Langmuir monolayers of cerebroside with different head groups originated from sea cucumber: Binary systems with dipalmitoylphosphatidylcholine (DPPC)

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A B S T R A C T

Surface properties (Langmuir monolayer) of two different cerebrosides which are extracted from the sea cucumber (Bohadschia argus) were investigated. A main difference in chemical structure of cerebroside between BAC–2a and BAC–4 is their head groups (glucose and galactose, respectively). Furthermore, miscibility and interaction between dipalmitoylphosphatidylcholine (DPPC) and cerebrosides (BAC–2a and BAC–4) in the monolayer have been systematically examined. The surface pressure (\(\pi\)) – area (A) surface potential (\(\Delta V\)) – A, and the dipole moment (\(\mu\)) – A isotherms for monolayers of DPPC, cerebrosides, and their binary combinations have been measured using the Wilhelmy method and the ionizing electrode method. BAC–4 forms a stable liquid-expanded (LE) monolayer, whereas BAC–2a has a first-order phase transition from the LE phase to the liquid-condensed (LC) state on 0.15 M NaCl at 298.2 K. The fundamental properties for each cerebroside monolayer were elucidated in terms of the surface dipole moment based on the three-layer model [R. J. Demchak, T. Fort Jr., J. Colloid Interface Sci. 46 (1974) 191–202] for both cerebrosides and the apparent molar quantity change (\(\Delta s^w\), \(\Delta h^w\), and \(\Delta u^w\)) for BAC–2a. In addition, their miscibility with DPPC was examined by the variation of the molecular areas and the surface potentials as a function of cerebroside mole fractions, the additivity rule. The miscibility was also confirmed by constructing the two-dimensional phase diagrams. The phase diagrams for the both binary systems were of negative azeotropic type. That is, the two-component DPPC/BAC–2a and DPPC/BAC–4 monolayers are miscible. Furthermore, the Joos equation for the analysis of the collapse pressure of binary monolayers allowed calculation of the interaction parameter and the interaction energy between the DPPC and cerebroside monolayers. The miscibility in the monolayer state was also confirmed by the morphological observation with Brewster angle microscopy (BAM), fluorescence microscopy (FM), and atomic force microscopy (AFM).

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1. Introduction

One of the most intriguing questions about cell membranes is why these supramolecular ensemble contain so many different types and species of lipids. After all, the chief purpose of membranes is to compartmentalize cells while serving as selective permeability barriers in the export and import of various cellular materials, and then only a handful of lipids should be necessary. Glycosphingolipids (GSLs) are the one of important components of membrane constituents. They are primarily located on the outer leaflet of the cellular membranes, especially animal cell plasma membranes, where they participate in the assembly of signaling molecules, modulation of cell adhesion, and cellular differentiation. Intracellular GSLs are also important for membrane protein trafficking.

Glycosphingolipids (GSLs), which consist of sugar chain and ceramide moiety, are thought to play a role in a number of cellular functions such as cell recognition \([1,2]\), cell differentiation \([3–5]\), signal transduction \([6,7]\), apoptosis \([8]\), and receptors for virus \([9]\). They may act to protect the membrane from harsh conditions like a low pH or degradative enzymes \([10]\). Recent cell biological studies show that GSLs in plasma membranes form cluster, so called a raft, with cholesterol and are relatively less in content of phospholipids than other parts of plasma membrane. GSLs can mediate the sig-
nal transduction pathway through interaction with these signaling proteins, circulate between the plasma membrane and intracellular organs, and also move laterally over the exoplasmic membrane. Such migration could be conducted by the raft [11,12]. A detailed description of the chemical, structural, and functional properties of GSLs has been summarized in a review article by Maggio [13].

GSLs are classified into gangliosides, globoseides, sulfatides, ceramides, dihexosides (lactosides), and cerebrosides based on constituent sugar chain types. Gangliosides are amphiphilic compounds containing sugar chains with one or more N-acetylneuraminic (sialic) acid residues and ceramide moieties. In addition, they are especially enriched in the brain and nervous tissues and constitute ~5–10% of the total lipid mass in nerve cells [14]. GSLs with N-acetylgalactosamine as the terminal sequence and tri- or tetrasaccharide containing head group, known as globoseides, are found in the erythrocyte membrane [15–17]. Sulfatides (sulfated derivatives of galactocerebrosides) is concerned with the HIV infection as a receptor [18]. Lactosylceramides are present in mature neutrophils as major components and serve as a receptor for bacteria and fungi [19–21]. Cerebrosides possess saccharide and ceramide moieties and are ubiquitous components of the plasma membrane of all eukaryotic cells [22,23]. Galactocerebrosides are major components of the myelin sheath [24–28]. Glucocerebrosides occur in all mammalian cells like sphingomyelins and are precursor of complex GSLs; for example, gangliosides, globoseides, and lactosylceramide [29]. GSLs show heterogeneity not only in their head group but also in their ceramide moieties. The biological significance of ceramide heterogeneity is not understood well, yet. However, the structure of fatty acid moieties in ceramides was found to influence the localization and function of GSLs in the plasma membrane, which is probably due to direct interaction with cholesterol, phospholipids, and the transmembrane domains of receptor proteins [30–33]. GSLs exist not only in the vertebrate but also in the mollusk [34,35], the plant [36–40], porifer [41–43], the echinoderm [44–50] and so on; for example, GSLs from the sea cucumbers [44,47–49].

The interfacial behavior of GSLs has been investigated by means of a Langmuir monolayer [51–58], bilayer [59], and liposome [60,61,62]. Langmuir monolayer is particularly used as a simplest model of biomembrane and can provide not only exact and detailed information on surface behavior but also interaction between film-forming materials. In addition, the phase behavior of monolayers at the air/water interface under lateral compression can be clarified morphologically as well as thermodynamically. The data obtained with optical techniques such as microscopy (BAM) [63–65], fluorescence microscopy (FM) [66–68], and atomic force microscopy [69–71] are commonly combined with the isothermal data in order to elucidate the monolayer behavior. Thus, the monolayer technique has been employed for studies on biological and biophysical functions at the interface, which are exerted by rafts [72], pulmonary surfactants [73], GSLs [57–60], and so on.

Cerebrosides are especially the simplest class of GSLs and are important surface-lying molecules found virtually in all cells. Galactocerebrosides and their metabolites have been shown to possess significant functions on promoting the regulation of nerve cell [74], on regulating protein kinase C activities [75], and on modulating the function of hormone receptors [76]. Glucocerebrosides have a relation with the precursor of gangliosides or the accumulated substrate of Gaucher disease. They have several activities; for example, anti-ulcerogenic activity [77], anti-tumor activity [35,78], and anti-microbial activity [36]. Therefore, it is expected that cerebrosides synthesized and/or extracted from natural products are utilized as a new medical resource.

In the previous studies, we have reported the surface behavior of glucocerebrosides (LMC-1, LMC-2, and LLC-2) [79–81] and gangliosides (DSG-1) [82]. The glucocerebrosides were completely miscible with dipalmitoylphosphatidylcholine (DPPC) within a monolayer state. However, the binary monolayers of the cerebrosides and dipalmitoylphosphatidylethanolamine (DPPE) or cholesterol (Ch) indicated immiscible or phase-separated behavior. The miscibility of GSLs with lipid components in biomembrane is considerably important for understanding the various functions in raft clusters. In order to know the detailed interactions of sphingolipids and their roles in the cell membrane, it is necessary to collect more information on their dependence on difference in the molecular structure; i.e. the number, location, and orientation of hydroxyl groups attached to acyl chains and so on. Also, it is expected that sphingolipids is utilized as a new medical resource from natural products.

The Langmuir monolayer properties of glycerocerebroside were reported from 1970s, whereas the molecular mechanisms underlying the cerebrosides from marine invertebrates have not been made clear yet. Here, we focus our attention on two different polar head groups of cerebrosides [glycerocerebroside (BAC-2a) and galactocerebroside (BAC-4)] isolated from sea cucumber (Bohadschia argus) [83]. BAC-2 and BAC-4 are molecular species, however, possessing almost same ceramide moiety. Comparing with BAC-2 and BAC-4, we are able to see whether the saccharide part is playing any difference sort of a role on the membrane surface.

Surface pressure (σ)—molecular area (A), surface potential (ΔΨ)—A, and surface dipole moment (μ⊥)–A isotherms were measured for DPPE, cerebrosides (BAC-2a and BAC-4), and their binary combinations. The mutual interaction of the two-component monolayers was analyzed in terms of additivity of molecular surface areas and surface potentials. An interaction parameter and an interaction energy between the two were evaluated from the Joos equation [84]. Furthermore, the phase behavior of the Langmuir monolayer states was confirmed by the morphological observation with BAM, FM, and AFM.

## 2. Experimental

### 2.1. Materials

The sea cucumber _B. argus_ (Janomenamako in Japanese) belongs to the kingdom Animalia, the phylum Echinodermata, the class Holothuroidea, and the family Holothuriidae. It was collected in the sea near Zaunamisaki in Okinawa (Japan) in 1999. The isolated cerebrosides (BAC-2a and BAC-4) from the sea cucumber were checked by 1H and 13C NMR, FAB-MS and GC–MS spectra after purification by column chromatography. The compositions of the hydrophobic acyl chain and long chain base (LCB) for BAC-2a and BAC-4 are given in Table 1. BAC-2a and BAC-4 are molecular species for hydrophobic parts. The chemical structures (Fig. 1) of BAC-2a and BAC-4 are 1-O-β-D-glucopyranosyl-β-ceramide and 1-O-β-D-galactopyranosyl-ceramide, respectively. That is, the structural difference between the two is mainly the species of their sugar moieties. More detail

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Acyl chain and LCB compositions of BAC-2a and BAC-4.</th>
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</thead>
<tbody>
<tr>
<td>Composition (%)</td>
<td>BAC-2a</td>
</tr>
<tr>
<td>Acyl chain</td>
<td>C14:0</td>
</tr>
<tr>
<td>C16:0</td>
<td>25.2</td>
</tr>
<tr>
<td>C18:0</td>
<td>29.7</td>
</tr>
<tr>
<td>C20:0</td>
<td>15.5</td>
</tr>
<tr>
<td>C22:0</td>
<td>38.8</td>
</tr>
<tr>
<td>LCB</td>
<td>C40:1</td>
</tr>
<tr>
<td>C42:1</td>
<td>59.5</td>
</tr>
<tr>
<td>C44:1</td>
<td>4.1</td>
</tr>
<tr>
<td>C46:1</td>
<td>12.0</td>
</tr>
</tbody>
</table>

a: C2: 2-hydroxyl fatty acid, C4: sphingosine type (1,3-dihydroxyl) long chain base.
information and structural determination for them were reported previously [82].
L-α-1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (DPPC; purity >99%) and 1-palmitoyl-2-[6-[[7-nitro-2-1,3-benzoxadiazol-4-yl]amino]hexanoyl]-sn-glycero-3-phosphocholine (NBD-PC; purity >99%) as a fluorescent probe were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). These lipids were used without further purification. n-Hexane (98.5%) and ethanol (99.5%) used as spreading solvents come from Cica-Merck (Uvasol, Tokyo, Japan) and nacalai tesque (Kyoto, Japan), respectively. Stock solutions of cerebrosides (0.5 mM) and DPPC (1.0 mM) were prepared with n-hexane/ethanol (7/3, v/v) mixture. The sodium chloride (nacalai tesque) was roasted at 1023 K for 24 h to remove any surface active organic impurity. The substrate solution was prepared using thrice distilled water (the surface tension = 71.96 mN m⁻¹) and the electrical resistivity = 18 MΩ cm.

2.2. Surface pressure (π)–area (A) isotherms

The surface pressure (π) of monolayers was measured using an automated homemade Wilhelmy film balance. The surface pressure balance (Mettler Toledo, AG-245) had a resolution of 0.01 mN m⁻¹. The surface pressure-measuring system was equipped with filter paper (Whatman 541, perimeter = 4.0 cm). The trough (effective area = 720 cm²) was made from Teflon-coated brass. The π–A isotherms were recorded mainly at 298.2 K and the electrical resistivity = 18 MΩ cm.

2.3. Surface potential (∆V) measurements

The surface potential (∆V) was simultaneously recorded with the surface pressure while the monolayer was compressed at the air/water interface. It was monitored by using an ionizing 241Am electrode at 1–2 mm above the interface while a reference electrode was dipped in the subphase. The electrometer (Keithley 614 and 6517) was used to measure the surface potential. The standard deviation for the surface potential was ∼5 mV.

2.4. Brewster angle microscopy (BAM)

The monolayer was directly visualized by a Brewster angle microscope (KSV Optrel BAM 300, KSV Instruments Ltd., Finland) coupled to a commercially available film balance systems (KSV Minitrough, KSV Instruments Ltd., Finland). The application of a 20 mW He–Ne laser emitting p-polarized light of 632.8 nm wavelength and a 10× objective lens allowed a lateral resolution of ∼2 μm. The angle of the incident beam to the air/water interface was fixed to the Brewster angle (53.1°) at 298.2 K. The reflected beam was recorded with a high grade charge coupled device (CCD) camera (EHDcamPro02, EHD Imaging GmbH, Germany), and then the BAM images were digitally saved to the computer hard disk.

2.5. Fluorescence microscopy (FM)

The film balance system (KSV Minitrough) was mounted on to the stage of the Olympus microscope BX51WI (Tokyo, Japan) equipped with 100 W mercury lamp (USH-1030L), an objective lens (SLMPlan50×, working distance = 15 mm), and a CCD camera with a camera control unit (IKTU51CU, Toshiba, Japan). The z-directional focus on the monolayer was exactly adjusted using an automation controller (MAC 5000, Ludl Electronic Products Ltd., NY, USA). A spreading solution of the samples was prepared as mixed solution doped with 1 mol% of a fluorescence probe (NBD-PC). The excitation (460 nm) and emission (534 nm) were selected by a mirror unit (U-MWIBA3); FM images were directly recorded with the hard disk via an online image processor (DVgate Plus, Sony Corp., Japan) connected to the microscope. Image processing and analysis were carried out by using the software, Scion Image Beta 4.02 for Windows (Scion Corp., Frederick, MD, USA). The total amount of ordered domains was evaluated and expressed as a percentage per frame by dividing the respective frame into dark and blight regions.

2.6. Atomic force microscopy (AFM)

Langmuir–Blodgett (LB) film preparations were carried out with a KSV Minitrough. Freshly cleaved mica (Okenshoji Co., Tokyo, Japan) was used as a supporting solid substrate for the film deposition. At selected surface pressures, a transfer velocity of 5 mm min⁻¹ was used for film-forming materials on a 0.15 M NaCl at 298.2 K. LB films with deposition rate of 1 were used in the experiments. AFM images were obtained using an SPA 400 instrument (Seiko Instruments Co., Chiba, Japan) at room temperature in a tapping mode, which provided both a topographical image and a phase contrast one. The tapping mode images (512 points per line) were collected with scan rates of 0.2–1.0 Hz. AFM images were obtained using an SPA 400 instrument (Seiko Instruments Co., Chiba, Japan) at room temperature in a tapping mode, which provided both a topographical image and a phase contrast one. The tapping mode images (512 points per line) were collected with scan rates of 0.2–1.0 Hz, using silicon tips (Olympus Co.) with nominal spring constant of 1.8 N m⁻¹ under the ambient conditions. The lateral and vertical resolutions were 0.2 and 0.1 nm, respectively. The transferred samples were checked for possible tri-induced deformation by zooming out after a region had been scanned.

3. Results and discussion

3.1. Surface behavior of pure DPPC and cerebrosides

3.1.1. Monolayer stability

The surface pressure (π)–time (t) isotherms for DPPC and cerebroside were measured on 0.15 M NaCl at 298.2 K to check the stability of these Langmuir monolayers. The monolayers were compressed up to 35 mN m⁻¹ and then the Teflon-barrier was stopped, after which relaxation of surface pressure was traced against time.
increase in surface pressure. Such phenomenon is often observed ∼almost identical. A DPPC monolayer has the transition pressure of the compositions of hydrophobic chains for both cerebrosides are BAC-4 one. The similarity at the close-packed state reveals that monolayer state. The extrapolated area of BAC-2a was they reached almost plateau values of 33 (BAC-2a) and 31 mN m−1 (BAC-4), respectively. It suggests that cerebrosides used here are less soluble in the subphase and are possible to be studied as an insoluble monolayer at the air/water interface.

3.1.2. π−A, ΔV−A and μ⊥−A isotherms

Fig. 3 shows the π−A isotherms of DPPC, BAC-2a, and BAC-4 monolayers spread on 0.15 M NaCl at 298.2 K. The π−A curve of BAC-2a showed a transition pressure (πeq) of 30 mN m−1, where the monolayer state changes from the liquid-expanded (LE) to the liquid-condensed (LC) phase. On further compression, the surface pressure increased to reach ∼43 mN m−1 (0.34 nm2) in the monolayer state. The extrapolated area of BAC-2a was ∼0.69 nm2. However, judging from the cross-sectional area of one saturated hydrocarbon chain (∼0.20 nm2), its area is too large. This is attributable to the larger cross-section of the unsaturated and branched acyl chains. As for BAC-4, on the other hand, it formed a typical LE monolayer up to 45 mN m−1 (0.37 nm2). Its extrapolated area is 0.72 nm2, which is almost the same value as the BAC-4 one. The similarity at the close-packed state reveals that the compositions of hydrophobic chains for both cerebrosides are almost identical. A DPPC monolayer has the transition pressure of ∼12 mN m−1 and collapses at ∼55 mN m−1, which was reported previously [79].

The surface potential (ΔV) is a measure of the electrostatic field gradient perpendicular to the surface and thus varies considerably with the molecular surface density. It generally reflects an orientational and conformational change of monolayers upon compression. The important feature of the surface potential is that it allows the Langmuir monolayer to be probed at much earlier stages of monolayer compression in comparison with π−A isotherms [85]. Such behavior is very clear in the case of DPPC. The behavior of ΔV−A isotherms for cerebrosides corresponds to the change of molecular orientation upon compression as shown in Fig. 3(b). The ΔV value of DPPC and cerebrosides is always positive. The ΔV−A isotherm of DPPC monolayer showed large variations with decreasing molecular area. As a result, the value reached ∼550 mV through the same transition states. On the contrary, the change in ΔV value for BAC-2a and BAC-4 is rather small in spite of the increase in surface pressure. Such phenomenon is often observed in the monolayers of molecular species like cerebrosides and gangliosides [79,81,82]. The maximum ΔV value of BAC-2a (285 mV) is larger than that of BAC-4 (250 mV) due to the formation of LC monolayer for BAC-2a.

The vertical component of surface dipole moment (μ⊥) was calculated from the measured ΔV values using the Helmholtz equation,

\[ \Delta V = \mu_{\perp} / e_0 \varepsilon A, \]

where ε0 is the permittivity of vacuum, ε is the mean permittivity of monolayer (which assumed to be 1), and A is the surface area occupied by the molecule. The ΔV values involve the resultant dipole moments accompanied by the subphase, the head group, the hydrophobic chain. Contrary to the ΔV behavior, the μ⊥ value of BAC-2a and BAC-4 monolayers steeply decreased from ∼550 (A = 1.0 nm2) to ∼200 mD (at closed-packed state) on compression (Fig. 3(c)). Analyses of the molecular behavior suggested that the μ⊥ value is more sensitive to molecular orientation and conformation than the ΔV value.

Fig. 3. The π−A (a), ΔV−A (b), and μ⊥−A (c) isotherms of pure DPPC, BAC-2a, and BAC-4 monolayers on 0.15 M NaCl at 298.2 K.
3.1.3. Surface dipole moments ($\mu_⊥$) of cerebrosides

We analyzed the surface potentials of the monolayers using the three-layer model proposed by Demchak and Fort [86]. This model postulates independent contribution of the subphase (layer 1), the polar head group (layer 2), and the hydrophobic chain (layer 3). Other models such as the Helmholtz model and the Vogel and Möbius model are also available [85(b)]. The estimation of polar head groups and hydrocarbon chains using the Demchak and Fort model assumes a condensed Langmuir monolayer, where its hydrophobic chains are closely packed [84,87]. Application of this model to the LE monolayer of BAC-4 may lead to a rough estimation. However, if this model is applied to the resultant value of the BAC-4 monolayer in the close-packed state, it is possible to induce a useful estimation, which can provide qualitative explanation of surface dipole moment behavior.

Thus we have substituted the experimental $\mu_⊥$ values of close-packed monolayers for the $\mu_⊥$ calc values of the three-layer model equation,

$$\mu_⊥\text{calc} = \frac{\mu_1}{\varepsilon_1} + \frac{\mu_2}{\varepsilon_2} + \frac{\mu_3}{\varepsilon_3},$$

where $\mu_1/\varepsilon_1$, $\mu_2/\varepsilon_2$, and $\mu_3/\varepsilon_3$ are the contribution of the subphase, the polar head group and the hydrophobic chain, respectively. The aim of the following analysis is to understand the difference in the contribution of the polar head group between BAC-2a and BAC-4 (glucose and galactose). First, the contribution of the hydrophobic chains needs to be determined.

The initial set of values proposed by Demchak and Fort ($\mu_1/\varepsilon_1 = 0.040$ D, $\mu_2/\varepsilon_2 = 7.6$ and $\mu_3/\varepsilon_3 = 5.3$) were determined for monolayers made from terphenyl derivatives and octadeyl nitrile on 4 M NaCl [86]. The other set was determined by Petrov et al. ($\mu_1/\varepsilon_1 = 0.025$ D, $\mu_2/\varepsilon_2 = 7.6$ and $\mu_3/\varepsilon_3 = 4.2$) for n-heptanol and 16-bromohexadecanol monolayer on 0.1 M phosphate buffer [88]. We have used a set of values introduced by Taylar and Olivia ($\mu_1/\varepsilon_1 = -0.065$ D, $\mu_2/\varepsilon_2 = 6.4$ and $\mu_3/\varepsilon_3 = 2.8$) for monolayers of ω-halogenated fatty acids and amines on water [89]. Furthermore, we have changed and utilized a combination of values ($\mu_1/\varepsilon_1 = 0.025$ D, $\mu_2/\varepsilon_2 = 7.6$ and $\mu_3/\varepsilon_3 = 2.8$) for BAC-2a and BAC-4 monolayers on 0.15 M NaCl due to the similarity of the subphase condition. The validity of the set was previously confirmed by applying to standard samples such as stearic acid and DPPC [79,81].

Here, we evaluate the contribution of the hydrophobic tail-end group for BAC-2a and BAC-4 monolayers. Although the hydrophobic moiety of BAC-2a is slightly different from that of the cerebrosides reported previously [74], the polar head group is the same as the previous cerebrosides. Therefore, we can evaluate the contribution of hydrophobic tail-end part using the value of 0.63 D for $\mu_2\text{calc}$ in Eq. (3).

$$\mu_⊥(\text{BAC-2a}) = \frac{\mu_1}{\varepsilon_1} + \frac{\mu_2^{\text{Glc}}}{\varepsilon_2} + \frac{\mu_3}{\varepsilon_3} = 0.26 \text{ D}$$

From the above equation, we obtained $\mu_⊥ = 0.43$ D.

Second, in order to evaluate the contribution of the hydrophilic group of BAC-4 (galactose), we assumed the contribution of hydrophobic tail-end group ($\mu_3 = 0.43$ D) to be the same for BAC-2a.

**Fig. 4.** AFM topographic images of LB films of BAC-2a (A) and BAC-4 (B) monolayers on 0.15 M NaCl at 258.2 K. The monolayers were transferred onto mica at 20, 30, and 40 mN m$^{-1}$. The scan area is $3 \times 3$ µm and the scale bar in the lower-right corner represents 1 µm. The cross-sectional profiles along the scanning line (white line) are drawn below each row. In (A) at 30 mN m$^{-1}$, white line circle means circular LC domain.
Table 2
Surface potential data for dipole moment evaluation.

<table>
<thead>
<tr>
<th></th>
<th>Area (nm²)</th>
<th>π (mN m⁻¹)</th>
<th>ΔV (mV)</th>
<th>μ₁ (D)</th>
<th>μ₂ (D)</th>
<th>μ₃ (D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAC-2a</td>
<td>0.360</td>
<td>40</td>
<td>273</td>
<td>0.26</td>
<td>0.63</td>
<td>0.43</td>
</tr>
<tr>
<td>BAC-4</td>
<td>0.399</td>
<td>40</td>
<td>249</td>
<td>0.26</td>
<td>0.65</td>
<td>0.43</td>
</tr>
</tbody>
</table>

and BAC-4 and assigned this value to Eq. (4).

\[
\mu_\perp(BAC-4) = \frac{\mu_1}{\varepsilon_1} + \frac{\mu_{Gal}}{\varepsilon_2} + \frac{\mu_3}{\varepsilon_3} = 0.26 \text{ D}
\] (4)

The contribution of the head group for BAC-4 is 0.65 D (Table 2). The \(\mu_2\) value of BAC-2a is almost the same as that of BAC-4. It is suggested therefore that the structural difference in head group between glucose and galactose does not contribute much to the surface dipole moment at the close-packed state.

3.1.4. Atomic force microscopy (AFM)

AFM has been performed for Langmuir–Blodgett (LB) film transferred onto mica to investigate the phase behavior of BAC-2a and BAC-4 on the nanometer level. The films transferred at 20, 30, and 40 mN m⁻¹ were examined in the tapping mode. In the case of BAC-2a monolayers (Fig. 4(A)), the AFM images at 20 mN m⁻¹ shows a coexistence of two different phases. Judging from the \(\pi–A\) isotherm as well as BAM and FM micrographs (latter sections) for BAC-2a, the higher regions with a height difference of ~1 nm from the surroundings are not LC domains but LE domains made of BAC-2a components with longer hydrophobic chains. The higher LE domains shrink in size by a surface pressure increase to 30 mN m⁻¹ which corresponds to the transition pressure to the LC domain. Nevertheless, there is no height difference between the higher LC domains and the circular LC domain. When the surface pressure increases further, the circular domains are finely fined by arrow (Fig. 5(b)). Shown in Fig. 6 are the plots of the curves have the first-order phase transitions, which are indicated by arrows (Fig. 5(b)). Shown in Fig. 6 are the plots of \(\pi^{eq}\) and \(\sigma_\beta\) against temperature. The transition pressures increased and the collapse pressure decreased with increasing temperature. The temperature-dependent behavior of \(\pi^{eq}\) and \(\sigma_\beta\) is very similar to that for standard samples such as myristic acid and DPPC [81,91].

The change of thermodynamic quantity on the phase transition of monolayers was calculated using the previous method [68].

Fig. 5. The temperature dependence of \(\pi–A(a)\) and \(\pi^{-1}–A(b)\) isotherms of BAC-2a monolayers on 0.15 M NaCl at typical temperatures.

which takes the contribution of the substrate to monolayers into account. The apparent molar entropy change (\(\Delta s^\alpha\)) of the phase transition was evaluated by following equation [92],

\[
\Delta s^\alpha(\alpha, \beta) = (a^\beta - a^\alpha) \left[ \frac{\partial \pi^{eq}}{\partial T} \right]_p - \left[ \frac{\partial \pi^{eq}}{\partial T} \right]_p
\] (5)

where \(\Delta s^\alpha\) is an apparent molar entropy change, \(a^\alpha\) and \(a^\beta\) are molecular area (in square nanometers, the superscript \(\alpha\) and \(\beta\) refer to phase states), \(\pi^{eq}\), the transition pressure from the \(a\) phase to the \(\beta\) phase, and the \(\gamma^\beta\) the surface tension of the substrate. \(a^\alpha\) and \(a^\beta\) are estimated as follows. \(a^\alpha\) is the area at the point where the

Fig. 6. Change of the transition pressures (\(\pi^{eq}\)) and collapse pressures (\(\pi^{-1}\)) for BAC-2a monolayers on 0.15 M NaCl as a function of temperature.
Table 3

<table>
<thead>
<tr>
<th></th>
<th>Δs° (J K⁻¹ mol⁻¹)</th>
<th>Δh° (kJ mol⁻¹)</th>
<th>Δu° (kJ mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAC-2a</td>
<td>−11</td>
<td>−3.1</td>
<td>−4.1</td>
</tr>
<tr>
<td>LLC-2-15</td>
<td>−70</td>
<td>−21</td>
<td>−22</td>
</tr>
</tbody>
</table>

* LLC-2-15: A pure cerebroside isolated from starfish Linckia laevigata [71].

The film starts to transform from the α to the β state. The a° value is determined following manner; when the point (a°, π°i) is moved parallel to the area axis to zero area, it comes into contact with the elongated line of the π–A isotherms of the solid state to the lower surface pressure. The intersection point gives the a° value. The inclinations of π°i and γ° against temperature are ∂π°i/∂T = 0.27 and ∂γ°/∂T = −0.16 mN m⁻¹ K⁻¹ (the value is quoted from the refs. [93–95]). Furthermore, the apparent molar enthalpy change (Δh°) and energy change (Δu°) of the phase transition are evaluated by using Eqs. (6) and (7), respectively [92].

\[
\Delta h°(\alpha, \beta) = T\Delta s°(\alpha, \beta) \quad (6)
\]

\[
\Delta u°(\alpha, \beta) = -(\pi°i - \gamma°)(a° - a°) + T\Delta s°(\alpha, \beta) \quad (7)
\]

The apparent molar quantity changes (Δs°, Δh°, and Δu°) of the phase transition for BAC-2a monolayers at 298.2 K on 0.15 M NaCl are shown in Table 3. The apparent molar enthalpy shows a negative value. Therefore, the transition from the LE phase to the LC one is exothermic. As for the apparent molar entropy, the value is also negative as expected. The apparent molar changes for BAC-2a are considerably smaller compared with those for a single cerebroside such as LLC-2-15 [81]. It means that the molecular species in hydrophobic chains exert the terminal resistance for the phase transition.
3.2. Two-component monolayers of DPPC and cerebrosides

3.2.1. Compression isotherms

The two-component monolayers composed of DPPC and cerebrosides (BAC-2a and BAC-4) have been studied in order to clarify the interaction and the miscibility between them in the monolayer state. The \( \pi - A \), \( \Delta V - A \), and \( \mu_{\perp} - A \) isotherms of DPPC/BAC-2a and DPPC/BAC-4 systems were systematically measured on 0.15 M NaCl at 298.2 K. For the \( \pi - A \) isotherms of DPPC/BAC-2a (Fig. 7(A)), all of the curves existed between those of the respective pure components. The transition pressure increases and becomes unclear with increasing \( X_{BAC-2a} \). Likewise, the \( \Delta V - A \) and \( \mu_{\perp} - A \) isotherms shift in between pure components with \( X_{BAC-2a} \). As for the DPPC/BAC-4 system, the similar isothermal variation and behavior are observed in Fig. 7(B), too. These results suggest that DPPC is miscible with both BAC-2a and BAC-4 in the monolayer state from the change in \( \pi^{eq} \) and \( \pi^{c} \) against the mole fraction \( X_{cerebrosides} \).

The interaction between DPPC and cerebrosides was investigated by examining whether the variation of the mean molecular areas and surface potentials as function of \( X_{cerebrosides} \) satisfied the additivity rule [96,97]. A comparison between the experimental mean molecular areas and the molecular areas based on ideal mixing at four surface pressures (5, 15, 25, and 35 mN m\(^{-1}\)) are shown in Fig. 8. For the DPPC/BAC-2a system (Fig. 8(A)), the \( A - X_{BAC-2a} \) curves show a positive deviation from the additivity line at all surface pressures except for 5 mN m\(^{-1}\), indicating repulsive interactions between DPPC and BAC-2a. At 5 mN m\(^{-1}\), the area values agree with ideal ones in \( 0 \leq X_{BAC-2a} \leq 0.5 \) and positively deviate from the ideal line in \( 0.5 \leq X_{BAC-2a} \leq 1 \). These positive deviations result from the steric hindrance of BAC-2a hydrophobic chains. In the case of the DPPC/BAC-4 system, the similar behavior (positive deviations) is also observed in Fig. 8(B).

Analysis of the surface potentials of the binary monolayers based on additivity rule is also performed in Fig. 9. Both of the DPPC/BAC-2a and DPPC/BAC-4 monolayers indicate a good agreement with ideal line at 5 mN m\(^{-1}\) and negative deviations at 15, 25, and 35 mN m\(^{-1}\). The negative deviations can be attributed to less ordered orientation induced by the steric hindrance of hydrophobic chains in cerebrosides.

3.2.2. Brewster angle microscopy (BAM)

FM measurements need an adequate amount of probes in the monolayer. There is a possibility that the dye molecule affects the original monolayer behavior, though the additional amount of FM probe is quit small (1 mol%). Thus, the phase behavior of the binary monolayers has been examined by BAM, which provided us an in situ direct monolayer image without FM probes. The representative BAM images are shown in Fig. 10. For the BAM images of DPPC monolayers (Fig. 10(a)), the image at 10 mN m\(^{-1}\) shows the homogeneous LE phase (dark contrast). On further compression, the LC domain (bright contrast) appears at 12 mN m\(^{-1}\), where the monolayer transfers from the LE phase to the LC one (data not shown). With an increase in surface pressure from 12 to 20 mN m\(^{-1}\), the LC domains grow in size. Beyond 20 mN m\(^{-1}\), the BAM image becomes homogeneous bright (data not shown).
The phase variation for DPPC monolayers is the same as reported previously [98].

When a small amount of BAC-2a ($X_{\text{BAC-2a}} = 0.1$) is added to DPPC monolayers (Fig. 10(b)), the LC domains become smaller in size and their shape also changes. Likewise, the addition of the equal amount ($X_{\text{BAC-4}} = 0.1$) exerts the similar phase variation (Fig. 10(c)). The variations of the domain shape and the transition pressure induced by the blend of cerebrosides support the miscibility of the binary systems within a monolayer.

3.2.3. Fluorescence microscopy (FM)

FM has higher resolution and magnification than BAM. Thus, the more detail phase behavior can be understood using FM. The FM contrast is generated due to the difference in solubility of FM probes between LE and LC phases. Shown in Fig. 11(A) are FM images for the DPPC/BAC-2a system. It is noticed that shape of DPPC LC domains observed in the FM images (Fig. 11) is the same as that observed in the BAM images (Fig. 10(a)). That is, the 1 mol% FM probes have no influence on the original phase behavior. For pure DPPC, the LC domains grow up in size on compression as similarly seen in the BAM images (Fig. 10(a)). The growth of the domains is also supported by the increase in percentage of them. The FM images for DPPC monolayers agree with the reported data [71].

For the DPPC/BAC-2a system (Fig. 11(A)), as the amount of BAC-2a in monolayer increases, the transition pressure increases and the LC domains become smaller in size. Correspondingly, the ratio of the LC domains reduces. As mentioned above, BAC-2a monolayers indicate the LE/LC transition at 30 mN m$^{-1}$. As for the FM images of pure BAC-2a (right column), the images at 15 and 20 mN m$^{-1}$ are homogeneously bright, indicating the LE phase. On further compression beyond the transition pressure, quite small LC domains appear in the FM images at 30 mN m$^{-1}$ (see inset).

In the case of the DPPC/BAC-4 systems (Fig. 11(B)), the LC domains become smaller with increasing $X_{\text{BAC-4}}$ similarly to the DPPC/BAC-2a system. The both systems being compared, the ratio of LC domains of the latter for $X = 0.1$ and 0.3 is smaller by ∼10%.
A mixed monolayer formed by DPPC, BAC-2a, and BAC-4 species, whereas "Balk" denotes a solid phase of DPPC, BAC-2a, and BAC-4.

DPPC/BAC-2a (A) and DPPC/BAC-4 (B). The dashed lines were calculated according to Eq. (8) for given composition of \( \Delta S^1 \), \( \Delta h^0 \), and \( \Delta u^0 \) of the phase transition for BAC-2a monolayers at 298.2 K were evaluated. It suggested that molecular species adjusting the interaction parameter in the above equation coincides with the experimental values. It is worth noting that the both systems exhibit a positive interaction parameter, although the other DPPC/cerebrosides systems reported previously result in a negative interaction parameter [79,81]. The new finders here are that the DPPC/BAC-2a system shows \( \xi = 0.25 \) and the DPPC/BAC-4 system indicated \( \xi = 0.90 \). The positive interaction parameter means that an interaction energy between different molecules is smaller in magnitude than the mean energy of interaction. The interaction energies \( (−ΔS^2 = −Δh^0/kT) \) were calculated to be \(-103 \text{ J mol}^{-1} \) (for DPPC/BAC-2a) and \(-372 \text{ J mol}^{-1} \) (for DPPC/BAC-4). As a result, the DPPC/BAC-2a and DPPC/BAC-4 systems are likely to be of negative azeotropic type.

The values of interaction parameters and interaction energies for DPPC/BAC-2a and DPPC/BAC-4 are completely opposite in sign to those of the other GSLs [79,81,82]. The steric hindrance of hydrophobic chains (cis-type unsaturation and branch) in cerebrosides investigated in the present study may lead to such the interactions with DPPC.

4. Conclusions

The two different cerebrosides (BAC-2a and BAC-4) isolated from sea cucumber can form a stable monolayer on 0.15 M NaCl at 298.2 K. BAC-4 forms a typical LE monolayer, whereas BAC-2a can form a stable monolayer on 0.15 M NaCl at 298.2 K. BAC-4 forms a typical LE monolayer, whereas BAC-2a from sea cucumber can form a stable monolayer on 0.15 M NaCl at 298.2 K.

3.2.4 Two-dimensional phase diagrams

Two-dimensional phase diagrams were constructed plotting the transition pressures and collapse pressures as a function of mole fraction of BAC-2a and BAC-4 in Fig. 12(A) and (B), respectively. The transition pressures for the binary systems change almost linearly with the mole fractions. Judging from the continuous change of the transition pressure, two components are miscible at all mole fractions. This behavior is an evidence of the miscibility of the two components in the monolayer state.

Assuming that the binary monolayers behave as a regular surface mixture with a hexagonal lattice, the coexistence phase boundary between the ordered monolayer phase (2D phase) and the bulk phase (3D phase) can be theoretically simulated by the Joos equation [84].

\[
1 = x_1^2 \exp \left( (\sigma_{m1} - \sigma_{m2})_1 \phi_1 / kT \right) \exp \left\{ \xi \left( \gamma_1^2 - \gamma_2^2 \right) \right\} \\
+ x_2^2 \exp \left( (\sigma_{m2} - \sigma_{m1})_2 \phi_2 / kT \right) \exp \left\{ \xi \left( \gamma_2^2 - \gamma_1^2 \right) \right\} 
\]

(8)

where \( x_1 \) and \( x_2 \) denote the mole fraction in the two-component monolayer of the components 1 and 2, respectively, and \( \sigma_{m1} \) and \( \sigma_{m2} \) are the corresponding collapse pressures of components 1 and 2. \( \phi_1 \) and \( \phi_2 \) are the corresponding limiting molecular surface areas at the collapse point. \( \gamma^1 \) and \( \gamma^2 \) are the surface activity coefficients at the collapse point. \( \xi \) is the interaction parameter, and \( kT \) is the product of the Boltzmann constant and the Kelvin temperature. The solid curve obtained by

at 40 mN m\(^{-1}\) than that for the former. It suggests that BAC-4 fluidized the LC domains to more extent at high surface pressures. This is supported by the fact that pure BAC-4 forms only a LE monolayer (right column). These results, the increase of \( \pi^0 \) and the variation of the domain shape against the mole fractions, provide an evidence of the miscibility of the both binary systems in the monolayer state.

Fig. 12. Phase diagrams from the variation of the transition pressure \( (\pi^0) \) and collapse pressure \( (\pi^c) \) on 0.15 M NaCl at 298.2 K as a function of cerebrosides mole fractions: DPPC/BAC-2a (A) and DPPC/BAC-4 (B). The dashed lines were calculated according to Eq. (8) for \( \xi = 0 \). The solid symbols represent experimental values. "Monolayer" indicates a mixed monolayer formed by DPPC, BAC-2a, and BAC-4 species, whereas "Bulk" denotes a solid phase of DPPC, BAC-2a, and BAC-4.
behavior was investigated to clarify the mutual miscibility and interaction. In terms of isothermal aspects, the miscibility for the binary systems was supported from the changes in transition pressure and collapse pressure of compression isotherms. In addition, the miscibility was morphologically confirmed also from the variations in domain shape and transition state with BAM and FM. The two-dimensional phase diagrams and the Joos equation yielded the interaction parameter ($\Delta\varepsilon$) and the interaction energy ($-\Delta\varepsilon$) between DPPC and cerebrosides. The negative azotropic types of two-dimensional phase diagrams were obtained, where an interaction energy between different molecules is smaller than the mean energy of interaction.

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References
