Lipids are important chemicals that can assemble in layers to form an interface. In particular, diacylglycerides (DAGs), which have one hydrophilic headgroup and two hydrophobic tailgroups, constitute an important category of lipids because they are the main components of naturally occurring biological membranes. The tailgroups of DAGs endow them with sufficient hydrophobicity to form a stable monolayer at the air/water interface. In an aqueous dispersion, DAGs assemble to lipid bilayers in the form of spherical vesicles or planar lamellae owing to the appropriate size balance between the hydrophilic headgroup and the hydrophobic tailgroups. Such bilayers of DAGs have many potential applications in biomedicine. For instance, the spherical vesicle structure has often been used to provide drug delivery vehicles.1−4 The flat lipid bilayer can be used as the interface of biosensors,5−7 the scaffolds for membrane-bound proteins,8,9 the surface modifier of microfluidic devices,5,10 etc.

We focused on phosphatidylcholines, which are naturally occurring DAGs, because they are the main component of biological membranes such as those of cells and microorganisms in the cytosol. Figure 1 depicts the chemical structure of 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC). The polar headgroup of phosphatidylcholine is phosphocholine (−PO4−−CH2CH2−N+(CH3)3), which has a large dipole moment because it is a zwitterionic group comprising a cationic choline moiety and an anionic phosphate ester. In contrast to naturally occurring lipids, DAGs with headgroup charges that are antiparallel to those of phosphatidylcholine have been synthesized recently by Szoka et al.11−15 and by our group.16 Such DAGs were named “inverse charge zwitterlipids (ICZLs)” on the basis of the arrangement of their headgroup charges. In a previous report, we demonstrated that

Comparison of Carboxybetaine with Sulfbetaine as Lipid Headgroup Involved in Intermolecular Interaction between Lipids in the Membrane

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ABSTRACT: Diacylglycerides (DAGs) constitute an important category of lipids owing to their ability to form a lipid membrane, which can be used in a wide variety of biomedical applications. DAGs often include a zwitterionic polar headgroup that can influence the properties of the lipid membrane (e.g., protein adsorption, ion binding, hydration, membrane fluidity, phase stability) and affect their applicability. To clarify the effect of the charge arrangement of zwitterionic headgroups on intermolecular interactions in the DAG bilayers, we investigated the intermolecular interaction between a naturally occurring DAG (1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC)) and synthetic DAGs (which is called “inverse charge zwitterlipids (ICZLs)” whose headgroup charges were antiparallel with respect to those of DPPC. We used 1,2-dipalmitoyl-sn-glycero-3-carboxybetaine (DPCB) and 1,2-dipalmitoyl-sn-glycero-3-sulfobetaine (DPSB) as ICZLs and compared two combinations of the lipids (DPPC–DPCB and DPPC–DPSB). We obtained surface pressure–area (π–A) isotherms to elucidate the intermolecular interaction between the lipids in the monolayer at the air/water interface. We found shrinkage of the area per molecule in both lipid combinations, indicating that mixing DPPC with ICZLs results in an attractive intermolecular force. As an overall trend, the degree of shrinkage of the mixed monolayer and the thermodynamic favorability of mixing were greater in the DPPC–DPCB combination than in the DPPC–DPSB combination. These trends were also observed in the lipid bilayers, as determined from the gel-to-liquid crystal phase transition temperature (Tc) of the aqueous dispersion of the lipid vesicles. In the highly compressed lipid monolayers and vesicles (lipid bilayer), the molar fractions of ICZLs, in which the intermolecular interaction reached a maximum, were 0.6–0.8 for the DPPC–DPCB combination and 0.5 (equimolar composition) for the DPPC–DPSB combination. Therefore, in the compressed monolayers and bilayers, the mechanism of intermolecular interaction between DPPC and DPCB is different from that between DPPC and DPSB.

INTRODUCTION

Lipids are important chemicals that can assemble in layers to form an interface. In particular, diacylglycerides (DAGs), which have one hydrophilic headgroup and two hydrophobic tailgroups, constitute an important category of lipids because they are the main components of naturally occurring biological membranes. The tailgroups of DAGs endow them with sufficient hydrophobicity to form a stable monolayer at the air/water interface. In an aqueous dispersion, DAGs assemble to lipid bilayers in the form of spherical vesicles or planar lamellae owing to the appropriate size balance between the hydrophilic headgroup and the hydrophobic tailgroups. Such bilayers of DAGs have many potential applications in biomedicine. For instance, the spherical vesicle structure has often been used to provide drug delivery vehicles.1−4 The flat lipid bilayer can be used as the interface of biosensors,5−7 the scaffolds for membrane-bound proteins,8,9 the surface modifier of microfluidic devices,5,10 etc.
We used two pairs of lipid combinations, DPPC–1,2-dipalmitoyl-sn-glycero-3-carboxybetaine (DPCB) and DPPC–DPSB, to further examine the interaction between zwitterionic headgroups with charges arranged antiparallel to each other in the DAGs. The chemical structures of these DAGs are shown in Figure 1. The intermolecular interaction between DPPC and ICZLs was investigated by obtaining the surface pressure–area (π–A) isotherm of the mixed lipid monolayer at the air/water interface. Furthermore, we investigated the thermotropic phase transition (gel-to-liquid crystal phase transition) of lipid vesicles composed of DPPC and ICZLs because the transition temperature reflects the intermolecular interaction of lipids.

**RESULTS AND DISCUSSION**

π–A Isotherms of the Lipid Mixture Monolayer. To examine the intermolecular interaction between DPPC and ICZLs, we acquired surface pressure–area (π–A) isotherms of the binary lipid monolayer at the air/water interface (Figure 2a). Because the lipids used in this study differed only in their headgroups (Figure 1), any modulation of the intermolecular interaction between the two molecules must have involved those headgroups. As an overall trend, the isotherms for the mixed monolayers comprising DPPC–DPCB or DPPC–DPSB were shifted to lower area. This result indicates that attractive interaction occurred between the lipids in both combinations. As discussed later, complexed intermolecular interactions that must have occurred between the headgroups (PC–SB and PC–CB) may have been involved in the attractive interaction between the lipids in the binary monolayer. The maximum surface pressure of the lipid monolayer comprising DPPC and DPCB ranged from 50 to 60 mN/m at a given molar fraction of DPCB, which was markedly higher than that of the monolayer comprising the DPPC–DPSB combination (40–50 mN/m). This result indicates that a more stable monolayer was formed from the DPPC–DPCB lipid combination than from the DPPC–DPSB combination.

In the isotherm of pure DPPC (Figure 2a; XICZL = 0), a plateau region resulting from a liquid expansion (LE)–liquid condensation (LC) coexistence phase was observed at 65–85 Å², which is the almost same with those found in the literatures.41–46 It is thought that the LE–LC coexistence phase arises from competition between attractive line tension and repulsive dipole interactions between the headgroups.41,44–46 In the π–A isotherms of the DPPC–DPCB combination at the higher molar fraction range of DPCB, X_{DPCB} = 0.7–1.0, plateau regions were observed at a surface pressure of ~40 mN/m (Figure 2a, arrows). As the molar fraction of DPCB decreased, these plateau regions narrowed and finally disappeared when X_{DPPC} was ≤0.6. There is a possibility that the monolayer undergoes a first-order phase transition in the plateau regions. However, the surface pressure of the plateau region observed in the pure DPCB isotherm was much higher than that in the pure DPPC isotherm (Figure 2a; X_{DPPC} = 1.0). Although the mechanism underlying the phase transition of the DPCB monolayer could not be determined at the present stage, we assumed that the tilt angle of CB headgroups in the monolayers is altered in the plateau region of the isotherms.

To clarify the effect of the headgroup combinations on lipid packing (shrinkage or expansion) in the monolayers, we acquired isotherms of the monolayers comprising different lipid combinations with parallel or antiparallel headgroup charges (Figure 2b). As discussed in our previous report,7 there was a marked reduction in area per molecule in the DPPC–DPSB...
Combination at the lower range of surface pressures ($\pi < \sim 30$ mN/m) compared to that in pure lipid systems of DPPC or DPSB (Figure 2b, green and blue plain lines and black plain line). At the higher surface pressure ($\pi > \sim 30$ mN/m), however, the isotherm of DPPC−DPSB resembled that of a pure lipid system. Considering this result, the intermolecular attraction between the antiparallel PC and SB headgroups seems to be effective only in the low surface pressure range. In the high surface pressure range, the intermolecular interaction between the headgroups was hidden for the DPPC−DPSB system. In a previous report, molecular dynamics simulations showed that SB moieties formed intermolecular interaction between them, whereas CB moieties did not.\(^{47}\) It can be assumed that the intermolecular interaction between the headgroups limits the mobility of the headgroups. Thus, the tilt angle of the SB headgroup in the monolayer may be less variable than that of the CB headgroup because of the stronger self-association between SB moieties. On the other hand, for the DPPC−DPCB combination, reduction of the area per molecule was observed over all ranges of surface pressure compared to the area per molecule values of the pure lipid systems, DPPC or DPCB (Figure 2b, magenta and blue plain lines and black dashed line). At the low surface pressure range ($\pi < \sim 40$ mN/m), the tilt angle of CB headgroups may be changed from parallel to perpendicular to the air/water interface. The isotherms were from two or more independent measurements. Data for the DPPC−DPSB combination reprinted with permission from ref 17. Copyright 2017 American Chemical Society.

Figure 2. Surface pressure−area ($\pi$−$A$) isotherms of lipid monolayers of DPPC in the presence of either DPCB (solid lines) or DPSB (dotted lines) with different molar fractions ($X_{ICZL}$) (a). Total lipid amount used in the monolayer experiments was 9 nmol (DPPC + ICZL). The arrows in (a) indicate plateau regions for the DPPC−DPCB combinations where the tilt angle of the DPCB headgroups should change from parallel to perpendicular to the air/water interface (as shown in (b)). Comparison of the $\pi$−$A$ isotherms of the equimolar mixtures composed of lipids whose headgroup charge arrangements were parallel or antiparallel (b). For all measurements of the isotherms, the subphase contained ultrapure water. The temperature of the subphase was 20 °C. The isotherms were from two or more independent measurements. Data for the DPPC−DPSB combination reprinted with permission from ref 17. Copyright 2017 American Chemical Society.
rearrangement of the CB headgroup orientation, which should contribute to further reduction of area per molecule of the DPPC–DPCB combination even at a high surface pressure region ($\pi > \sim 40$ mN/m). Headgroup mobility should be one of distinguished differences between DPCB and DPSB. Interestingly, the $\pi$–$A$ isotherm of the DPCB–DPSB system (Figure 2b, dotted line), which is a combination of lipids with parallel headgroup charges, was shifted to lower area slightly compared to the isotherms of the pure lipids (Figure 2b, green and magenta lines). This result indicates that the SB headgroups attractively interact with the CB headgroups slightly even though they have parallel charge orientation to each other. Such attractive interaction may occur only when these headgroups orient parallel to the air/water interface. This parallel orientation to the air/water interface is the cause of the relatively large molecular area of the DPCB–DPSB combination (Figure 2b, dotted line) than that of the antiparallel combinations (Figure 2b, black dashed line and black plain line). Comparing $\pi$–$A$ isotherms at $\pi < 40$ mN/m, the area per molecule of pure DPSB was smaller than that of pure DPCB (Figure 2b, green and magenta lines), which indicates that the intermolecular attraction occurred between SB headgroups.

According to previous literature data, multiple factors are related to zwitterionic associations. To explain intermolecular interactions between the lipids with zwitterionic headgroups, the following factors should be considered, in particular, (i) charge densities of cationic and anionic groups in the zwitterion, (ii) the carbon spacer length (CSL) between the cationic and anionic groups in the zwitterion, and (iii) the hydration state of the zwitterionic group. Moreover, these factors are related to each other. For instance, charge densities of the charged group are modulated by the CSL.48 The charge density is critical to the hydration of the zwitterions.9 The interaction between zwitterionic groups is inversely related to the degree of hydration of the zwitterion.35,41 To dictate the zwitterionic association, Shao et al. have proposed a concept in terms of matching degree of charge densities of zwitterions.47 They reported that the charge densities of the cationic and anionic groups in sulfobetaine (CSL = 3) and carboxybetaine (CSL = 2) were $+3.0$ and $-4.5$ e/nm$^3$ and $+3.0$ and $-5.3$ e/nm$^3$, respectively. In their report, the intermolecular self-association of these zwitterions is stronger as the difference in the absolute values of charge densities between the cationic and anionic groups becomes smaller. Thus, in this case, the intermolecular self-association of the sulfobetaines should occur more favorably than that of the carboxybietaines.

In addition to the concept of charge density, CSL should also be considered. It has been reported that the charge densities in the zwitterion decrease with the decreasing CSL.48 In particular, the charge density of the anionic group in the zwitterion is more affected by the CSL than that of the cationic group. Moreover, the large difference in the charge density is observed when CSL $\leq 3$. In the present study, CSL of the DPCB is shorter than that used in the literature.47 Therefore, zwitterionic groups in DPCB should be less charged. Thus, there is a possibility that matching charge densities between CB and PC headgroups might be higher than those between SB and PC headgroups (charge densities in cationic and anionic groups in PC were calculated to be $+3.0$ and $-3.0$ e/nm$^3$, respectively47). However, it should be noted that the charge density theory is not completely adequate to dictate zwitterionic association. For example, on the basis of the charge density theory, the intermolecular interaction between phosphocholines is stronger than that in sulfobetaines and carboxybietaines. The $\pi$–$A$ isotherm of pure DPPC in the present study, however, showed that this is not the case. The area per molecule of DPPC at low surface pressure ($\pi \leq 10$ mN/m) was larger than that of DPSB (Figure 2b, blue and green plain lines). This suggests that the intermolecular interaction between PC headgroups should be weaker than the estimated degree from the charge density theory. Zwitterionic association may be dominated by not only degree of the matching charge densities but also hydration state of the zwitterionic groups.

It is known that the hydration state of the zwitterion group is related to the intermolecular interaction between the zwitterions. The intermolecular interaction between zwitterions is weaker (stronger) with increasing (decreasing) hydration strength of the zwitterions.50,51 The hydration state tends to be changed depending on charge densities of zwitterionic groups.48 The number of water molecules in the coordination shell of the zwitterion increases with increasing charge densities of the cationic and anionic moieties. Simulation studies revealed that sulfonate and carboxylate anions of zwitterions (CSL = 2) have 7.08 and 5.94 water molecules in their coordination shells, respectively.52 Therefore, the number of coordinated water molecules of the sulfonate moiety in DPSB should be larger than 7.08 because of their higher charge density arising from the longer CSL compared to that in the literature.53 On the other hand, carbohydrate moieties of DPCB should have less water molecules because of its shorter CSL. It can be assumed that the less hydration water in the zwitterion in DPCB may contribute to a more compressed state observed in the monolayer when mixing with DPPC.

**Extrapolated Molecular Area of Lipids.** The extrapolated area ($A_L$) represents the mean cross-sectional area of a single lipid molecule that is free from the external pressure exerted by the surrounding lipids in the condensed lipid monolayer; it can be used as an indicator of the geometry of the lipid and for evaluating the intermolecular interactions between lipids in the mixed lipid monolayer. The $A_L$ values for the different lipid combinations are shown in Figure 3 (open circles for DPPC–DPSB and filled symbols for DPPC–DPCB). In both lipid combinations, negative deviation from the ideal additivity of a mixed system was observed at any $X_{ICZL}$. This indicates that attractive interaction dominated in both lipid combinations. In addition, the $A_L$ values of the DPPC–DPSB combinations for any molar fractions were higher than those of the DPPC–DPCB combinations. This indicates that the intermolecular interaction between DPPC and DPCB was more favorable than that between DPPC and DPSB.

In the mixed lipid monolayer comprising the DPPC–DPSB combination, the minimum value of $A_L$ was observed at $X_{DPSB} = 0.5$ (Figure 3, open circles). This indicates that a combination of lipids with antiparallel headgroup charges formed an equimolar complex (1:1 molar ratio) in the lipid layer. Although such equimolar complex formation seems to be a general feature in lipid combinations that have antiparallel headgroups, the $A_L$ data for the DPPC–DPCB combination negated the generality of equimolar complex formation. At $X_{DPPC} = 0.8$, the $A_L$ of the lipid monolayer comprising DPPC and DPCB exhibited a minimum value (41.5 ± 0.5 Å$^2$) (Figure 3, filled circle). This indicates that the mixed lipid monolayer was mostly shrunk at the molar composition of DPCB:DPPC = 4:1. At $X_{DPCB} = 0.2$ and 0.5, negative peaks in the $A_L$ were observed, indicating that the mixed monolayer was shrunk at...
these molar fractions as well. At these molar fractions, DPCB and DPPC form complexes with the molar composition of DPCB/DPPC = 1:4 and 1:1, which may be a metastable state because values of the excess free energy of mixing ($\Delta G^{\text{exc}}$) at $X_{\text{DPCB}} = 0.2$ and 0.5 were not minimum. Such complex formation at $X_{\text{DPCB}} = 0.2$ or 0.8 (not equimolar composition) indicates that complicated interaction being relevant to charge densities and/or hydration state of the zwitterionic groups should contribute to the interaction between DPPC and DPCB.

Because there were plateau regions in isotherms of the DPPC–DPCB combination at $X_{\text{DPCB}} = 0.7$–1, we could obtain two series of $A_t$ values corresponding to both sides of the plateau regions. The $A_t$ value of pure DPCB was $61.2 \pm 2.8 \text{ Å}^2$ at the lower surface pressure (Figure 3, triangle, $X_{\text{DPCB}} = 1$). This value was not surprising considering that the glycerolipid in the monolayer had a dipalmitoyl tailgroup. For example, the cross-sectional area of DPPC in the LE phase is $63.9$–$67.7 \text{ Å}^2$ at $25 \degree C$. As the higher surface pressure region, the $A_t$ value of pure DPCB was $44.8 \pm 1.0 \text{ Å}^2$ (Figure 3, filled circle, $X_{\text{DPCB}} = 1$). This value was slightly lower than that of DPPC in the LC phase ($44$–$47.9 \text{ Å}^2$ at $20 \degree C$). As discussed in the previous section, there is a possibility that DPCB undergoes phase transition at the plateau regions in the isotherms of DPCB. Changes in the CB headgroup orientation may involve the phase transition. If DPCB changes the headgroup orientation in response to lateral compression, the tilt angles of the headgroup at the lower $A_t$ should be more perpendicular than those at the higher $A_t$ values.

**Figure 3.** Extrapolated molecular area ($A_t$) of the lipid comprising the lipid monolayer. The open circles represent the $A_t$ values of the DPPC and DPSB system. The filled symbols represent the $A_t$ values of the DPPC and DPCB system. There were two series of $A_t$ values in the DPPC–DPCB system, which were determined at high (filled circles) and low (triangles) surface pressures. The $\pi–A$ isotherms for DPPC–DPSB mixtures do not have plateau regions (Figure 2a). This indicates that the tilt angle of DPSB does not change even though the lipid monolayer is laterally compressed. Thus, $A_t$ for the DPPC–DPSB mixtures was determined as a single value at each lipid composition. The dotted lines represent the ideal additivity for both systems. The $p^*$ values for all series of $A_t$ were $<0.01$ (one-way ANOVA, $n \geq 3$). Data for the DPPC–DPSB combination reprinted with permission from ref 17. Copyright 2017 American Chemical Society.

**Effect of Mixing DPCB on Compressibility Modulus of the Lipid Monolayer.** To investigate the extent of the spontaneous mixing of ICZLs and DPPC in the monolayer, we calculated the excess free energy of mixing ($\Delta G^{\text{exc}}$) at $X_{\text{DPCB}} = 0.2$ and 0.5. As discussed in the previous section, there is a possibility that DPCB undergoes phase transition at the plateau regions in the isotherms of DPCB. Changes in the CB headgroup orientation may involve the phase transition. If DPCB changes the headgroup orientation in response to lateral compression, the tilt angles of the headgroup at the lower $A_t$ should be more perpendicular than those at the higher $A_t$ values. Although we clearly observed a temporary uplift of $C_4^{-1}$ that corresponded to the phase transition from the LE phase to the LE–LC phase in pure DPPC, the uplift disappeared when mixing with even a small amount of ICZL. We observed $C_4^{-1}$ peaks at relatively small $A$ regions in the DPPC–DPCB combination (Figure 4a, filled symbols) compared with those in the DPPC–DPSB combination (Figure 4a, open symbols). The maximum compressibility modulus ($\Delta G^{\text{exc}}$) did not change at any $X_{\text{DPCB}}$ and remained in the ranges 280–350 and 200–240 mN/m in the high and low surface pressure regions, respectively (Figure 4b, filled symbols). The unexpected result indicates that mixing DPCB with DPPC did not have a strong influence on the maximum compressibility modulus of the monolayer, whereas mixing these lipids caused shrinkage of the monolayer. Even at $X_{\text{DPCB}} = 0.8$, when the lipids may form a complex, we did not observe any increment of $\Delta G^{\text{exc}}$. In contrast to that of the DPPC–DPCB combination, the $C_4^{-1}$ of the DPPC–DPSB combination increased slightly, although the difference was not significant.

To investigate the extent of the spontaneous mixing of ICZLs and DPPC in the monolayer, we calculated the excess free energy of mixing ($\Delta G^{\text{exc}}$) between DPCB and DPPC system. There were two series of $\Delta G^{\text{exc}}$ values ranging 220–240 and 280–350 mN/m (Figure 4b). These two series of $\Delta G^{\text{exc}}$ values correspond to low ($\sim 40 \text{ Å}^2$) and high ($\sim 50 \text{ Å}^2$) area per molecule, respectively (Figure 4a, indicated with arrows). According to a review of the DPPC monolayer, the phase state of the monolayer can be classified from the $C_4^{-1}$ value, that is, the LE phase occurs at $10$–$50 \text{ mN/m}$, the LC phase occurs at $100$–$250 \text{ mN/m}$, and the solid phase occurs at $>250 \text{ mN/m}$. According to such a classification, the observed plateau regions of the isotherms at $X_{\text{DPCB}} = 0.7$–1 (Figure 2a) may be attributed to the transition from the LC phase to the solid phase.
Effect of Mixing DPCB on the Gel-to-Liquid Crystal Phase Transition Temperature of the Lipid Dispersion.

The gel-to-liquid crystal phase transition temperature ($T_c$) reflects the favorability of the intermolecular interaction between the lipid molecules in the bilayer membrane. To minimize the effect of the curvature of the vesicles on the phase transition temperature, large vesicles were used for differential scanning calorimetry (DSC) measurements in the present study. Figure 6a shows the representative DSC thermograms and mean values of $T_c$ for the aqueous dispersion of vesicles comprising DPPC and DPCB. For the mixed lipid system, the $T_c$ significantly increased with increasing $X_{DPCB}$ until $X_{DPCB}$ was 0.6 (Figure 6b). In the range of $X_{DPCB} = 0.6−0.8$, the value of $T_c$ remained at the higher level. This suggests that the intermolecular interaction between lipids was greatest at $X_{DPCB} = 0.6−0.8$ in the lipid bilayer. This was consistent with the results for the lipid monolayer discussed in the previous sections. Moreover, at $X_{ICZL} = 0.5−0.9$, the values of $T_c$ for the DPPC–DPCB combination were significantly larger than those for the DPPC–DPSB combination. This indicates that the intermolecular interaction between PC and CB combinations is more favorable than that between PC and SB combinations at $X_{ICZL} = 0.5−0.9$.

The sharpness of the endothermic peaks reflects the mixing state of the lipid in the bilayer in the gel phase. At lower ICZL content ($X_{ICZL} = 0.1−0.3$), the endothermic peaks were broad (Figure 6a). This indicates that lipids comprising the bilayer exist in a poorly mixed state. With regard to the thermogram of pure DPSB, there were two endothermic peaks at 40.8 and 56.8 °C (Figure 6a, $X_{DPSB} = 1$). This indicates that pure DPSB forms lipid domains that have different lipid associations with each other, as we discussed in our previous report,17 whereas the thermogram for pure DPCB exhibited a single endothermic peak at 52.0 ± 0.1 °C. This indicates that DPCB existed in a
single state in the bilayer where the lipid molecules were uniformly mixed and interacted evenly with each other without domain formation.

Figure 6a,c illustrates a comparison of the DSC thermograms of the pure lipids with those of each equimolar lipid combination comprising parallel or antiparallel headgroup charges. With regard to the combinations of PC−CB and PC−SB, which had antiparallel headgroups, the $T_c$ values of the equimolar mixtures were higher than those of the pure lipids, which indicates enhancement of the intermolecular interaction.
between the lipids. With regard to the mixtures comprising lipids with parallel headgroup charges, the $T_c$ values were intermediate between DPSB and DPCB. This indicates that a combination of parallel headgroup charges does not enhance the intermolecular interaction of lipids.

**CONCLUSIONS**

In the present work, we revealed that binary lipid combinations with antiparallel headgroup charges can exert attractive intermolecular interactions in the lipid monolayer and bilayer. A lipid combination comprising 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC) and 1,2-dipalmitoyl-sn-glycero-3-carboxybetaine (DPCB) had more favorable intermolecular interaction than that comprising DPPC and 1,2-dipalmitoyl-sn-glycero-3-sulfobetaine (DPSB). This was supported by the excess free energy of the mixed monolayer and the gel-to-liquid crystal phase transition temperature of the mixed bilayer. Such attractive interaction in both lipid combinations results from the interaction between the headgroups (PC−CB and PC−SB), which are antiparallel to each other. In the highly compressed lipid monolayers and vesicles (lipid bilayer), the molar fractions of ICZLs, in which the intermolecular interaction reached a maximum, were 0.6−0.8 for the DPPC−DPCB combination and 0.5 (equimolar composition) for the DPPC−DPSB combination. Therefore, for the compressed monolayers and bilayers, the mechanism of intermolecular interaction between DPPC and DPCB is different from that between DPPC and DPSB. Different charge densities and/or hydration states between SB and CB headgroups should be attributed to different interactions observed in their binary mixture with DPPC. At a highly compressed state of the DPPC−DPCB combination, the changing orientation of the CB headgroup should contribute to further shrinkage of the monolayer.

**MATERIALS AND METHODS**

**Materials.** DPPC (>99% purity) was purchased from Yuka Sangyo (Tokyo, Japan) and used without further purification. DPCB and DPSB were synthesized according to previously published methods (refs 12 and 17 for DPCB and DPSB, respectively). The purity of these lipids was confirmed by thin layer chromatography (DPCB; $R_f = 0.64$, CHCl$_3$/MeOH = 9:1, visualized by iodine or bromocresol green, DPSB; $R_f = 0.60$, CHCl$_3$/MeOH = 8:2, visualized by iodine). To prepare lipid stock solutions, a given lipid was dried under reduced pressure at room temperature and then dissolved in organic solvent (CHCl$_3$ for the DPCB mixture, CHCl$_3$/MeOH = 8:2 for the DPSB mixture). Ultrapure water was obtained using the Milli-Q system (resistivity: 18.2 MΩ cm at 25 °C; Merck-Millipore).

**Acquisition of π−A Isotherms.** Surface pressure−area ($\pi−A$) isotherms were acquired using a trough equipped with a Wilhelmy plate (KSV NIMA Small; Biolin Scientific, Stockholm, Sweden). To prepare sample solutions, the lipid stock solution containing ICZL (DPSB or DPCB) was mixed with DPPC stock solution at a desired molar fraction of ICZL with respect to DPPC (total lipid concentration: 0.5 mM). To form monolayers at the air/water interface, 17 μL of the sample solution was spread on the subphase filled with ultrapure water using a microsyringe. After spreading the lipid, the monolayer was equilibrated on the subphase for 15 min at 20 °C. The isotherms were acquired following the following conditions: the barrier speed was 5 mm/min and the temperature of the subphase was maintained at 20 °C. The extrapolated area ($A_x$) represents the cross-sectional area of a single lipid molecule, which is free from external pressure from the surrounding lipids in the condensed lipid monolayer.\(^{17}\) The compressibility modulus ($C_{\pi-1}$) of the lipid monolayers was calculated using eq 1\(^{19}\)

$$C_{\pi-1} = -A(d\pi/dA) \tag{1}$$

where $\pi$ is the surface pressure of the lipid monolayer at the air/water interface and $A$ is the occupied molecular area per single molecule constituting the lipid monolayer. The miscibility of lipids in the mixed monolayer and the interactions between lipid components were analyzed quantitatively on the basis of the excess free energy of mixing ($\Delta G^{\text{mix}}$) calculated for the obtained π−A isotherms, as defined in eq 2

$$\Delta G^{\text{mix}} = N_\Lambda \int_0^\pi A_{12} - (A_1 X_1 + A_2 X_2) \, d\pi \tag{2}$$

where $A_{12}$ is the occupied molecular area for a particular composition of a mixed monolayer at a given surface pressure; $A_1$ and $A_2$ are the occupied molecular areas for pure monolayers of components 1 and 2 at the same surface pressure, respectively; $X_1$ and $X_2$ indicate the molar fractions of the components in the mixed monolayer; and $N_\Lambda$ is Avogadro’s number.\(^{20}\)

**Differential Scanning Calorimetry (DSC).** To determine the gel-to-liquid crystal phase transition temperature ($T_c$) of the aqueous dispersion composed of pure or mixed lipids, DSC was carried out using a DSC 1 calorimeter (Mettler Toledo, Switzerland). The lipid dispersions for DSC were prepared as follows. To prepare the sample for DSC measurements, the lipid stock solution containing ICZL (DPSB or DPCB) was mixed with DPPC stock solution at a desired molar fraction of ICZL with respect to DPPC. The sample solution was placed in a 10 mL round-bottomed flask. The solvent was then removed by rotary evaporation to form a thin lipid layer at the bottom. The obtained lipid film was hydrated in ultrapure water for 2 min at 80 °C with gentle agitation. The total lipid concentration in the dispersion was 20 mM. The hydrated lipid was agitated using a AZU-6D bath-type sonicator (As One, Osaka, Japan) for 1 min at 80 °C. The obtained lipid dispersion (40 μL) was added to a 100 μL aluminum pan and sealed tightly with a lid. The sample temperature was set at 2 °C/min during the heating and cooling processes. $T_c$ was defined as the onset temperature of the endothermic peaks.

**Statistical Analysis.** The $A_x$, $C_{\pi-1}$, $\Delta G^{\text{mix}}$, and $T_c$ as functions of the molar fraction of ICZL in DPPC ($X_{\text{ICZL}}$) are represented as mean ± standard deviation (SD). Statistical analysis of these data was carried out by one-way analysis of variance (ANOVA) using Excel 2016 software (Microsoft). Significant difference was defined as $p < 0.05$.

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**Notes**

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