may be understood in terms of the headgroup contribution to the overall intermolecular interaction. In particular, by examining ρ_0 values estimated for some phospholipid species of different headgroups, we will be able to compare the relative contribution from headgroup interaction for different phospholipid species. It should be noted here that the reproducibility of experimental phase boundaries by Eqs. (1) and (2) is rather poor for the mixtures composed of two lipid components differing in their chain length by four methylene units (see Fig. 4 and Refs. [23–25]). This may be attributed to their highly non-ideal nature of mixing to such an extent as exceeding the limitation of the Bragg–Williams approximation; it is reasonably understood that the non-ideality of mixing increases with the increase in the chain length difference of the two lipid components. The ρ_0 values estimated for these mixtures may include some uncertainty originating from the poor approximation adopted in the present thermodynamic treatment. Therefore, in comparing ρ_0 values derived for homologous mixtures of various phospholipid species with different headgroups, it will be safe to restrict the discussion within those obtained for the mixtures of the components differing in their chain length by two methylene units, for which the present thermodynamic treatment reproduces the experimental phase boundaries quite satisfactorily.

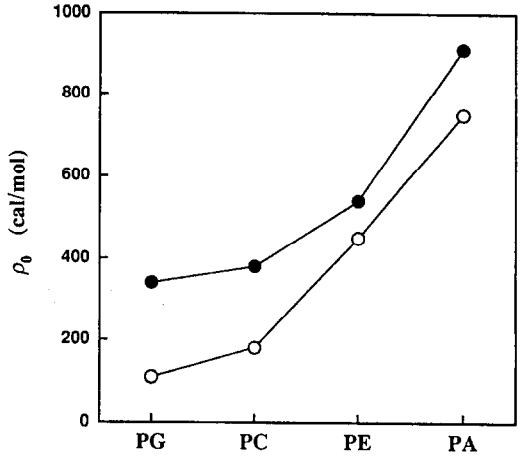

Fig. 5. Dependence of ρ_0 values obtained for mixtures of homologous phospholipids differing in the acyl chain length by two methylene units on the lipid headgroup. (●) Gel state bilayer and (○) liquid–crystalline state bilayer.

Fig. 5 illustrates the variation of $\rho_0^{(L)}$ and $\rho_0^{(S)}$ with the headgroup type of homologous phospholipid mixtures with the chain length difference by two methylene units, i.e., dipalmitoyl/distearoyl mixtures for PC, PG [25], and PE [24], and dimyristoyl/dipalmitoyl mixture for PA [23]. There should be something mentioned here about the electrical charge of headgroups of these phospholipid species. In a series of experiments, neither the control of pH nor the external addition of inorganic salts was made for water which was used to produce the hydrated lipid bilayers. Under this condition, the headgroup charge for respective lipids is considered as follows: PC is zwitterionic; PE is also zwitterionic ($pK_a(PO_4) \approx 1$ and $pK_a(NH_3^+) \approx 10$–11 [41]); PA and PG has a monovalent negative charge (for PA, $pK_a(PO_4) \approx 3.5$ and 9.0 [41], and for PG, $pK_a(PO_4) \approx 3$ [41]).

It can be seen in Fig. 5 that $\rho_0^{(L)}$ is smaller than $\rho_0^{(S)}$ for all phospholipid species. This means that the liquid–crystalline bilayer exhibits a higher miscibility than the gel phase bilayer regardless of the type of lipid headgroup. The difference in the miscibility property between the two bilayer phases may be interpreted in terms of the difference in the conformational states of the lipid acyl chain assumed in the respective phases. In liquid–crystalline bilayer, the

lipid acyl chain has a disordered 'liquid-like' structure including many gauche conformers, while in the gel phase bilayer, it has an ordered all-trans conformation. The difference in pair-interaction energies between like-pair and mixed-pair formed in the lipid bilayer would become more significant for the components with rigid 'solid-like' acyl chains than for those with flexible 'liquid-like' acyl chains. This may bring about larger ρ_0 values or higher non-ideality of mixing in gel state bilayers than in liquid–crystalline state bilayer.

An interesting feature of Fig. 5 is the dependence of ρ_0 on the type of lipid headgroup. Both $\rho_0^{(L)}$ and $\rho_0^{(S)}$ increase in the order of PG ≈ PC < PE < PA. According to the above-mentioned interpretation of ρ_0 in terms of the pair-interaction energies, this sequence of ρ_0 means that the relative contribution of the headgroup interaction to the overall intermolecular interaction increases in the order of PA < PE < PC ≈ PG. The difference in the acyl chain-length of the two components in the mixtures are identical for all cases, i.e., palmitoyl and stearoyl for PG, PC, and PE, and myristoyl and palmitoyl for PA. The chain–chain interactions in the hydrated bilayer may be similar even for phospholipid species with different headgroups as long as the bilayer is in a given phase, say, liquid–crystalline, because both the conformational state and the packing state of lipid acyl chains are similar in the same bilayer phase. Therefore, the headgroup contribution in the overall intermolecular interaction may be regarded to reflect the strength of the headgroup interaction acting in hydrated bilayers.

The difference in the strength of the headgroup interaction among different phospholipid species may be ascribed to the substituent group on the phosphate moiety attached to the glycerol backbone of the phospholipid molecule. It seems reasonable that PA exhibits the weakest headgroup interaction, since it has no additional substituent group. It is regarded for other phospholipid species that the substituent groups on a phosphate moiety act to strengthen the headgroup interaction within the hydrated bilayer. In addition, the results in Fig. 5 suggest that the substituent effect of glycerol on the headgroup interaction is nearly identical with that of choline, and is considerably larger than that of ethanolamine. A highly miscible nature reported for PC/PG mixtures with identical acyl chains [18] is understood considering the similarity of headgroup interaction in the hydrated bilayer for PC and PG species. On the other hand, the mixtures of PC and PE have been found to exhibit a considerably non-ideal mixing behavior even when the two components have the same acyl chain [3–6,9,11,12,14,15]. This may be attributed to the difference in the headgroup interaction within the bilayer between the two phospholipid species, as clearly demonstrated by the difference in ρ_0 values revealed in the present study.

Acknowledgements

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