Anomerism is a common feature of nearly all glycoconjugates. However, the impact of anomerism on their functional diversities has been rarely investigated. In our opinion, glycoconjugates are expected to dope into liposomes or vesicles for adjusting property or function. In this paper, we investigated comparatively the interfacial behaviour of a phospholipid separately doped with a pair of anomeric (α or β) galactolipid by using the Langmuir-Blodgett technique. The β-galactolipid was found to be more erective than the α-counterpart at the air-water interface. The molecular interaction of the α-galacto-monolayer might be stronger than the β-counterpart. Results showed an unprecedented observation that anomerism can largely impact the properties of glycolipid embedded phospholipid monolayers. The intermolecular interaction and phase state of the mixed membranes had significant influence on their interfacial behaviours. This study offers a useful insight into the more sophisticated development of these functional glyco-materials.

**Key word:** Interfacial behaviour, biomembrane, glycolipid, Langmuir-Blodgett technique
glycolipids show distinct monolayer properties by using the Langmuir-Blodgett (LB) technique He et al. (2011) and Rigopoulou et al. (2012). The information of their molecular behaviours in LB monolayer should be a valuable reference for discussing those of their self-assembly vesicles or particles in solutions. The membrane properties of these self-assembly would be related directly to the cellular reactions.

In this paper, a pair of anomic galactolipids (α-, β-galactolipid) (Figure 1, left) that can be recognized by the asialoglycoprotein receptors (ASGP-Rs) expressed by hepatocytes and hepatomas are selected. Anomerism is a common identity of nearly all glycoconjugates. Despite the increasing interest in the use of glycolipids in a number of biochemical applications, investigations as regards the impact of anomerism on the functional diversity of glycolipids have been elusive. In our opinion, glycoconjugates are expected to dope into various drug carriers, such as liposomes or vesicles, for adjusting their property or function. Here, the number of alkyl group of the galactolipid is designed to be 16 similar to that of phospholipid. The monolayer properties of a phospholipid separately doped with the pair of anomic (α- or β-) galactolipids are investigated comparatively (Figure 1, right).

MATERIALS AND METHODS

Materials:

1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE) was purchased from lipoid (Germany). The anomic α- (1) or β- (2) galactolipids were synthesized according to a previous study (Song et al., 2012). Their molecular structures are shown in Figure 1 (left).

**π-A isotherm measurement**

DPPE, galactolipids and DPPE doped with the galactolipids with different molecular fractions (10-90%) were dissolved in chloroform to obtain a solution with a whole concentration of 5 × 10⁻⁴ mol/L. Then, 100 μL of the lipid solution was spread on pure MilliQ water surface using a microsyringe and equilibrated for 10 min to allow the solvent to evaporate completely. The π-A isotherms were obtained at 25 ± 0.1 °C by a model 612D computer-controlled Langmuir film balance (Nima Technology, Coventry, UK). The subphase temperature was maintained by water circulated from a thermostat. Measurements were run 4 times for each sample to ensure the overall reproducibility.

Their excess Gibbs free energy of mixing (ΔGex) was determined by the following equation: Haro et al.(2005)

$$\Delta G_{\text{ex}} = \int_{0}^{\pi} A_{12} \, d\pi - \int_{0}^{\pi} X_{1} A_{1} \, d\pi - \int_{0}^{\pi} X_{2} A_{2} \, d\pi \tag{1}$$

where $A_{12}$ is the experimental area per molecule of the mixed monolayer at a given surface pressure, $X_{1}$ and $X_{2}$ are the molar fractions of the two components, $A_{1}$ and $A_{2}$ are the areas per molecule at that surface pressure for pure monolayer.

**Preparation of LB monolayer**

The LB monolayers were transferred onto mica under a
Figure 2: (a) π-A isotherm of pure DPPE, 1 and 2. (b) Cartoon describing the plausible interfacial morphology of 1 and 2.

Table 1. The values of AL and πC of the pure galactolipid 1, 2, DPPE and their mixtures

<table>
<thead>
<tr>
<th>Compound</th>
<th>$A_L$ ($\text{Å}^2$)</th>
<th>$\pi_C$ (mN/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 α-galactolipid</td>
<td>134</td>
<td>40</td>
</tr>
<tr>
<td>2 β-galactolipid</td>
<td>88</td>
<td>37</td>
</tr>
<tr>
<td>DPPE</td>
<td>90</td>
<td>48</td>
</tr>
<tr>
<td>DPPE·1</td>
<td>82</td>
<td>52</td>
</tr>
<tr>
<td>DPPE·2</td>
<td>70</td>
<td>59</td>
</tr>
</tbody>
</table>

*a: DPPE doped with 20% (molar fraction) of galactolipid.

constant surface pressure of 32 mN/m. A freshly cleaved mica was immersed in the subphase and then the lipid solution was spread onto water surface. The experiment was performed by pulling the mica up vertically from the Langmuir trough and then the samples were dried in desiccators overnight.

The morphology of LB monolayer

The morphology of LB monolayer was observed by using atomic force microscopy AFM method (NT-MDT Co., Moscow, Russia) with a tapping mode in the air under room temperature. Five to six spots were selected from the entire mica surface to observe the morphology of membranes and one of which representatively shown for each group.

RESULTS AND DISCUSSION

α- (1) and β-galactolipid (2) with a 16-carbon lipid chain (Figure 1) had been prepared by a glycosylation reaction in a previous study He et al. (2011). DPPE, also tailed with 16-carbon lipid chain, was used to be doped separately with the anomic galactolipids. The π-A isotherms of galactolipids, DPPE with or without the galactolipids were obtained in various doping ratios (galactolipid molar ratio: 0-100%) using the LB technique to evaluate their interfacial properties at the air-water interface.

π-A isotherm of pure lipid

The π-A isotherm curves of pure DPPE and anemic galactolipids were obtained and shown in Figure 2. Their values of liftoff area ($A_L$, the molecular occupation area where the isotherm rises just from the baseline) are listed in Table 1 with collapse pressure ($\pi_C$).

In order to explore the influence of anomers, the π-A isotherm of β-galactolipid (2) was also obtained to compare with that of α-galactolipid (1). It is easy to find even if they had the same length of carbon chain, their molecular
covering areas are totally different. And, β-galactolipid (2) has lower values of $A_L$ and $\pi_c$, which might just be caused by the configurational difference at the anomeric carbon; the β-galactolipids might be more erective at the interface as shown in Figure 2 (right). This difference in interfacial behaviour of these galactolipids might give significant influence on the properties and biofunctions of the membrane.

Figure 2 also shows the π-A isotherm of pure DPPE which is steeper than those of the pure galactolipids, suggesting that the compressibility of the former is lower than the latter. The $\pi_c$ value of pure DPPE (about 48 mN/m) is much larger than those of galactolipids, showing a significant difference in compressive strength. Interestingly, while doped with the galactolipids, the interfacial properties of DPPE monolayer have unconceivable change. Hereinafter, how the anomerism of galactolipid effect the interfacial behaviour of the mixed monolayer will be explored.

π-A isotherm of DPPE doped with galactolipid

The π-A isotherm of DPPE doped by galactolipid in various doping ratios were obtained and shown in Figure 3. Although the π-A isotherms of pure lipids are quite different, those of DPPE-1 in all ratios (10-90%) are found to be almost overlapping under the most pressures, indicating a tendency to form membranes in similar molecular density. In the meanwhile, their $A_L$ values are not between the values of the two pure lipids, but near to that of DPPE. However, the π-A isotherms of DPPE-2 changes gradually with the increase of galactolipid ratio. The $A_L$ values of DPPE-2 decreases from 88 to 70 Å$^2$.

The $\pi_c$ values of the two pure galactolipids are very low comparing with that of pure DPPE, showing a slightly unstable monolayer. When 1 mixed with DPPE, the $\pi_c$ value increases from 40 to 52mN/m. And the same phenomenon could be found in galactolipid 2. Results suggest the capacity of resistance external pressure of both isomer monolayers have been enhanced after being doped with DPPE. Among them, the $\pi_c$ values of the mixture with doping ratio of 20% are the highest and exceeded to that of pure DPPE (48mN/m), showing a most stable monolayer structure. Their detail data were also listed in Table 1.

Effect of isomers on π-A isotherm

In the future, galactolipids are going to be doped with phospholipids to prepare hybrid liposomes for targeting drug carriers. A content of galacolipids more than 20% might destroy the liposomal stability. Then, in the following, we selected the mixed monolayers with galactolipid doping ratio of 20% to discuss the isomeric effect. Figure 4 (a) shows the π-A isotherms of the DPPE monolayers mixed with isomer. The $A_L$ values of 1-doped (82 Å$^2$) and 2-doped (70 Å$^2$) are smaller than that of DPPE (90 Å$^2$), whereas the $\pi_c$ values of which (52 mN/m for 1-doped and 59 mN/m for 2-doped DPPE monolayer as shown in Table 1) are larger than those of DPPE alone (48, 40 and 39 mN/m for DPPE, 1 and 2 respectively). These data suggest that doping of the galactolipid with DPPE could increase the molecular density and compressive strength of the monolayer. Under elevated pressures (above 52 mN/m), however, 2 (β)-doped DPPE monolayer became more stable than 1 (α), which probably implies the overall better tolerance of the former against external pressures.
Figure 4: (a) \(\pi-A\) isotherm of DPPE and DPPE doped with 20\% (molar fraction) of 1 and 2. (b) The compressibility curves of monolayer formed by DPPE, DPPE doped with 20\% of 1 and 2.

Figure 5: (a) Plotting the averaged area per molecule as a function of the molar fraction of 1 and 2 embedded into DPPE at a water subphase with a surface pressure of 32 mM\( m^{-1}\) (the straight line represents the ideal molecular area). (b) Plotting the excess Gibbs energy of mixing as a function of the molar fraction of galactolipid.

As the lateral pressure of biomembranes has been estimated to be around 32 mN/m, Calvez et al. (2009), Oguchi et al. (2010) the data under this pressure are worthy of attention. Under this pressure, both pure anomeric molecules occupied less area than the pure DPPE (Figure 2) at the air-water interface, and the monolayer of pure 2 has a minimum value. However, the molecular occupation area of both anomer-doped DPPE was almost identical beyond expectation (Figure 4a). The two mixed monolayers have similar molecular arranging density under this surface pressure seems to suggest the different molecular interaction between anomeric glycolipids and phospholipid.

The \(\pi-\kappa\) curves of the monolayers were obtained by plotting various surface pressures as a function of compressibility (\(\kappa\)) calculated according to a previous study. He et al. (2011) From the \(\kappa\) values of 1- and 2-doped DPPE shown in Figure 5(a), we determined that both were in liquid-condensed phase (0.01 > \(\kappa\) > 0.005), Miñones et al. (2009), Sarkar et al. (2010) similar to that of pure DPPE. The slightly higher \(\kappa\) value shown by 1-doped DPPE monolayer suggests its higher compressibility than the
other two in the whole pressure range. 2-doped DPPE monolayer shows a character closing to that of a solid membrane, which might contribute to its higher stability against external pressure.

As the excess molecular area ($A_{ex}$) is often used to quantitatively analyze the miscibility and molecular interaction of mixed monolayer, the $A_{ex}$ values under a surface pressure of 32 mN/m were calculated by comparing the average area per molecule of the mixed monolayers with that of an ideal mixed monolayer (deviated from the ideal straight line, Figure 5a). When the two components are immiscible or form an ideal mixed monolayer, $A_{ex}$ will be zero (akin to an ideal mixed monolayer) (Chou and Chang, 2000, Li et al., 2008). As shown in Figure 5(a), 1-doped DPPE monolayer shows negative deviations for all compositions, while 2-doped DPPE monolayer shows an inversion at a 0.6 molar fraction of glycolipid. These results suggest that the anomers adopted different intermolecular behaviours with DPPE at the interface. The $A_{ex}$ values of 1- and 2-DPPE at molar fractions of glycolipid ranging from 0 to 0.4 are negative, meaning that both mixed monolayers are miscible and adopt non-ideal mixing behaviour. The incorporation of glycolipid into DPPE monolayer may increase the interaction between molecules in the monolayer. Figure 5(a) also shows the $A_{ex}$ values of the 1-doped DPPE monolayer are smaller than that of 2-doped at a 0.1 molar fraction of galactolipid, but became larger than that of 1-doped monolayer with the molar fraction range of 0.2-0.3. These results suggest that the intermolecular interactions and the molecular packing are affected significantly by the molar fraction of galactolipids.

To better understand the thermodynamic properties of the mixed monolayers, their excess Gibbs free energy of mixing ($\Delta G_{ex}$) was determined and shown in Figure 5(b). The $\Delta G_{ex}$ value is zero for an ideal mixed monolayer. As shown in Figure 5b, $\Delta G_{ex}$ values of both monolayers deviate from zero. The $\Delta G_{ex}$ values of 2 (β)-doped DPPE monolayer possess two minima at 0.2 and 0.4 molar fractions of glycolipid, while those for 1 (α)-doped DPPE monolayer appear at molar fractions of 0.3 and 0.8. These results suggest that a phase separation might take place between the two minima according to the thermodynamic principle. Focusing on the results of the mixed monolayers with 20% galactolipid-doped, we infer that the molecular interaction of both galactolipids increased after embedding into the DPPE monolayer. The interaction within the 2 (β)-doped monolayer seems to be weaker than the 1 (α)-doped. This may result in a phase separation within 20% β-galactolipid monolayer and then increase its compressive strength.

Atomic force microscope (AFM) was then used to scrutinize the interfacial behaviour of the monolayers by transferring DPPE with 20% of isomerous galactolipids onto mica surface. The AFM images obtained show that, under the same lateral pressure (32 mN/m), the monolayers of DPPE (Figure 6a) and 1-doped DPPE (Figure 6b) are relatively flat, but that of 2-doped DPPE displays extruded areas (Figure 6c, indicated by arrows). From these results, the β-galactolipid-doped mixed monolayer seems to show a phase separation. We attempt to obtain the phase images, however, we did not observe any separation pattern in the images which suggested that the two parts with different heights probably possessed similar properties. Since the LB monolayer investigation suggested that the galactolipids and DPPE are miscible, therefore, we sought to reason the height difference by defining the monolayer as a partially miscible system. As a result, the higher (extruded area) and lower parts of the monolayer could probably be assigned to the galactolipid-rich and DPPE-rich domains, respectively.

Results from the LB technique collectively suggest that: 1) the β-galacto-monolayer might be more erective than the α-counterpart under a pressure of biomembranes; 2) DPPE monolayers doped with the anomeric galactolipids show increased molecular density and stability; 3) the molecular interaction of the β-galacto-monolayer might be stronger than the α-counterpart. Differences in membrane property induced by isomers should attract attention. Membrane composed of phospholipid and/or galactolipid is an appropriate model of biomembrane, including cytomembrane, karyotheca and organelle, etc. The kind of lipid and their molecular interfacial behaviour act an important role in many physiological processes. The relationship between LB membrane property and their
membrane property/biofunction in solution should be researched further.

CONCLUSION

To conclude, using Langmuir-Blodgett technique we determined that β-galacolipids might be more erective at air-water interface than the α-counterpart. The molecular arranging density and the stability of these pure lipids are different. When doped into DPPE monolayer separately, not like 2 (β)-doped mixture, the 1 (α)-doped ones show similar interfacial property regardless of doping ratio. The intermolecular interaction of DPPE monolayer doped with the β-galacolipids is weaker than the α-counterpart, leading to a more solid-like state and stable monolayer. The small configurational difference of anomeric glycolipids impacts the micro-structure and properties of the LB membrane. To find out the relationship between molecular interfacial behavior and biofunction should be an important task for understanding the physiological processes, such as endocytosis, membrane fusion. The study offers a useful insight into the more sophisticated development of these functional glyco-materials.

ACKNOWLEDGMENTS

This work is supported by the 973 Project (2013CB733700), the National Natural Science Foundation of China (21202045, 21276074, 91334203), the Key Project of Shanghai Science and Technology Commission (21202045, 21276074, 91334203), the Key Project (2013CB733700), the National Natural Science Foundation of China (13NM1400900) and the Fundamental Research Funds for the Central Universities.

REFERENCES


Neef AB, Schultz C (2009). Selective fluorescence labeling of...


