Dynamic Reorganization and Correlation among Lipid Raft Components

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Supporting Information

ABSTRACT: Lipid rafts are widely believed to be an essential organizational motif in cell membranes. However, direct evidence for interactions among lipid and/or protein components believed to be associated with rafts is quite limited owing, in part, to the small size and intrinsically dynamic interactions that lead to raft formation. Here, we exploit the single negative charge on the monosialoganglioside G_{M1}, commonly associated with rafts, to create a gradient of G_{M1} in response to an electric field applied parallel to a patterned supported lipid bilayer. The composition of this gradient is visualized by imaging mass spectrometry using a NanoSIMS. Using this analytical method, added cholesterol and sphingomyelin, both neutral and not themselves displaced by the electric field, are observed to reorganize with G_{M1}. This dynamic reorganization provides direct evidence for an attractive interaction among these raft components into some sort of cluster. At steady state we obtain an estimate for the composition of this cluster.

INTRODUCTION

Many biological membrane components, in particular lipids, are observed to diffuse within the plane of the membrane.¹ By itself, this fluidity suggests there can be no long-range order; however, it does not preclude the possibility of short-range order. The idea that short-range order exists, and is in fact crucial to many functions of the cell membrane, has led to the concept of lipid rafts.²−⁷ In particular, nanoscale clusters of sphingolipids, cholesterol, some GPI-linked and acylated proteins, and the gangliosides are widely regarded as providing a platform for signaling, viral budding, and many other cellular functions that require the association of multiple membrane-localized components.

Raft domains are typically not directly visualized in plasma membranes due to their putative small size and dynamic nature but are inferred by specific resistance to detergent extraction, functional effects of cholesterol depletion, diffusion of dye-labeled lipids, and colocalization of certain proteins. By contrast, larger-scale lipid domains have been extensively documented in model systems such as monolayers at the air−water interface,²⁸ giant unilamellar vesicles (GUVs)²⁹ and supported lipid bilayers (SLBs),¹⁰ along with detailed equilibrium phase diagrams.¹¹ While these equilibrium phase diagrams support the notion that certain components tend to interact (attractively or repulsively), the relevance of their compositions to rafts in the plasma membrane is unclear. Given the presumed dynamic nature of rafts, several groups have used measurements of lateral diffusion in cell membranes and the effect of cholesterol extraction on diffusion as a means to infer the existence, size, and dynamics of rafts.¹²−¹⁵ Recent comparisons of the lipid composition of enveloped viruses and the host membranes from which they were derived provide a particularly compelling argument for the functional importance of raft compositions.¹⁶,¹⁷ The large differences between these membrane compositions and the enrichment in the viral membranes of raft-related lipids suggest a degree of preorganization of lipid and viral protein components in the plasma membrane prior to budding.

In the following, we take a new approach to interrogating the interactions between the ganglioside G_{M1}, typically visualized by binding fluorescently labeled cholera toxin B, and cholesterol and/or sphingomyelin, components believed to be associated with rafts. We exploit the fact that G_{M1} has a single negative charge due to its sialic acid moiety, whereas cholesterol and sphingomyelin are neutral at physiological pH. Charged lipid components move in response to an electric field imposed parallel to the plane of the membrane, and when barriers to diffusion are imposed in an SLB, the competition between this electrophoretic force and random diffusion leads to a gradient of the charged component.¹⁸,¹⁹ Other groups have used membrane electrophoresis as an analytical technique to separate charged membrane components.²⁰−²³ The concept of the experiments reported here, illustrated schematically in Figure 1, is to selectively move G_{M1} with the electric field and then determine by imaging mass spectrometry using isotopic and atom labels (Figure 2) whether other neutral membrane components also move, i.e., whether they dynamically reorganize in concert with G_{M1} which would demonstrate the presence of a specific, attractive interaction.

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Previous work by our group and others has used membrane electrophoresis to study the thermodynamics of simple lipid mixtures, imaged indirectly by fluorescence, but here we extend the concept to measure specific interactions between different biologically relevant lipid species whose concentrations are analyzed directly by mass spectrometry. Note that local interactions among components may be present prior to electrophoretic reorganization, but the length scale is such that no current method can visualize this, whereas a perturbation in composition, provided by the selective movement of GM₁, can establish whether components tend to move together.

We emphasize the importance of using an analytical technique like imaging mass spectrometry as dye-labeled lipids can behave very differently from unlabeled lipids. Furthermore, mass spectrometry can be used to obtain quantitative estimates for the mole percent of each membrane component from which information on the average stoichiometry of the interactions can be obtained.

### RESULTS

#### Reorganization of GM₁ by Membrane Electrophoresis.

In order to determine whether other components also move and to avoid the potentially complicating effects of dye-labeled components, we use imaging mass spectrometry with components selectively labeled with rare stable isotopes or other atoms: GM₁ (18F), cholesterol (CHOL, 13C), 1,2-dioleoyl-snglycerol-3-phosphocholine (DOPC, 15N), and palmitoyl sphingomyelin (PSM, 2H) (Figure 2). The GM₁ concentration gradient produced by electrophoresis (Figure 3, see also Figure S4) can be directly visualized by the unique $F^−$ signal, and interestingly, neutral DOPC is displaced from the region where GM₁ accumulates, thus conserving the average area per molecule. This conservation of molecular area in a fluid bilayer is expected but has never been directly visualized before.

#### Correlated Reorganization of GM₁ and Other Components.

NanoSIMS analysis was then used to address whether CHOL and/or PSM rearrange as GM₁ is reorganized by an electric field. As shown in the control experiment in Figures S5 and S6, in the absence of GM₁, application of an electric field leaves the composition of neutral CHOL, PSM, and DOPC across the corral unchanged. By contrast, as shown in Figure 4, when GM₁ is present the neutral CHOL does reorganize in parallel with GM₁ upon the application of an electric field while DOPC is displaced in the opposite direction. In the quaternary mixture (Figure 5), PSM also reorganizes with GM₁ and CHOL, while DOPC is displaced. We attempted to test the association
Analysis of Lipid Compositions. The overall composition of an SLB that has been reorganized within a corralled area by an applied electric field is calculated by NanoSIMS quantitative analysis. Briefly, counts for each ion species are summed, and then the ratios J10F/12C2, J13C/12C2, JH/12C2, and J12C/12C14N are calculated. Calibration curves obtained from standard samples are then employed (see Figure S3 and Table S1) to determine the percent molar content for each component of interest. Analysis of eight corralled SLBs (see Table S2) suggests that the overall composition (±uncertainty) of the corralled SLB in Figure 5 is 9.2 ± 0.9 mol % of PSM, 8.8 ± 0.3 mol % of GM1, 26.2 ± 1.8 mol % of CHOL, and 78.4 ± 2.4 mol % of DOPC. We note that the application of an electric field does not affect SLB composition (data not shown).

DISCUSSION

There are several immediate qualitative conclusions from the NanoSIMS concentration profiles: (1) in the absence of GM1, the neutral CHOL, PSM, and DOPC components do not move in an electric field (Figures S4 and S5); (2) GM1 does move in an applied electric field toward the positive electrode and forms a concentration gradient (Figures 3 and S4); (3) when CHOL and GM1 are present and GM1 moves in an electric field, cholesterol also moves and in the same direction (Figure 4); (4) when CHOL, PSM, and GM1 are present and GM1 moves in an electric field, both CHOL and PSM move in the same direction as GM1 (Figure 5); and (5) neutral background DOPC is always displaced from the region where GM1 and other components accumulate under an applied electric field.

Taken together, these results demonstrate that both CHOL and PSM dynamically reorganize when GM1 reorganizes, consistent with the concept that they interact to form some sort of nanoscale cluster. We note that the shapes of the gradients in the case of the quaternary mixture (Figure 5) appear different from those in the simpler mixtures (Figures 3 and 4), and the latter are more similar to those observed for simple charged dye-labeled lipids. The origin(s) of this difference is not yet understood; however, the purpose of this report is to establish that components interact and codistribute and, as described in the following, to obtain an estimate for the stoichiometry of the interaction under specific conditions.

There has been considerable discussion in the literature about possible clusters formed between lipid raft components. The word cluster is used here to describe an average association of the clusters of lipids that we observe represent true phases or different from those in the simpler mixtures (Figures 3 and 4), and the latter are more similar to those observed for simple charged dye-labeled lipids. The origin(s) of this difference is not yet understood; however, the purpose of this report is to establish that components interact and codistribute and, as described in the following, to obtain an estimate for the stoichiometry of the interaction under specific conditions.

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Table of Contents Entry

The overall composition of an SLB that has been reorganized within a corralled area by an applied electric field is calculated by NanoSIMS quantitative analysis. Briefly, counts for each ion species are summed, and then the ratios J10F/12C2, J13C/12C2, JH/12C2, and J12C/12C14N are calculated. Calibration curves obtained from standard samples are then employed (see Figure S3 and Table S1) to determine the percent molar content for each component of interest. Analysis of eight corralled SLBs (see Table S2) suggests that the average (±uncertainty) overall composition of this SLB population is 9.6 ± 1.3 mol % of PSM, 7.6 ± 1.5 mol % of GM1, 3.6 ± 0.9 mol % of CHOL, and 79.3 ± 2.8 mol % of DOPC. For example, the overall composition (±uncertainty) of the corralled SLB in Figure 5 is 9.2 ± 0.9 mol % of PSM, 8.8 ± 0.3 mol % of GM1, 26.2 ± 1.8 mol % of CHOL, and 78.4 ± 2.4 mol % of DOPC. We note that the application of an electric field does not affect SLB composition (data not shown).

Figure 4. Correlated motion of GM1 and CHOL when GM1 is moved by an electric field. NanoSIMS images and corresponding concentration profiles of a patterned supported lipid bilayer (nominal composition 83 mol % 15N-DOPC, 1 mol % 19F-GM1, and 16 mol % 13C-cholesterol) after an electric field was applied prior to sample freezing.

Figure 5. Correlated motion of GM1, CHOL, and PSM when GM1 is moved by an electric field. NanoSIMS images and corresponding concentration profiles of a patterned supported lipid bilayer after an electric field was applied prior to sample freezing.
these plots, we observe that PSM, G_{MI}, and CHOL account for 23 ± 2, 15 ± 1, and 6 ± 1 mol %, respectively, at the edge closest to the cathode with the balance (54 ± 3 mol %) filled by DOPC. The average composition in this region corresponds to the average reorganization of 4 PSM, 2 G_{MI}, and 1 CHOL molecules in response to an electric field.

The ratio of bilayer components estimated from the flat region of the mole fraction profile in Figure 6 is somewhat different from suggestions in the literature.\textsuperscript{30–32} We briefly consider issues, in particular related to the possibility of G_{MI} leaflet asymmetry and possible flip–flop of components, which might affect the quantification of these values. It should be noted that the value that we calculate for the average stoichiometry of the clusters is made possible because one of the components (PSM) was the limiting reactant; if this were not the case, we would not be able to distinguish between clustered and unclustered components which would lead to an incorrect estimate in the stoichiometry calculations.

Additionally, the ratios could be different if different lipid species (e.g., POPC vs DOPC or DPPC vs PSM) were used. The average numbers of associated molecules (4:2:1, PSM/G/CHOL) estimated above from analysis of the compositional gradients for the colocalizing components are calculated from the composition of both leaflets of the SLB as theNanoSIMS cannot distinguish the composition of the upper and lower leaflets. We assume that there is no asymmetry in leaflet composition in the GUVs used to form SLBs, but G_{MI} asymmetry in supported bilayers formed from small unilamellar vesicles has previously been reported.\textsuperscript{33–35} While lipid bilayer asymmetry would not change the qualitative finding that CHOL and PSM reorganize with G_{MI}, it would change the quantitative interpretation of the data. In the most extreme case were all G_{MI} and associated CHOL and PSM in one leaflet, one would predict phase separation based on the phase diagram for the ternary DOPC/CHOL/PSM mixture.\textsuperscript{36} Since our bilayers appear microscopically uniform, we conclude that G_{MI} is not completely asymmetrical distributed (a more detailed discussion can be found in the SI).

The existence of dynamic subdiffraction limited clusters or complexes of CHOL and saturated phospholipids has been inferred from NMR and FRET experiments and based on thermodynamic arguments from phase diagrams.\textsuperscript{31,37–39} In these experiments, complexes are indirectly analyzed from fatty acid chain order parameters, changes in FRET efficiency of different membrane-associated dyes, and changes in molecular areas. For monolayers of mixtures of CHOL and saturated phospholipids, the stoichiometry of complexes appears to be approximately 1:2 CHOL/phospholipid. In monolayers, DPPC and G_{MI} form a complex with an apparent stoichiometry of 3:1 DPPC/G_{MI}.\textsuperscript{40} Similarly, DMPC and DSPE-PEG200 form a complex in monolayers with a stoichiometry of 3:1 DMPC/DSPE-PEG200.\textsuperscript{51} To the best of our knowledge, these analyses have not been carried out with both G_{MI} and cholesterol in bilayers. It is not surprising that given the apparent cooperative nature of cluster formation in this quaternary mixture, the stoichiometry of complexes is different from that in simpler binary and ternary mixtures. On the other hand, analysis of the composition of detergent resistant membrane fractions suggests a more than 2:1 CHOL/PSM ratio.\textsuperscript{32} However, this value may be skewed by effects of the detergent or by the relatively high concentration of cholesterol in the plasma membranes of most cells.\textsuperscript{41} Although our experiments were carried out with model membranes, the existence of clusters containing CHOL and PSM in this system supports the physical possibility of nanometer-scale clusters like those proposed by the lipid raft hypothesis and provides estimates of their composition.

\section*{METHODS}

Materials. 99.9% \textsuperscript{13}C\textsubscript{6}-glucose was purchased from Cambridge Isotope Laboratories. 98% \textsuperscript{15}N-choline chloride was from Sigma, N-Palmitoyl-d31-D-erythro-sphingosylphosphorylcholine (9H-D-sphingomyelin) and 1,2-dioleoyl-sn-glycerol-3-phosphate (sodium salt) (DOPA) were from Avanti Polar Lipids. All other reagents were from Fisher and were used as supplied.

Biosynthesis and Purification of \textsuperscript{13}C-Cholesterol. Saccharomyces cerevisiae strain RH6829, which produces cholesterol as its primary sterol, was obtained from Prof. Riezman (University of Geneva).\textsuperscript{31} Cholesterol was metabolically labeled with \textsuperscript{13}C\textsubscript{6}-glucose and purified with HPLC according to the procedure of Shivapurkar et al.\textsuperscript{32} Briefly, RH6829 yeast were grown in minimal media with 1.5% \textsuperscript{13}C\textsubscript{6}-glucose (99 atom %) as the sole carbon source. After being shaken at 230 rpm at 30 °C for 2 days, cells were subjected to alkaline methanolysis. Lipids were extracted with petroleum ether, and cholesterol was purified from the lipid extract with HPLC. The extent of labeling and purity was assessed with GC–MS (see inset, Figure 2). Approximately 5 mg of labeled cholesterol per liter of media was obtained under these conditions.

\textbf{Synthesis of \textsuperscript{13}C-N-DOPC.} \textsuperscript{13}C-N-DOPC was synthesized by esterifying DOPA with \textsuperscript{13}C-choline as previously described.\textsuperscript{26,65} Briefly, 515 mg of \textsuperscript{13}C\textsubscript{6}-choline chloride, 264 mg of DOPA, and 20 mL of anhydrous pyridine were added to a round-bottom flask with a stir bar. A 3.47 mL portion of trichloroacetonitrile was slowly added to the flask, and the mixture was stirred at 60 °C overnight. The solution was cooled, filtered, and concentrated via rotary evaporation. The resulting brown residue was dissolved in 40 mL of CH\textsubscript{2}OH/CHCl\textsubscript{3}(1:1) and again concentrated via rotary evaporation. The product was purified via column chromatography on IWT TMD-8 ion-exchange resin (50% tetrahydrofuran in water), followed by column chromatography on silica gel (CHCl\textsubscript{3}/CH\textsubscript{2}OH/H\textsubscript{2}O 65:25:4) and column chromatography on octadecyl-functionalized silica gel (CH\textsubscript{2}Cl\textsubscript{2}/CH\textsubscript{3}OH 5:95), yielding the pure phosphatidylcholine (92.9 mg, 32.2% yield) as a white solid.

\textbf{Synthesis of 18-F-GM1.} Monofluorinated G\textsubscript{MI} (18-F-G\textsubscript{MI}) was synthesized by coupling lyso-G\textsubscript{MI} with the corresponding fluorinated stearic acid.\textsuperscript{26,66} Briefly, lyso-G\textsubscript{MI} was obtained by alkaline hydrolysis of native G\textsubscript{MI} and then treated with the N-hydroxysuccinimide (NHS) ester of the 18-monofluorinated stearic acid in diisopropylamine to yield the fluorinated G\textsubscript{MI}. The product was purified by flash column chromatography (CH\textsubscript{3}OH/CH\textsubscript{2}OH/H\textsubscript{2}O, 60:40:5). Note that it was shown in previous work that the 18-F-G\textsubscript{MI} behaves identically in biological assays as native G\textsubscript{MI}.\textsuperscript{26}

\textbf{SLB Formation and Electrophoresis.} Patterned supported lipid bilayers were formed from giant unilamellar vesicles (GUVs, typically approximately 1 μm) that were incubated in a buffer solution. The vesicles were then electrophoresed at a constant voltage, and the resulting patterned bilayers were attached to a coverslip using a thin layer of agarose. This process was repeated until the desired pattern was obtained. The resulting bilayers were then imaged using a confocal microscope to confirm their patterned nature. In some cases, the bilayers were further processed for additional analysis, such as mass spectrometry or fluorescence microscopy, to provide additional information about their composition and structure.
measurements. All experiments were performed at room temperature. 

Note that substantially higher supply (current <0.001 mA which produced a negligible amount of resistive heating). The cell was rinsed thoroughly to remove residual salt deposits. An electroformed by spreading $10^{-4}$ molar percentages with tens of microns in diameter) that were deposited on chrome-patterned platinum wire electrodes 6 cm apart and a glass coverslip arranged to create fields can be applied during the electrophoresis experiment. The negative charge on the electron microscopy were applied.45 

**Freeze-Drying Samples.** Because NanoSIMS analysis takes place in an ultrahigh vacuum, lipid bilayer samples must be dehydrated. To preserve the lateral organization of lipid bilayers formed in an aqueous environment, techniques from electron microscopy were applied.13,14 Briefly, supported lipid bilayer samples on the NanoSIMS supports were carefully removed from their aqueous environment with tweezers and flash-frozen by plunging quickly into a chamber filled with liquid N$_2$. The frozen samples were then subjected to reduced pressures (70–80 μbar) generated by an oil-free scroll pump equipped with a liquid N$_2$ trap for at least 12 h to sublime vitreous ice. 

The membrane electrophoresis cell consisted of two 0.5 mm platinum wire electrodes 6 cm apart and a glass coverslip arranged to form a bridge between the electrodes. Electrical connection was achieved through water (Milli-Q, resistivity = 18.2 MΩ cm) contact. Note that the time between the field and end of application of the electric field and rastered over sample areas that were between 35 μm × 35 and 50 μm × 50 μm. The images consisted of 10 scans (long enough to collect a signal of quality). Fluorescence imaging was performed using a Nikon Eclipse 80i epifluorescence microscope equipped with an Andor Clara camera. 

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**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

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**REFERENCES**

Supplementary Figures:

**Figure S1. Schematic of the NanoSIMS experiments.** A cesium ion beam is rastered across a surface generating secondary ions ($^2$H$^-$, $^{19}$F$^-$, $^{12}$C$^-$, $^{12}$C$^{13}$N$^-$, and $^{12}$C$^{15}$N$^-$) from the components of a supported lipid bilayer shown as a mixture of $^{13}$C$_{27}$-cholesterol, $^2$H$_{31}$-palmitoyl-sphingomyelin, 18-F-GM1, and $^{15}$N-DOPC (see Figure 2 for chemical structures).

**Figure S2. NanoSIMS High Mass Resolving Spectra.** Mass spectra (secondary ion counts per second vs. atomic mass) obtained using the NanoSIMS 50L at Stanford University set at high mass resolving power (MRP) for species at masses 2 (MRP~1000), 19 (MRP~7300), 24 (MRP~6200), 25 (MRP~6500), 26 (MRP~6300), 27 (MRP~6600) demonstrating the ability to separate interfering isobars from the species of interest.
Figure S3. Calibration curves from standard samples. Data points (circles) represent single NanoSIMS measurements as a function of composition. Solid lines represent best-fit lines for $r = mx + b$ (i.e. $^2$H-Sphingomyelin, where $r$ represents ratio between two ion species) or $R = mx + b$ (i.e. $^{19}$F- GM1, $^{13}$C-CHOL and $^{15}$N-DOPC, where $R = r^{1/2}$). Upper and lower dashed lines represent upper and lower regression bands (see Table S1 for best-fit line parameters).

Figure S4: GM1 reorganizes in response to an electric field visualized by binding to fluorescently labeled cholera toxin B subunit. Epi-fluorescence image of a patterned SLB comprised of 97 mol% egg phosphatidylcholine, 2 mol% GM1, and 1 mol% neutral PC tail labeled BODIPY 581/591 before (a) and after (b) the application of an electric field (33V/cm, 62 min) followed by immediate addition of cholera toxin B subunit (labeled with Alexa 488). The BODIPY 581/591 was imaged through a Texas Red filter set while the Alexa 488 was imaged through an NBD filter set, the images were colored red and green respectively and overlapped to give the image. The cholera toxin (green) moves in the opposite direction of the electric field, while the neutral BODIPY (red) does not move. The SLB was assembled from small unilamellar vesicles on glass that was pre-patterned with Alexa 488 labeled bovine serum albumin.
Figure S5. NanoSIMS imaging of a three-component (16:8:76 CHOL:PSM:DOPC without G\textsubscript{M1}) supported lipid bilayer after the application of an electric field (7 V/cm for 20 min) demonstrating that molecule-specific concentration gradients do not form. Color bars represent molar percentages from quantitative analysis.

Figure S6. Molecule-specific line-scans (averaged perpendicular to the electric field direction) from supported lipid bilayer in Figure S4. The compositions of each component are essentially flat across the coral, indicating that CHOL and PSM are not moved by the application of an electric field.
### Table S1. Best-fit line parameters for calibration curves in Figure S6.

<table>
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<tr>
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<th>$^2$H-PSM</th>
<th>$^{19}$F-GM1</th>
<th>$^{13}$C-CHOL</th>
<th>$^{15}$N-DOPC</th>
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### Table S2. Compositional analysis of a corralled supported lipid bilayer population (total of 8). Molar composition (± uncertainty) for individual samples was calculated from quantitative analysis using standard samples.

<table>
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<tr>
<th>Corralled SLB</th>
<th>mol% PSM</th>
<th>mol% GM1</th>
<th>mol% CHOL</th>
<th>mol% DOPC</th>
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<td>1 (Fig 5)</td>
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<td>3.6 ± 1.8</td>
<td>78.4 ± 2.4</td>
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<td>8.9 ± 0.2</td>
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<td>79.9 ± 1.4</td>
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<tr>
<td>8</td>
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<td>5.7 ± 0.2</td>
<td>2.3 ± 1.3</td>
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<tr>
<td>Avg. ± Std. Dev</td>
<td>9.6 ± 1.3</td>
<td>7.6 ± 1.5</td>
<td>3.6 ± 0.9</td>
<td>79.3 ± 2.8</td>
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### Table S3. Stoichiometry calculation for 8 corralled supported lipid bilayers. The average (± one standard deviation) stoichiometry coefficients a and b, for c=1, from individual samples were obtained from the constant fraction regime (i.e. $a = Y_{\text{PSM}}/Y_{\text{CHOL}}$ and $b = Y_{\text{GM1}}/Y_{\text{CHOL}}$, for c=1 and d < 10µm).

<table>
<thead>
<tr>
<th>Corralled SLB</th>
<th>PSM:CHOL</th>
<th>GM1:CHOL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$a \pm 1\sigma$</td>
<td>$b \pm 1\sigma$</td>
</tr>
<tr>
<td>1 (Fig. 5 and Fig. 6)</td>
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<td>2.6 ± 0.2</td>
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<tr>
<td>2</td>
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<tr>
<td>3</td>
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<td>2.1 ± 0.1</td>
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<td>8</td>
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<tr>
<td>Avg. ± Std. Dev</td>
<td>3.7 ± 0.8</td>
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Supplementary Discussion:

Overall Composition of SLB Populations

The average overall composition for this SLB population is significantly different, especially for G\textsubscript{M1} and cholesterol, from that of the lipid mixture used to prepare GUVs for SLB formation (8 mol\% PSM, 1 mol\% G\textsubscript{M1}, 16 mol\% cholesterol and 75 mol\% DOPC). The difference in composition between the starting lipid mixture and the final reorganized SLB has been documented in earlier work for a mixture prepared with the same components, but at different molar proportions to yield a phase separated SLB at room temperature.\textsuperscript{1} The standard deviations measured for each component (i.e. ±1.3 mol\% PSM, ±1.5 mol\% G\textsubscript{M1}, ±0.9 mol\% CHOL, and ±2.8 mol\% DOPC) in this SLB population indicate minimal SLB-to-SLB compositional variation. This result is quite different from what we reported on phase separated SLB patches prepared with these components.\textsuperscript{1} In the phase separated case we observed four to ten times larger standard deviations for all components except DOPC, indicating significant sample-to-sample variation (i.e. ±12.4 mol\% sphingomyelin, ±10.9 mol\% G\textsubscript{M1}, ±4.1 mol\% cholesterol, and ±2.9 mol\% DOPC). This suggests that samples prepared with lipid mixtures that yield microscopically single phase SLBs as described here have minimal sample-to-sample variations whereas lipid mixtures that yield phase separated bilayers have significant sample-to-sample variations.

Potential Leaflet Asymmetry and Phase Behavior

As discussed in the text, there is evidence that G\textsubscript{M1} is found largely in the distal leaflet when SLBs are formed from small unilamellar vesicles.\textsuperscript{2} This asymmetric distribution of G\textsubscript{M1} presumably occurs when these small vesicle fuse to the surface or form islands in order to minimize charge repulsion between G\textsubscript{M1} and the support. The SLBs in our experiments are formed from single GUVs, so the edges, where the exchange is most likely to occur are much further away on average, though transfer could occur through defects. If G\textsubscript{M1} did flip-flop upon SLB formation, it could also bring PSM and/or CHOL with it, causing these components to also become asymmetrically distributed. We consider the phase behavior for the leaflet compositions that would result from PSM/CHOL flip-flop by mapping them onto a phase diagram obtained from fluorescence imaging analysis of GUVs containing DOPC/PSM/CHOL.\textsuperscript{3} In this analysis, we assume the phase behavior is dictated only by DOPC/PSM/CHOL content (i.e. the effect of a small concentration of G\textsubscript{M1} on phase behavior is assumed to be negligible). The composition used for the analysis of complex stoichiometry lies near a phase boundary on the ternary phase diagram for DOPC/PSM/CHOL. As a result, significant flip-flop of PSM, CHOL, or both PSM and cholesterol should push the composition of the distal leaflet across the phase boundary into phase separating regimes of the phase diagram. As no phase separation is observed with fluorescence microscopy or NanoSIMS, we conclude that flip-flop of PSM and CHOL is not significant. While the analysis presented here is at best suggestive, we are led to the most likely scenario that the average stoichiometry for the cluster with this lipid composition is 4:2:1 DOPC:PSM:CHOL.
References