



9-17-2013

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Frey, Shelli L. and Ka Yee C. Lee. "Number of Sialic Acid Residues in Ganglioside Headgroup Affects Interactions with Neighboring Lipids." *Biophysical Journal* 105.6 (2013): 1421-1431.

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Abstract

Monolayers of binary mixtures of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and asialo-(GA₁), disialo-(GD_{1b}) and trisialo-(GT_{1b}) gangliosides were used to determine the effect of ganglioside headgroup charge and geometry on its interactions with the neighboring zwitterionic lipid. Surface pressure versus molecular area isotherm measurements along with concurrent fluorescence microscopy of the monolayers at the air-water interface were complemented with atomic force microscopy imaging of monolayers deposited on solid substrates. Results were used to further develop a proposed geometric packing model that the complementary geometry of DPPC and monosialoganglioside GM₁ headgroups affects their close molecular packing, inducing condensation of the layer at small mol % of ganglioside. For GA₁, GD_{1b}, and GT_{1b}, a similar condensing effect, followed by a fluidizing effect is seen that varies with glycosphingolipid concentration, but results do not directly follow from geometric arguments because less DPPC is needed to condense ganglioside molecules with larger cross-sectional areas. The variations in critical packing mole ratios can be explained by global effects of headgroup charge and resultant dipole moments within the monolayer. Atomic force microscopy micrographs further support the model of ganglioside-induced DPPC condensation with condensed domains composed of a striped phase of condensed DPPC and DPPC/ganglioside geometrically packed complexes at low concentrations.

Disciplines

Chemistry

Number of Sialic Acid Residues in Ganglioside Headgroup Affects Interactions with Neighboring Lipids

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ABSTRACT Monolayers of binary mixtures of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and asialo-(GA₁), disialo-(GD_{1b}) and trisialo-(GT_{1b}) gangliosides were used to determine the effect of ganglioside headgroup charge and geometry on its interactions with the neighboring zwitterionic lipid. Surface pressure versus molecular area isotherm measurements along with concurrent fluorescence microscopy of the monolayers at the air-water interface were complemented with atomic force microscopy imaging of monolayers deposited on solid substrates. Results were used to further develop a proposed geometric packing model that the complementary geometry of DPPC and monosialoganglioside GM₁ headgroups affects their close molecular packing, inducing condensation of the layer at small mol % of ganglioside. For GA₁, GD_{1b}, and GT_{1b}, a similar condensing effect, followed by a fluidizing effect is seen that varies with glycosphingolipid concentration, but results do not directly follow from geometric arguments because less DPPC is needed to condense ganglioside molecules with larger cross-sectional areas. The variations in critical packing mole ratios can be explained by global effects of headgroup charge and resultant dipole moments within the monolayer. Atomic force microscopy micrographs further support the model of ganglioside-induced DPPC condensation with condensed domains composed of a striped phase of condensed DPPC and DPPC/ganglioside geometrically packed complexes at low concentrations.

INTRODUCTION

Glycolipids, or lipid molecules containing sugar groups, are a large and heterogeneous family of amphipathic molecules present in the extracellular leaflet, which contribute to the complexity of the glycocalyx and are thought to regulate various physiological events at the cell surface. Cells interact via their outer surfaces with the glycolipids presenting a dense region of sugar polymers that have the potential for a high degree of structural diversity. The most complex form of glycosphingolipids are gangliosides, which are characterized by a variable number of negatively charged sialic acid residues attached to hydrophilic sugar moieties. Gangliosides have been shown to play roles in a number of cellular functions, including cell recognition and adhesion (1–3), signal transduction (3), and cell growth regulation (4,5). Although a minor component in most cells, they constitute up to 10% of the total lipid mass in nerve cells (6). Moreover, because they reside primarily on the outer leaflet, the external surfaces of certain cells contain 10–20 mol % gangliosides. The chemical and structural properties of gangliosides and other glycosphingolipids have been reviewed extensively (7,8).

Despite the abundance of glycolipids in the cell, little is known about their lateral structural organization and their influence on neighboring lipids in the outer leaflet of the biological membrane. The raft hypothesis proposes that naturally occurring lipids such as sphingolipids, glycosphingolipids, and cholesterol specifically aggregate in the plane

of the membrane, driven primarily by saturated hydrocarbon chains of the sphingolipids, which allow cholesterol to be tightly intercalated (9). Although the presence and biological role of lipid rafts is still under debate, these ordered regions are postulated to play important roles in protein lateral transport and signal transduction (9,10). Due to their small dimensions and likely dynamic nature, it is challenging to directly assess them in living cells. This establishes a need to determine how individual lipid components physically and chemically interact with and affect each other to understand the principles behind the structure and dynamics of cell membranes.

The most prevalent ganglioside, monosialoganglioside GM₁, is a surfactant molecule with a bulky headgroup composed of four sugar groups and one sialic acid that preferentially resides in caveolae and lipid rafts. When pure GM₁ monolayers are formed at the air-water interface at 30°C, surface pressure versus molecular area isotherms, fluorescence microscopy (FM), and grazing incidence x-ray diffraction indicate that GM₁ is completely fluid at all compressions and surface pressures (8,11). In binary mixtures with dipalmitoylphosphatidylcholine (DPPC), GM₁ condenses the monolayer at low concentrations (<25 mol %), whereas further addition (>25 mol %) causes the monolayer to become more fluid. This has been attributed to the different geometry of the two molecules where the wedge-shaped GM₁ aligns at the interface and forms an open pocket into which the complementarily shaped DPPC molecule can reside, forcing the tail regions of the two molecules to align and condense (11). The driving force for this condensation effect is likely intermolecular

Submitted April 19, 2013, and accepted for publication July 19, 2013.

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Editor: Andreas Engel.

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0006-3495/13/09/1421/11 \$2.00



<http://dx.doi.org/10.1016/j.bpj.2013.07.051>

hydrogen bonding between ganglioside sugar groups and, in the more general case of ganglioside-phospholipid condensation, has also been attributed to alignment of the dipole moment of DPPC with the negatively charged sialic acid residues, which can counteract the electrostatic repulsion (8,12).

Within model membrane systems, conclusions about the lateral organization of various gangliosides and interactions with neighboring phospholipids have been inconsistent, dependent on techniques employed, gangliosides under study, and environmental conditions; this makes meaningful comparisons difficult (13–17). The main motivation of this work is to further understand the influence of gangliosides on the surrounding lipids, and determine if the characterization previously presented for the binary DPPC/GM₁ system (11) can be generalized for gangliosides of different structures. The gangliosides investigated (GA₁, GD_{1b}, and GT_{1b}) have the same ceramide backbone region and display headgroups with zero, two, or three sialic acid residues in branched chains (Fig. 1).

Comparing the effect of different headgroup structures is useful from a biological as well as a more physical viewpoint. In vivo, each ganglioside can be de novo synthesized (or recycled) by the stepwise addition of sugar units to a ceramide backbone structure, catalyzed by membrane-bound glycosyltransferases in the endoplasmic reticulum and the Golgi complex (8,18,19). Different glycosphingolipids localize to the external plasma membrane of a variety of cell types and serve diverse roles ranging from modulators of transmembrane signal transducers that result in cell proliferation regulation to mediators for cell-cell recogni-

tion (20). Therefore, understanding how the structure of each ganglioside dictates organization will illuminate the structure-function relationship. The availability of various structures allows one to systematically modify the ratio of sialic acid residues to sugar groups and consequently characterize how geometry and varying degrees of hydrogen bonding and electrostatic repulsion affect different physical interactions with the lipid molecules. Lipid monolayers were used to model the outer leaflet of a cellular bilayer membrane, which enables us to use a full range of ganglioside concentrations and amplify any weak ganglioside-lipid interactions. Although mixtures with >20 mol % ganglioside will not be found within a cell membrane, the higher amounts used in our study allow us to test and establish a molecular model that is applicable to systems at lower, more realistic concentrations.

We report the behavior of binary DPPC-ganglioside mixtures of various mole ratios by means of surface pressure versus molecular area isotherms and concurrent FM measurements at the air-water interface. Each monolayer mixture was subsequently deposited from the air-water interface onto a solid support, and atomic force microscopy (AFM) imaging was performed to study submicron resolution morphology and phase separation. Ganglioside molecules with different headgroup geometries due to an increasing number of sialic acids show the same general behavior previously reported for GM₁ (an increase in ganglioside concentration causes condensation followed by fluidization (11)), but there are subtle differences, which indicate the condensation effect is more complicated and therefore includes more variables than the sterically hindered headgroup geometry of the ganglioside requiring a specific number of complementary shaped DPPC molecules to pack tightly. Ganglioside and phospholipid organization can also be attributed to electrostatic interactions between dipole moments within their headgroups and the tendency of gangliosides to have cooperative interactions qualitatively attributed to hydrogen bond formation between sugar residues of adjacent molecules.

MATERIALS AND METHODS

Lipids and subphase

1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) was obtained in powder form from Avanti Polar Lipids (Alabaster, AL) and used without further purification. Asialoganglioside GA₁ prepared as previously described (21) was donated by Tadeusz Pacuszk, whereas disialoganglioside GD_{1b} and trisialoganglioside GT_{1b} (Fig. 1) were purchased from Sigma-Aldrich (St. Louis, MO) in powder form. The fluorescent probe used for visualization with FM was Texas Red labeled 1,2-dihexadecanoyl-*sn*-glycerol-3-phosphoethanolamine (TR-DHPE) (Molecular Probes, Eugene, OR). Stock solutions of DPPC were made in chloroform, GA₁ in 9:1 chloroform/methanol, and GD_{1b} and GT_{1b} in 5:5 chloroform/methanol (HPLC grade, Fisher Scientific, Pittsburg, PA). Monolayer spreading solutions were prepared by dissolving in either chloroform (e.g., DPPC) or chloroform containing 10% methanol (HPLC grade, Fisher Scientific)

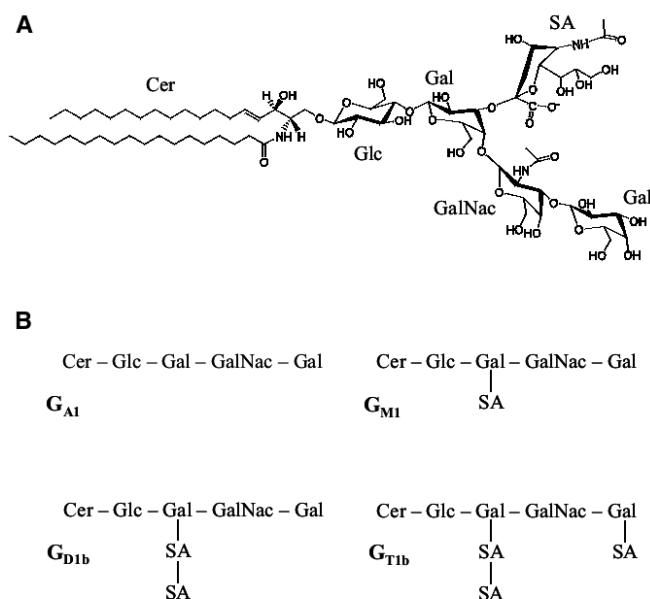


FIGURE 1 (A) Ganglioside GM₁ with ceramide hydrophobic backbone, four sugar groups, and sialic acid. Each pertinent residue is labeled. (B) Skeletal structures of GA₁, GM₁, GD_{1b}, and GT_{1b}.

at a concentration of 0.2 mg/ml and adding 0.5 mol % of TR-DHPE. Lipid solutions were stored at -20°C in glass vials. For all Langmuir trough experiments, the subphase was ultrapure water (resistivity $\geq 18\text{ M}\Omega\text{cm}$) processed by a Milli-Q ultrapurification system (A-10 gradient, Millipore, Bedford, MA).

Instrument setup

Details of the Langmuir trough setup have been discussed previously (11) and are included in the [Supporting Material](#) along with protocols for monolayer lateral compression experiments and subsequent AFM performed on monolayers transferred to solid supports from the air/water interface.

RESULTS AND ANALYSIS

Absence of sialic acid, GA_1

Isotherms

Surface pressure versus molecular area isotherms were measured for DPPC, GA_1 , and binary DPPC/ GA_1 monolayers at the air-water interface at 30°C while concurrently imaging with FM. [Fig. 2](#) shows the overlay of the resulting isotherms. The pure DPPC isotherm is in agreement with published data (22,23) and the phase transitions have been discussed at length elsewhere (24,25). DPPC goes through the expected gas/liquid expanded (LE) coexistence to lift-off in the LE phase at $95\text{ \AA}^2/\text{molecule}$, and then a coexistence plateau where condensed (C) domains form at $\sim 20\text{ mN/m}$, followed by a rapid rise in pressure until the collapse of the monolayer at $\sim 70\text{ mN/m}$. These phase changes can be correlated with concurrently obtained FM images.

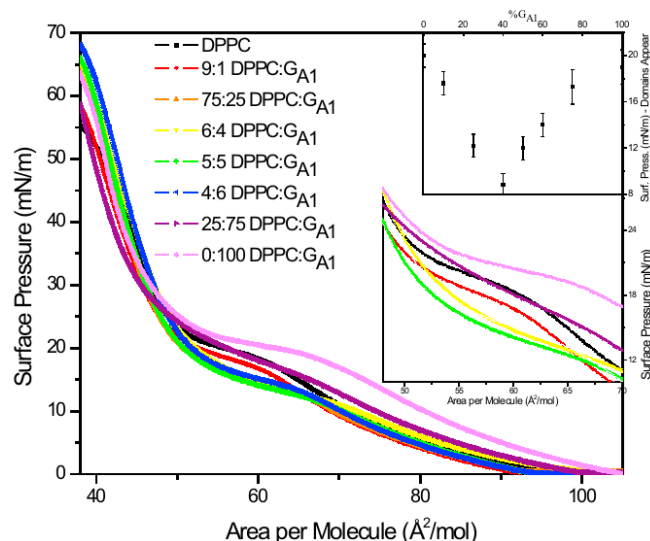


FIGURE 2 Monolayer compression isotherms of pure DPPC, pure GA_1 , and binary mixtures of 9:1, 75:25, 6:4, 5:5, 4:6, and 25:75 mol % DPPC/ GA_1 at 30°C . (*lower inset*) Plateau region of isotherm for subset of mixtures (*upper inset*) Surface pressure at which condensed domains appear in monolayers composed of DPPC and GA_1 , plotted as a function of GA_1 concentration. Domain formation was visualized using FM with TR-DHPE probe partitioning into the more fluid phase.

The glycosphingolipid GA_1 is structurally identical to GM_1 , except for deletion of the sialic acid residue, resulting in a headgroup composed of four sugar groups and rendering it charge neutral and smaller in diameter ([Fig. 1](#)). The isotherm for a monolayer of pure GA_1 ([Fig. 2](#)) has a lift-off area of $\sim 105\text{ \AA}^2/\text{mol}$, which shows that the oligosaccharide chain extending into the subphase has only a slightly large cross-sectional area compared to DPPC, in agreement with published data (26,27). A broad plateau region begins at $\sim 18\text{ mN/m}$ and C domains visualized by FM appear in the vicinity of 20 mN/m . The morphology of the GA_1 C domains is different from that typically seen with phospholipids and is characterized by long, thin hair-like structures that grow in length until forming a mesh of C phase at $\sim 23\text{ mN/m}$; the interlocking mesh morphology is shown in [Fig. S2 D](#) in the [Supporting Material](#). This is in contrast to work by Rosetti et al. (27) that showed fluorescence images of a GA_1 monolayer doped with fluorescently labeled NBD-PE on a 145 mM NaCl subphase at room temperature to remain homogenous (reportedly in the LE state) throughout compression, but this is likely due to their use of a $20\times$ objective that would not be capable of resolving the mesh morphology. At the end of the plateau, the GA_1 monolayer has low compressibility as indicated by a steep increase in surface pressure until collapse at $\sim 70\text{ mN/m}$ and a similar area per molecule ($35\text{--}40\text{ \AA}^2$) to DPPC in agreement with earlier studies (27). This contrasts with GM_1 , which is a fluid monolayer until collapse at a surface pressure of $\sim 63\text{ mN/m}$ and a molecular area of 55 \AA^2 (11). These differences can be attributed to the electrostatic repulsion of the negatively charged GM_1 headgroups and the larger cross-sectional area due to the branching in the structure; the smaller cross-sectional area of the GA_1 headgroup allows alignment of the hydrocarbon tails resulting in the visualized condensation.

In the case of binary mixtures of the individual components, one can see that at 10 mol % GA_1 , the isotherm shifts slightly to the left of pure DPPC at lift-off, suggesting a condensing effect. For the intermediate ratios, 75:25, 6:4, 5:5, and 4:6, the isotherms are nearly superimposable, with a lift-off at an area per molecule similar to that of pure DPPC, whereas the 25:75 DPPC/ GA_1 isotherm shifts toward that of the pure GA_1 monolayer ([Fig. 2](#) and *lower inset*). Because the isotherms of the pure components are not drastically different, changes in average area per molecule for the binary mixtures to determine the ideality of mixing are difficult to quantitatively compare. A more accurate means to assess the intermolecular packing is the correlation of surface pressure at which C domains form to the DPPC/ GA_1 ratio ([Fig. 2](#), *upper inset*). There is a strong dependence on the ratio of components, but the minimum pressure, or most condensed mixture, is at $\sim 6:4$ (or $3:2$) DPPC/ GA_1 . It should be noted that the point where further addition of GA_1 leads to expansion of the monolayer is at a higher concentration than that found for GM_1 (11). This

may follow from a simple geometric argument that for a glycosphingolipid with a small cross-sectional area, more of it can be incorporated into the DPPC film before a fully condensed state of the film is reached. Although a pure monolayer of GA₁ forms C domains upon compression, addition of DPPC significantly lowers the surface pressure of this phase transition; the monolayer becomes more condensed, up to a 3:2 DPPC/GA₁ ratio, as the binary mixture of molecules pack into a more favorable geometry.

FM

Fluorescence images of binary mixtures of DPPC/GA₁ at 20 mN/m are shown in Fig. S2. A surface pressure of 20 mN/m was chosen to highlight the differences between DPPC, which forms condensed domains at ~18–20 mNm, and the DPPC/ganglioside mixture, which typically form condensed domains at a lower pressure; by 30 mN/m at which lipid packing reaches a physiologically relevant level, domain growth and coarsening masks these differences. At low concentrations of GA₁, 75:25 and 6:4 DPPC/GA₁, C domains, similar in morphology to that of DPPC (Fig. S1) (11) but varying in size, form at low pressures; upon further compression, the interstitial region between domains becomes gray as seen with FM (Fig. S2, A and B). This intermediate gray phase is due to a roughening at the boundary between LE and C phases as a result of the formation of narrow protrusions beyond the resolution of optical microscopy from the domain boundaries, arising from an edge instability caused by a lowering of the line tension or potentially, differing elastic properties of the two phases (28). The core condensed region from which the protrusions arise is still visible up to high surface pressures. This contrasts with the 5:5 DPPC/GA₁ monolayer at 20 mN/m (Fig. S2 C), which is similar in morphology to that of GA₁ with a homogenous C-phase mesh at the interface (Fig. S2 D).

AFM

To gain insight about the molecular organization, AFM was performed on deposited monolayers of pure DPPC and pure GA₁ as well as binary mixtures of the two at varying mole ratios. Deposition was performed at 30 mN/m, chosen as it is the approximate bilayer equivalent pressure (29), from a water subphase via an inverse Langmuir-Schaefer technique (see the Supporting Material) onto an atomically flat, hydrophilic mica substrate. At 30 mN/m, the majority of the binary DPPC/ganglioside monolayers are condensed enough to deposit onto a solid support without artifacts due to liquid flow; morphology of each monolayer was monitored to ensure that the fidelity of the film was preserved during the deposition process. Each deposited monolayer sample was imaged using AFM contact mode in air after ensuring the engagement force did not affect the morphology.

A deposited monolayer of pure DPPC (Fig. 3 A) is composed of an intricate pattern of C domain stripes interspersed with the LE phase, which reflects the roughening at the LE/C boundary visualized with FM. The ~0.8 nm height difference between the two phases is consistent with that reported in the literature (30). The AFM micrograph of pure GA₁ (see Fig. 3 D) shows a morphology of 100 nm thick long hair-like structures that are tangled together and 1 nm greater in height than the surrounding deposited layer. This mesh of C phase GA₁ extends across the deposited layer. Based on the relatively cylindrical structure of GA₁, it is plausible that the tallest material is condensed GA₁ such that the hydrocarbon tails and sugar groups are extended normal to the interface, whereas the surrounding, lower material is more fluid or LE GA₁.

The addition of a small amount of ganglioside to a DPPC monolayer to obtain a 75:25 DPPC/GA₁ mixture (Fig. 3 B) does not have a large effect on the micron-scale morphology as compared to pure phospholipid. This concentration of GA₁ corresponds to a point left of the minimum on the plot of surface pressure when C domains form (*inset* of Fig. 2), meaning that the addition of GA₁ would further condense the layer. In agreement with the FM image shown in Fig. S2 A, there are small C domains, ~5 μ m in diameter, as indicated by material of a single height surrounded by a pattern of C domain stripes reminiscent of the pure DPPC film in height and width, but with a higher degree of curvature. The existence of a central core region of the C domain that does not result in a LE/C stripe coexistence seen for DPPC provides further evidence of a binary mixture

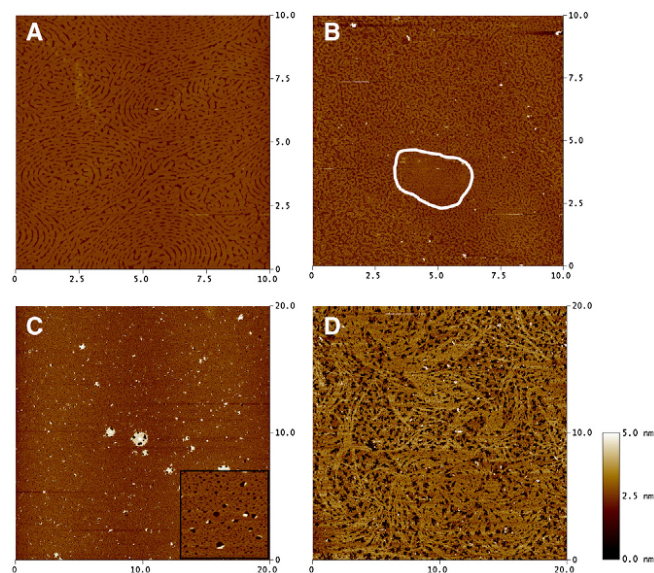


FIGURE 3 AFM topographic images of (A) DPPC; (B) 75:25 DPPC/GA₁; (C) 3:7 DPPC/GA₁, and (D) GA₁ monolayers transferred at 30 mN/m (*z* scale 5 nm). The outline in (B) surrounds the core of the condensed domain and is included for clarity. The inset in (C) is a 1 μ m \times 1 μ m region of 3:7 DPPC/GA₁ to show the pores in the surface. Scan size in μ m is indicated on each image.

monolayer that is more tightly packed than the pure lipid. Based on domain morphology and heights, the 75:25 DPPC/GA₁ monolayer most resembles DPPC with no evidence of GA₁-rich regions or clusters taller than the surrounding layer. This is in contrast to the DPPC/GM₁ mixed monolayer case at a low mol % of GM₁, where there was a striped phase in the C domain due to a thicker GM₁-rich region (11).

At a high concentration of GA₁, 3:7 DPPC/GA₁, the monolayer contains round depressions (Fig. 3 C, see inset for higher resolution) that are 1.9 nm lower than the surrounding background (height cross section in Fig. S3). There are also scattered circular raised portions with structures 4–7 nm taller than the background. Because these heights are too tall to correspond to a monolayer, they are attributed to multilayer structures extruded from the monolayer either during compression or the deposition process.

GA₁ is a glycosphingolipid that is structurally unique compared to other gangliosides investigated in this study because its hydrophilic headgroup does not contain any negatively charged sialic acid residues. As seen from isotherm measurements, there is a driving force for this bulky molecule in pure monolayers to form condensed domains, which can be attributed to the hydrogen bonding between the hydrated sugar groups and a headgroup with a cross-sectional area that is more commensurate with the area occupied by the hydrophobic tail region, allowing the molecules to pack well together during compression. Though pure GA₁ monolayers form C domains, the addition of DPPC to the monolayer further condenses the layer, due to packing of 3 DPPC molecules and 2 GA₁ molecules, as illustrated by the minimum defined surface pressure when domains develop (Fig. 2, inset). The AFM micrographs show no evidence for sequestration of GA₁ in taller clusters within the C phase or inducement of a LE phase, which is seen in the DPPC/GM₁ binary systems. This suggests that the absence of the charged sialic acid electrostatic repulsions and subsequent alteration of the headgroup geometry are responsible for these phenomena.

Two sialic acids, GD_{1b}

Isotherms

Disialoganglioside GD_{1b} is a derivative of monosialoganglioside GM₁ on the biosynthetic pathway and varies structurally by the addition of a sialic acid residue on the same galactosyl residue as the existing negatively charged group (18) (Fig. 1). This increases the negative charge to -2 and also increases the effective cross-sectional area of the headgroup region. A monolayer of pure GD_{1b} has a lift-off area per molecule of 140–150 Å² (Fig. 4) similar to that reported earlier (31,32). Though there is an inflection in the isotherm at ~30 mN/m, FM images indicate a pure GD_{1b} monolayer remains fluid until collapse (data not shown). This is similar

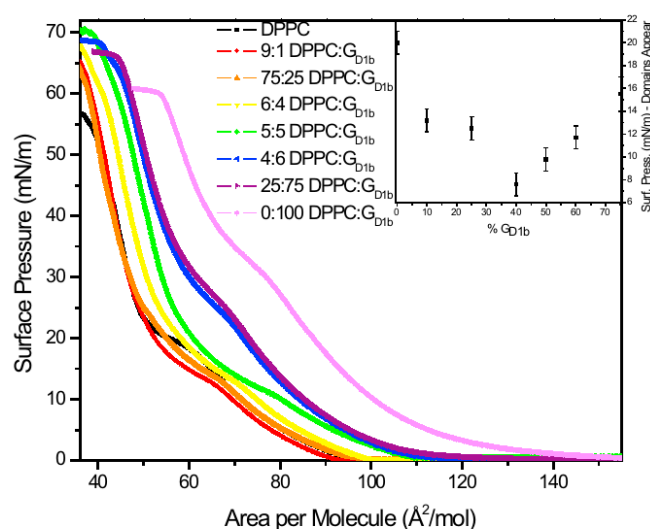


FIGURE 4 Monolayer compression isotherms of pure DPPC, pure GD_{1b}, and binary mixtures of 9:1, 75:25, 6:4, 5:5, 4:6, and 25:75 mol % DPPC/GD_{1b} at 30°C. (inset) Surface pressure at which condensed domains appear in monolayers composed of DPPC and GD_{1b}, plotted as a function of GD_{1b} concentration. Domain formation was visualized using FM with the TR-DHPE probe partitioning into the more fluid phase.

to GM₁, which has a shoulder in the isotherm at 40 mN/m; surface potential measurements have attributed the slight plateau to changes in electrostatic interaction between the charged headgroups (33), although polarization modulation infrared reflection adsorption spectroscopy studies show a reorientation of the sugar residues and network of hydrogen bonds (34), not formation of ordered domains. Collapse of the pure GD_{1b} monolayer occurs at 60 mN/m and an area of 60 Å²/mol, which stems from the smallest compressible cross-sectional area of the headgroup compounded with the multivalent negative charge.

For the binary mixtures, only the 9:1 DPPC/GD_{1b} isotherm shifts appreciably to the left of the pure phospholipid trace and the 75:25 mixture has a comparable lift-off area to DPPC. Further addition of GD_{1b} raises the lift-off area as expected for the addition of a fluid molecule to the monolayer. As the mol % of GD_{1b} is increased in the pure DPPC monolayer, the pressure of the LE/C coexistence plateau is depressed up to a point as discussed below, but the slope of the plateau region of each subsequent mixture increases to more closely resemble that of pure GD_{1b}. For monolayers with a low mol % of GD_{1b}, collapse occurs at ~70 mN/m, but the collapse pressure decreases as the ratio of ganglioside is increased. Using Crisp's surface phase rule (35), collapse pressure that varies systematically with composition indicates the two species, DPPC and GD_{1b}, are miscible and do not completely phase separate.

To better determine how the addition of GD_{1b} affects the phase behavior of DPPC, the surface pressure at which C domains appear was plotted with respect to the relative concentration of ganglioside (Fig. 4, inset) and the minimum or

most compact monolayer is ~ 40 mol % GD_{1b} . This value of 3 DPPC molecules and 2 GD_{1b} molecules matches the GA_1 series, which is surprising because the disialoganglioside molecule has a larger cross-sectional area, as evidenced by the area per molecule at lift-off, and the net negative charge that would ideally be spaced further apart due to steric hindrance and electrostatic repulsion of the headgroups. In addition to geometrically packing with the ganglioside headgroups, we propose that DPPC acts as a spacer to reduce electrostatic repulsion between the negatively charged sialic groups on neighboring gangliosides. This is supported by experiments where the addition of salt (phosphate buffer, pH 7.2, with KCl and NaCl) to the DPPC/ganglioside system, which would act to screen electrostatic repulsion, lessens the DPPC-induced condensation effect as evidenced by a higher surface pressure when condensed domains form (data not shown).

FM

FM images of various mixed DPPC/ GD_{1b} monolayers at a surface pressure of 20 mN/m are shown in Fig. S4. Pure GD_{1b} monolayers are in a LE phase at 20 mN/m (Fig. S4 D) and continue to be fluid until collapse. Similar to DPPC/ GM_1 (11), the disialoganglioside GD_{1b} mixed monolayers form domains with a flower-like morphology at low pressures that grow in size upon compression. Though there is no evidence of another plateau in the isotherms, a second set of C domains consistently appears 5–8 mN/m after the initial nucleation of C phase (Fig. S4, B–C). Although these domains are smaller in diameter due to having formed later, they have a similar morphology and usually merge with larger C domains by 30 mN/m, but the difference in surface pressure of formation is unusual. The second set of domains can likely be attributed to an enrichment of ganglioside in the remaining liquid expanded phase after the initial nucleation event forms condensed domains with the preferred lipid/ganglioside ratio.

AFM

To determine the identity of components in the LE and C phases as determined by FM, binary mixed monolayers of DPPC/ GD_{1b} were deposited onto solid substrates from the air-water interface and imaged with AFM (Fig. 5). Though GD_{1b} is a fluid monolayer at 30 mN/m, attempts to deposit the monolayer on a substrate were made, which resulted in a heterogeneous layer with structures at various heights (data not shown), likely the result of depositing a fluid, highly negatively charged layer on a negatively charged mica substrate.

At low concentrations of ganglioside, 9:1 DPPC/ GD_{1b} (Fig. 5 A), the C domains contain a striped region with height variations of ~ 1 nm (Fig. 5 A, inset) corresponding to the difference between the approximate height of ganglioside with perpendicularly extended headgroup and surrounding condensed DPPC. The C domain edges appear

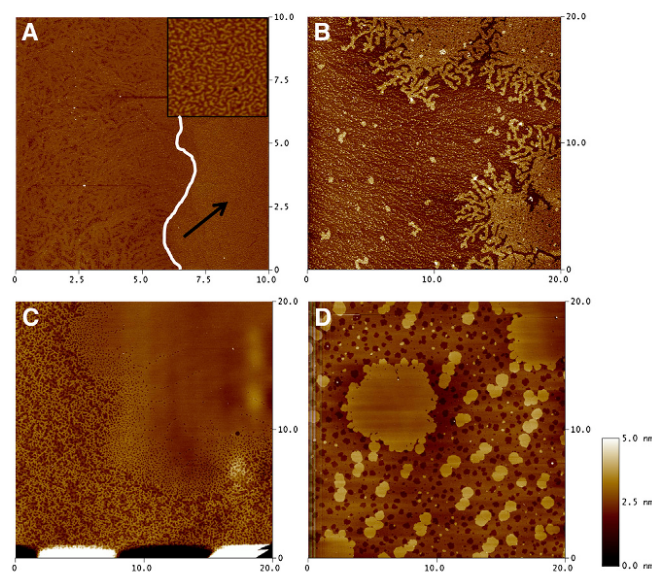


FIGURE 5 AFM topographic images of (A) 9:1 DPPC/ GD_{1b} ; (B) 75:25 DPPC/ GD_{1b} ; (C) 6:4 DPPC/ GD_{1b} ; and (D) 25:75 DPPC/ GD_{1b} monolayers transferred at 30 mN/m (z scale 5 nm). The inset in (A) is a $1 \mu\text{m} \times 1 \mu\text{m}$ region of the location indicated by the arrow in (A). The outline in (A) highlights the boundary between the condensed domain (right side) and the more fluid liquid expanded phase. Scan size in μm is indicated on each image.

blurred in that there is no clear-cut delineation from C phase to LE phase because the material of the tallest height, presumably geometrically packed clusters of DPPC/ GD_{1b} , mixes into the LE boundary region. As more ganglioside is added to make a 75:25 DPPC/ GD_{1b} monolayer, the C domains are composed primarily of the tallest component, presumably DPPC/ GD_{1b} geometric packed complexes based on the surface area of coverage, and some of these ordered structures spread into the more fluid LE phase (Fig. 5 B). The edges of the C domains are marked with thin protrusions of the C phase into the LE phase, reminiscent of pure lipid DPPC finger-like structures seen at ~ 23 mN/m (Fig. 3 A).

For the 6:4 DPPC/ GD_{1b} layer, most condensed as determined by FM, the C domain is homogeneous and consists of the tallest component (*height cross section* in Fig. S5), corresponding to the proposed DPPC/ GD_{1b} complex (Fig. 5 C). The LE phase is now composed of material of three different heights, paralleling results seen in the DPPC/ GM_1 monolayers when additional GM_1 begins to fluidize the layer (11). The heights in the LE phase roughly correlate to that expected for different phases of the binary mixture, with a 1.4 nm step from the lowest layer (fluid DPPC) to the middle (fluid GD_{1b}) and a 2.1 nm height difference between the lowest layer and the highest (condensed DPPC/ GD_{1b}) (*height cross section* in Fig. S5). At the highest concentration of GD_{1b} , 25:75 DPPC/ GD_{1b} , the flower-shaped condensed domains are smaller, $<10 \mu\text{m}$ in diameter, and are mixed with smaller domains of the same

height with similar morphology (Fig. 5 D), which correlate to the second set of C domains seen with FM forming at a surface pressure 5–8 mN/m higher than the initial C domain nucleation. This may be due to the heterogeneity of the monolayer in that at a low surface pressure, large condensed domains of GD_{1b} and DPPC form, but upon further compression, some of the GD_{1b} in the expanded phase reaches a critical density point where it can nucleate additional domains, similar in packing geometry, with neighboring DPPC molecules. Though GD_{1b} has a larger cross-sectional area and is more negatively charged than GM₁, the binary monolayer is most condensed at a ratio of 3 DPPC molecules to 2 ganglioside molecules, or less DPPC than that needed to pack most favorably with GM₁.

Three sialic acids, GT_{1b}

Isotherms

Trisialoganglioside GT_{1b} is a derivative of disialoganglioside GD_{1b} on the biosynthetic pathway and varies by the addition of a sialic acid residue on the terminal galactose sugar group resulting in a highly negatively (−3) charged molecule. A monolayer of pure GT_{1b} lifts off between 180 and 200 Å², but the increase in surface pressure upon compression is gradual until 120 Å²/mol (Fig. 6). The monolayer has phase behavior similar to that of GD_{1b} in that it is fluid until reaching a collapse pressure of 60 mN/m at an area of ~60 Å²/mol. It has been reported that the limiting cross-sectional area of GD_{1b} and GT_{1b} are similar despite the structural difference due to the width of the solid angle required by the disialosyl chain, such that addition of the

third sialic acid does not have a large effect (5). Therefore, differences between the molecules can be attributed to electrostatic repulsion and the resultant dipole moments. Trends similar to that of the disialoganglioside GD_{1b} are also seen in the GT_{1b} binary mixtures in that 95:5 DPPC/GT_{1b} has a lift-off at slightly lower area per molecule than the pure lipid, DPPC, but further addition of the bulky ganglioside shifts the isotherms toward the right or higher average molecular areas. The plateaus occur at lower pressures as ganglioside is added to the layer (up to ~30 mol %), but the plateau region also becomes shorter and less pronounced with a slope approaching that of the GT_{1b} monolayer. For monolayers with a low mol % of GT_{1b}, collapse occurs at 70 mN/m, but collapse pressure decreases slightly as the ratio of ganglioside is increased. Because the collapse pressure varies systematically with composition, this indicates the two species, DPPC and GT_{1b}, are miscible and do not completely phase separate (35). The minimum in the plot of surface pressure of domain formation (Fig. 6, inset) is ~28 mol % GT_{1b} or a ganglioside concentration lower than that for the most condensed DPPC/GD_{1b} layer, but greater than the binary mixture of DPPC/GM₁.

FM

FM images of various binary mixture monolayers of DPPC/GT_{1b} at 20 mN/m are shown in Fig. S6. A pure monolayer of GT_{1b} is in the LE phase and remains fluid upon further compression to collapse (Fig. S6 D). The images of the two-component systems parallel the results reported for GD_{1b} with formation of C domains with a flower-like morphology, supplanted by an additional set of domains that nucleate 5–8 mN/m higher in surface pressure. The smaller domains are more prevalent in monolayers with a higher mol % of GT_{1b}, but exist in all mixtures. Although the size of the C domains varies between the mixtures, the surface area coverage of C domains is greatest for monolayers where the components are the most condensed (20–40 mol % GT_{1b}).

AFM

To determine the identity of components in the LE and C phases as determined by FM, binary monolayers of DPPC/GT_{1b} were deposited onto solid substrates from the air-water interface and imaged with AFM (Fig. 7). There are C domains in the deposited 9:1 DPPC/GT_{1b} monolayer that contain stripes of material that are 1 nm taller in height than the surrounding condensed lipid, presumably due to condensed DPPC/GT_{1b} geometric complexes in coexistence with condensed DPPC (Fig. 7 A and inset). These C domains are bordered by a well-defined fence of taller material with height similar to the geometric complexes. The driving force for these entities to line the border even at areas of high curvature is unclear.

The monolayer composed of 7:3 DPPC/GT_{1b} has well defined, round C domains composed entirely of a single

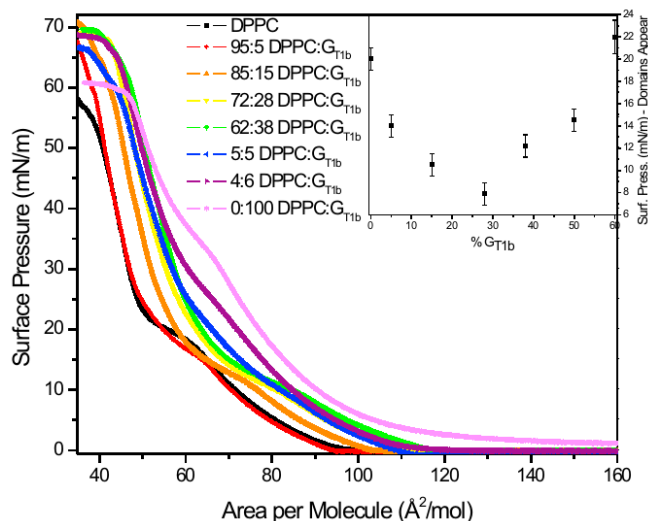


FIGURE 6 Monolayer compression isotherms of pure DPPC, pure GT_{1b}, and binary mixtures of 95:5, 85:15, 72:28, 62:38, 5:5, and 4:6 mol % DPPC/GT_{1b} at 30°C. (inset) Surface pressure at which condensed domains appear in monolayers composed of DPPC and GT_{1b}, plotted as a function of GT_{1b} concentration. Domain formation was visualized using FM with TR-DHPE probe partitioning into the more fluid phase.

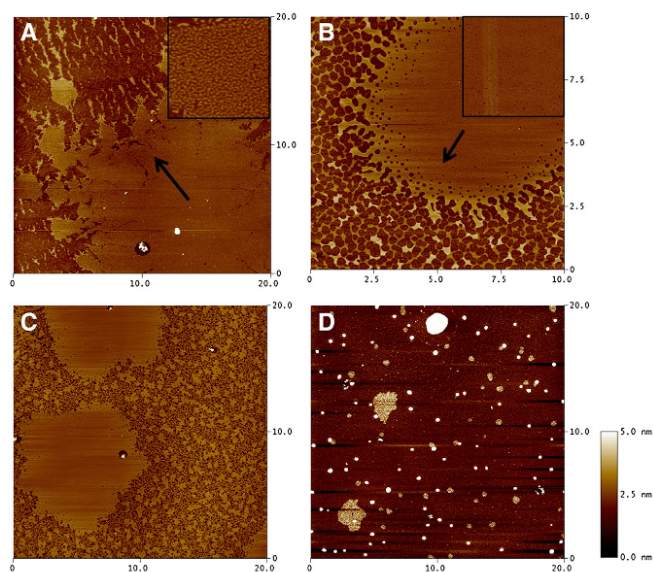


FIGURE 7 AFM topographic images of (A) 9:1 DPPC/GT_{1b}; (B) 7:3 DPPC/GT_{1b}; (C) 6:4 DPPC/GT_{1b}; and (D) 25:75 DPPC/GT_{1b} monolayers transferred at 30 mN/m (z scale 5 nm). The insets in (A) and (B) are $1\ \mu\text{m} \times 1\ \mu\text{m}$ regions of the location indicated by the arrow in (A) and (B) to show morphology of the condensed phase. Scan size in μm is indicated on each image.

height material, the tallest DPPC/GT_{1b} complexes, whereas the LE phase has three heights (with differences of 1.5 and 2.3 nm, *height cross sections* shown in Fig. S7) similar to those seen in GM₁ and GD_{1b} as the ganglioside partitions to the fluid region (Fig. 7 B and inset). This is also seen in the 6:4 DPPC/GT_{1b} monolayer, but there are also a large number of smaller condensed domains (also visible with FM) spread evenly throughout the LE phase of the monolayer (Fig. 7 C). At high GT_{1b} concentrations, the monolayer transfer process was difficult (this was reflected in an inability to successfully deposit the highly fluid and polyanionic GT_{1b}) and this resulted in a deposited monolayer of 25:75 DPPC/GT_{1b} that had large protrusions of material 15–25 nm in height above the background (Fig. 7 D). It is unclear if this is a product of the deposition process due to the negatively charged substrate or if it reflects the actual morphology of the monolayer at the air-water interface. If the latter is true, these structures could be indicative of fluid-like collapse that occurs at low surface pressures where fluid sections of the monolayer protruding into the subphase in the form of vesicles were caught upon deposition.

DISCUSSION AND CONCLUSIONS

In our earlier work, a geometric packing model between DPPC and GM₁ in monolayers at the air-water interface was proposed where complementary differences in the size and shape of the phospholipid and ganglioside hydrophilic headgroups led to a preferred tighter packing up to

25 mol % ganglioside after which additional GM₁ partitioned to the fluid phase and caused expansion of the layer (11). Molecular dynamics simulations of DPPC bilayers containing GM₁ show decreases in surface area and increases in both order parameter and bilayer thickness, indicating condensation with increasing concentration of GM₁ up to 20 mol %. The protrusion of sugar residues from the layer increases with an increasing concentration of GM₁ supporting a geometric packing argument where DPPC serves as a wedge molecule between gangliosides to allow for tighter packing (36).

The current work focuses on determining if this condensation phenomena, which may play a role in the formation of lipid rafts, can be generalized to all phosphatidylcholine/ganglioside mixtures. Experiments performed with different gangliosides using a variety of environments have shown diverse and conflicting results as summarized below that make a systematic comparison between ganglioside structures with varying numbers of sugar groups and sialic acid residues difficult. Vyas et al. (16) used fluorescence resonance energy transfer measurements on both monolayers and cell membranes to determine that GM₁ molecules spontaneously self-associate within the membrane, although GD₃ (a disialoganglioside related to GD_{1b} without the two terminal sugar groups) does not cluster or associate with the same regions as GM₁; they attributed this phenomena to differences in carbohydrate interactions. In mixed monolayer experiments between DPPC and several different gangliosides on a 145 mM NaCl, pH 5.6 interface (well above pK_a for sialic acid = 2.6), expansion or fluidization was seen at all DPPC/GA₁ and DPPC/GM₁ ratios, whereas gangliosides with a higher number of sialic acid residues (GD_{1a} and GT₁) resulted in condensation of the layer (37). Conversely, Bordi et al. (32) ran monolayer experiments under similar conditions of 145 mM NaCl, pH = 7.2 to show that monosialogangliosides mixed with DPPC showed positive deviations from ideality at low concentrations and negative deviations at high concentrations, whereas disialogangliosides showed positive deviations, or expansion, over the entire composition range. In sphingomyelin/GM₃ monolayers (a monosialoganglioside related to GM₁ without the two terminal sugar residues), the components mix ideally at compositions below 30 mol % GM₃; condensation is seen at higher GM₃ amounts and was attributed to a change in orientation of GM₃ at the interface above this concentration threshold (17). Luckham et al. (38) performed monolayer isotherm experiments with DPPC/GM₁ and DPPC/GT_{1b} mixtures on pure water subphases and saw negative deviations from ideal mixing, or condensation, at all pressures and mole ratios, but concluded that the two components mixtures were completely miscible with one another.

Here, data from numerous techniques performed on DPPC/ganglioside monolayers that have been systematically varied at the air-water interface and also deposited

on a solid substrate have been presented to determine how the structure of the ganglioside headgroup affects its lateral ordering and phase behavior in DPPC monolayers. As the 145 mM NaCl subphase used in several experiments outlined previously provides electrostatic screening to ameliorate the repulsive charge effects between the ganglioside headgroups, a water subphase was used. The results from each technique show that the basic structure of a glycosphingolipid molecule with a ceramide backbone and a hydrophilic headgroup composed of four sugars is sufficient to condense a DPPC monolayer, or pack more tightly than either individual component. The surface pressure at which condensed domains form is used as an indicator of material properties within the layer with the mixture having a minimum in pressure correlating to the most tightly packed layer; DPPC/GA₁ was most condensed at a 6:4 ratio (domains form at 9 mN/m), DPPC/GM₁ at 75:25 (~9 mN/m) (11), DPPC/GD_{1b} at 6:4 (~8 mN/m), and DPPC/GT_{1b} at 7:3 (~8 mN/m). Further addition of ganglioside serves to expand the layer closer to an ideal, noninteracting mixing scenario. Additionally, we have examined more biologically relevant fluid monolayers and found that similar to DPPC, the addition of gangliosides to either more fluid dimyristoylphosphatidylcholine or palmitoylcholinephosphatidylcholine results in film condensation at low ganglioside concentration, followed by expansion of the film as the mole fraction of ganglioside increases. Fluorescence microscopy shows that condensed domains are formed upon the addition of gangliosides at low concentrations; both single component monolayers remain fluid upon compression until collapse (data not shown). This suggests that the condensation effect of gangliosides explored in this work can be generalized and may play a physiological role.

The structure of GA₁ is fundamentally different from the gangliosides studied due to its lack of sialic acid, which results in a smaller molecular cross-sectional area and lack of electrostatic repulsion between headgroups. These structural characteristics contribute to pure monolayers of GA₁ forming C domains upon compression, visible with FM at the air-water interface and AFM on deposited layers. Though pure GA₁ forms C regions, addition of DPPC results in a more condensed monolayer compared to either individual component. Because the orientation of the sugar headgroup is sensitive to cross-sectional area available to each molecule, the oligosaccharide portion of a glycosphingolipid orients itself more parallel to the interface at high area per molecule. Upon compression, once the sugar groups begin to interact, they extend into the aqueous phase perpendicular to the air-water interface (31,34). In the case of DPPC/GA₁ binary mixtures, the complementary geometry of the DPPC molecules allows the acyl chains of the mixture to align at a lower surface pressure than would occur for the pure glycosphingolipid, but the overall condensation effect is less than seen with the negatively charged gangliosides. Based on a geometric packing model, a smaller headgroup

cross-sectional area compared to GM₁ would require fewer lipid molecules to fill the pockets of space to allow the hydrocarbon chains to align and condense. This is reflected in the results where the DPPC/glycosphingolipid ratio for the most condensed layer is 3:2 for GA₁ and 3:1 for GM₁.

Di- and trisialogangliosides each require less DPPC to fully condense compared to monosialoganglioside, a smaller molecule, when simple geometric space-filling arguments would predict a higher mol % of lipid. Adding more monosaccharides or sialic acid residues to a ganglioside headgroup, essentially increasing the complexity, causes the monolayer to become more fluid (27,31). Therefore, because the most condensed binary monolayer mixtures form condensed domains at 8–9 mN/m regardless of the number of sialic acid residues, there is a larger global condensation effect with the di- and trisialoganglioside molecules. We propose this may be explained by a combination of molecular geometry and the electrostatic interactions and alignment of the resultant dipole moments of the individual components. To provide further insight into the cross-sectional area of the molecules, compressions of ganglioside monolayers can be performed at pH = 1.2, below the 2.6 pKa of sialic acid to remove the electrostatic repulsions between the sialic residues. Neutral GM₁, GM₂, GD_{1a}, and GT₁ showed similar areas per molecule (31). This supports the idea that the physical cross-sectional area of the ganglioside headgroup is likely determined at the first sugar group branching point (Fig. 1).

From surface potential versus molecular area measurements made on monolayers, the overall dipole moment in the direction perpendicular to the interface, μ_{\perp} , can be determined for a surfactant molecule resulting from the contribution of the dipole moments from the oriented water molecules, the polar headgroup, and from the hydrocarbon chains (39). On a 145 mM NaCl pH = 5.6 subphase, the ceramide backbone, equivalent to ganglioside without an oligosaccharide headgroup, has a considerable vertical dipole moment of ~400 mD arising from the closely packed fatty acyl chains with the sign defined as the positive end of the dipole oriented toward the air (31,35). The first sialic acid group along with the four sugar residues of GM₁ orient to introduce a net dipole moment in the opposite direction, causing a lowering of the molecular μ_{\perp} to ~50 mD. The second and the third sialic acid residues (for GD_{1b} and GT_{1b}) introduce resultant dipoles of similar magnitude to the first sialic group, but in the opposite direction resulting in overall μ_{\perp} = ~200 and 400 mD for GD_{1b} and GT_{1b}, respectively (31,40). DPPC has an overall negative perpendicular dipole of ~500 mD (opposite in direction from the ganglioside dipole) due to the relative positioning of a negatively charged phosphate group and positively charged trimethylammonium group (41,42).

We propose a model where there are a number of competing interactions, including molecular dipoles and the generic bulky headgroup geometry of a ganglioside,

which determine the number of DPPC molecules that will ideally pack or condense a ganglioside film. The hydrophilic sugar moieties and ceramide backbone in the ganglioside structure have a high propensity to form intermolecular hydrogen bonds. Due to differences in the cross-sectional area between the bulky headgroup and the hydrophobic acyl chain, a ganglioside will remain fluid up to high compressions, including at molecular densities that correlate to a cell membrane, unless a smaller spacer molecule like DPPC can participate in a closer geometrically driven packing (11,43). Using electrical conductivity measurements, Cametti et al. (44) showed that in mixtures of DPPC and gangliosides, clusters of the glycosphingolipid molecules somehow neutralize the electrical dipole moment of the ganglioside headgroups to minimize the repulsive headgroup-headgroup interactions. This suggests that rearrangement and alignment of the sugar residues, which would change the positioning of the dipoles within the headgroup region, can lead to surface depolarization, allowing for tighter packing. A zwitterionic DPPC headgroup with a predominant dipole moment in the opposite direction could serve to play a similar depolarization role.

As more sialic acids are added to the ganglioside, the electrostatic repulsion increases and this increases the cross-sectional area, but the perpendicular dipole moment is also dramatically affected. In the case of GM₁, the μ_{\perp} is small and so packing is primarily determined by the geometry of the molecule; alignment of the DPPC and GM₁ molecular dipoles does not provide a strong degree of stabilization. When comparing gangliosides with more sizeable dipole moments, GD_{1b} and GT_{1b}, the molecules with the larger positive μ_{\perp} require a smaller relative proportion of DPPC (with a negative μ_{\perp}) to ameliorate some of the electrostatic repulsion, regardless of molecular cross section (12). This idea is supported by experiments performed at pH = 1.2, which show protonation of the negative charge of the sialyl group (pK_a for free sialic acid = 2.6) allows a closer packing of the pure ganglioside monolayers by diminishing electrostatic repulsions (31). Additional support for the surface depolarization due to the alignment of dipole moments in opposite directions is reflected in the edge morphology of the condensed domains as visualized by AFM. At high DPPC concentrations, the domains display a number of protrusions indicating that dipole repulsions override the effect of line tension, but as the dipole moments are canceled by the addition of gangliosides and resultant reorganization of headgroups, the line tension element becomes relatively more important and the edges of the domains smooth.

Ganglioside molecules with headgroup geometries that vary due to an increasing number of sialic acids show the general behavior reported for GM₁ in that an increase in ganglioside concentration in binary phosphatidylcholine/ganglioside monolayers causes condensation followed by fluidization. The condensation effect can be explained via

electrostatic interactions between and alignment of dipole moments within their headgroups and the tendency of gangliosides to have cooperative interactions qualitatively attributed to hydrogen bond formation between adjacent sugar residues.

SUPPORTING MATERIAL

Seven figures and references (45,46) are available at [http://www.biophysj.org/biophysj/supplemental/S0006-3495\(13\)00877-1](http://www.biophysj.org/biophysj/supplemental/S0006-3495(13)00877-1).

This work was made possible by a grant from National Science Foundation (MCB-0920316). S.L.F. and K.Y.C.L. thank Tadeusz Pacuska for GA₁ samples and Jarek Majewski for facilitating this collaboration. S.L.F. thanks Gettysburg College for financial support and is grateful for the support of a National Science Foundation Graduate Research Fellowship at the University of Chicago. S.L.F. and K.Y.C.L. acknowledge the use of the Shared Facilities supported by the University of Chicago NSF-MRSEC program (DMR-0820054).

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