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Ganglioside Influence on Phospholipid Films Investigated with Single Molecule Fluorescence Measurements

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Abstract

Single molecule fluorescence measurements are used to probe the effects of GM1 in DPPC monolayers. Langmuir-Blodgett films of GM1 and DPPC were doped with ~10^{-8} mol% of the fluorescent lipid probe, BODIPY-PC, and transferred onto glass substrates at 23 mN/m. As shown previously, the individual orientation of each BODIPY-PC probe in the membrane can be measured using defocused polarized total internal reflection fluorescence microscopy, revealing changes in film properties at the molecular level. Here, BODIPY-PC tilt angle histograms are used to characterize the effects of GM1 in DPPC films from 0.05 mol% to 100 mol% GM1. At high GM1 levels (>5 mol% GM1), trends in the single molecule measurements agree with previous bulk measurements showing the turnover from condensing to expanding influence of GM1 at ~20 mol%, thus validating the single molecule approach. At biologically relevant, low concentrations of GM1 (<5 mol% GM1), where bulk fluorescence measurements are less informative, the single molecule measurements reveal a marked influence of GM1 on film properties. The addition of trace amounts of GM1 to DPPC films leads to an expansion of the film which continues to 0.10 mol% GM1, above which the trend reverses and the condensing effect previously noted is observed.

Keywords

GM1; DPPC; fluorescence; partitioning; Langmuir-Blodgett films; total internal reflection

INTRODUCTION

Understanding the functional roles of lipids in biological membranes has continued to evolve since the introduction of the fluid mosaic model in 1972.[1] Far from being passive matrices that simply support membrane constituents, lipids now appear to provide active roles in organizing and modulating events at the cellular membrane. One of the more intriguing and enigmatic roles that has emerged from these studies involves the formation of nanometric lipid domains termed lipid rafts.[2, 3] These small, dynamic domains represent compositional heterogeneities in the membrane that are thought to be important in signaling and organizational processes.[4, 5]

The formation of domains in complex mixtures, such as those found in biomembranes, is neither surprising nor unexpected. Characterizing their physical, chemical, and biological properties, however, has proven daunting in natural membranes given the complexity of the system and often lack of clear controls. Moreover, the small size and dynamic nature of lipid rafts makes them difficult to probe directly in intact membranes, which has led to some

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controversy in the literature.[5–7] These challenges have led to the widespread use of model membranes where specific interactions can be probed in highly controlled environments.[8, 9] These studies have proven invaluable for understanding how putative raft components interact in various lipid environments and in identifying key interactions that modify membrane structure. For example, these studies have characterized the formation of domains in lipid membranes,[10, 11] elucidated the effect of cholesterol on membrane fluidity,[12] and characterized the complex partitioning of lipid raft components.[13] For the latter, studies involving ganglioside GM1, a putative raft component, illustrate the complexity that can be encountered even in simple membrane mixtures.[14–21]

GM1 is part of a larger class of glycolipids found predominantly in the outer leaflet of animal cell membranes. GM1 is a member of the ganglioside family whose members contain oligosaccharides with one or more sialic acid residues, giving them a net negative charge. Over 100 gangliosides have been identified in vertebrate cells and their incorporation into membranes appears ubiquitous.[22] While they represent only a minor component in most cells, they are enriched in neuronal membranes where they can comprise ~2 to 10% of the total lipid content.[23, 24] Among other functions, they appear to play roles in cell recognition, signal transduction, and as receptors for viruses and toxins.[25–28] Ganglioside GM1, for example, is a cell-surface receptor for cholera toxin, the bacterial toxin which leads to the incapacitating diarrhea of cholera.

GM1 is anchored in the external leaflet of cellular bilayers through its hydrophobic ceramide group while its bulky oligosaccharide region is exposed to the extracellular milieu. Structural diversity within the ceramide group has recently been shown to influence how GM1 traffics cholera toxin from the plasma membrane to the endoplasmic reticulum, illustrating the complex structure/function relationships associated with GM1.[29] This complexity is also manifest in model membrane studies which have revealed complex partitioning of GM1 between lipid phases.[14–21]

Atomic force microscopy (AFM) studies of model membranes have shown that GM1 partitions into condensed domains at low GM1 concentrations.[14–19] In Langmuir-Blodgett (LB) mixed monolayers of DOPC and DPPC, for example, the addition of GM1 (0.2 mol% to 1 mol%) leads to the formation of nanometric domains within condensed regions of the film.[18] As the GM1 concentration is increased to 4 mol%, the number of these domains increases accompanied by the formation of elongated domains along the condensed phase boundaries. These studies also identified small domains in the expanded membrane regions at higher GM1 concentrations.

Studies on more complicated mixtures have found essentially the same trends. AFM studies on supported membranes of sphingomyelin, DOPC, and cholesterol found evidence for GM1 partitioning into condensed domains rich in cholesterol and sphingomyelin.[15] As GM1 was increased to 5 mol%, additional GM1 rich condensed microdomains were observed in the expanded regions of the films. These and related studies largely agree that GM1 segregates into condensed domains at low mole percentages and experiences expanded phase partitioning at higher concentrations.

Isotherm and X-ray diffraction studies have shown that pure GM1 monolayers remain fluid at all surface pressures, which is attributed to the bulky charged headgroup of GM1.[14, 30] Even when the GM1 headgroups are tightly packed at high surface pressures, there remains sufficient freedom for the acyl tails to adopt disordered configurations. These observations raise intriguing questions regarding the mechanism that drives GM1 to partition within condensed domains in mixed membranes. In mixed monolayers with the zwitterionic lipid DPPC, for example, GM1 has a condensing effect on the monolayer at low mole

percentages.[14] Fluorescence studies of phase partitioning have found a gradual growth in the liquid condensed (LC) domains with increasing GM1 concentration from 10 mol% to 25 mol%. Interestingly, as the GM1 component is increased above 25 mol%, the fluorescence measurements revealed a reversal in the trend. At the higher GM1 levels, the LC domains gradually diminish as the film becomes expanded, eventually becoming purely liquid expanded (LE) at high GM1 concentration. The transition from condensing to expanding effect observed at 25 mol% was interpreted as reflecting a stoichiometric interaction between GM1 and DPPC that leads to an optimal packing in the membrane. Once the GM1 component exceeds this stoichiometric limit, additional GM1 expands the membrane.

These measurements reveal the complicated influence that GM1 can have on membrane structure. Due to experimental constraints, however, most studies have focused on relatively high GM1 concentrations, which are not reflective of the typically low levels found in most natural membranes. The phase structure observed in fluorescence measurements at low GM1 levels, for instance, is ill-defined which limits the amount of useful information that can be extracted from these measurements. This places a practical limit of ~5 mol% GM1 in these studies. Recently, we have shown that single molecule fluorescence measurements can provide a complementary view into membrane structure.[31–36] These studies characterize the orientation of fluorescent lipid analogs doped into membranes at trace levels which have been shown to be sensitive to the surrounding lipid matrix. These measurements have been used to characterize changes in membrane structure due to surface pressure, the addition of additives such as cholesterol, and ambient humidity levels.[31–34] Here we use this approach to probe changes in DPPC structure at the molecular level with the addition of GM1.

Single molecule fluorescence measurements on Langmuir-Blodgett (LB) mixed monolayers of DPPC and GM1 are studied over the GM1 concentration range of 0.05 mol% to 100 mol %. The low concentration range is several orders of magnitude lower than previous fluorescence studies of GM1 influence and reveals significant changes occur in the monolayers even at these low levels. At the higher concentration range, the single molecule measurements of membrane structure reveal complicated trends with GM1 that are consistent with previous bulk studies in model systems.[14, 15, 18] The trends observed from 0.05 mol% to 0.10 mol% GM1 in DPPC suggest that GM1 partitions into the expanded phase at low concentrations and only above 0.10 mol% begins to partition into the condensed regions as observed previously. Thus, the single molecule orientation measurements presented here offer new insight into the complex phase partitioning of GM1 at the low concentrations typically observed in most biological cell types.

EXPERIMENTAL METHODS

Dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylserine (DPPS), and ganglioside GM1 (GM1) (Avanti Polar Lipids, Alabaster, AL) were obtained at >99% purity and used without further purification. Lipid stock solutions of DPPC were prepared at 1 mg/mL in chloroform. Stock solutions containing DPPS or GM1 were prepared at 1 mg/mL in a 65:35 volume mixture of chloroform and methanol. The fluorescent lipid analog 2-(5-butyl-4,4-difluoro-4-bora-3a,4a-diaza-s-indacene-3-nonanoyl)-1-hexadecanoyl-sn-glycero-3-phosphocholine (BODIPY-PC) (Invitrogen Corporation, Carlsbad, CA) was diluted in methanol to obtain appropriate working concentrations. The chemical structures for the lipids employed in this study are shown in Figure 1.

Lipid monolayers prepared for bulk fluorescence studies were doped with 0.5 mol% BODIPY-PC dye, while monolayers prepared for single molecule measurements were doped with ~10^{-8} mol% of the reporter dye. Approximately 50 μL of the appropriate lipid solution was dispersed on an 18MΩ water subphase in a Langmuir-Blodgett trough (Type 611, Nima

Technology, Coventry, England). The solvent was allowed to evaporate for at least 15 min prior to initiating compression/expansion cycles to anneal the film. Each monolayer was subjected to two compression/expansion cycles between surface pressures of 40 mN/m and 10 mN/m. The barrier rate during these cycles was held at 100 cm^2/min. Following the last expansion, the monolayers were compressed to the desired target pressure and held for ~10 min prior to transfer onto a solid substrate. Monolayers were transferred on to Piranha-cleaned glass coverslips in a headgroup down arrangement using a dipping velocity of 5 mm/min. All monolayers were transferred and imaged at 22 °C.

Monolayers were imaged with a total internal reflection microscope (TIRF-M) (Olympus IX71, Olympus, Center Valley, PA) equipped with a 100x, 1.45 NA objective (Achromat, Olympus, Center Valley, PA) for single molecule imaging, and a 60x, 1.45 NA (Achromat, Olympus) objective for bulk fluorescence imaging. P-polarized excitation was generated at the sample by directing the 514nm line from an argon ion laser (Coherent Innova 90, Coherent Inc., Santa Clara, CA) through half-wave and quarter-wave plates (Newport, Irvine, CA). Bulk and single molecule fluorescence images were collected in an epi-illumination configuration with a cooled CCD camera (Retiga 1300, Q Imaging, Surrey, BC, Canada). Single molecule orientation measurements were collected with the optics defocused ~500nm using a piezo focusing collar (Mad City Labs Inc., Madison, WI) on the objective. The defocus distance was consistent with single molecule emission pattern simulations using MATLAB (Mathworks, Natick, MA). The TIRF angle was controlled by positioning the excitation light in the back aperture of the objective to achieve an incident angle just greater than the critical angle of 41.4°. Image collection was controlled using Slidebook software (Intelligent Imaging Innovations, Denver, CO) with 500 ms integrations and no binning used in the single molecule captures. Bulk fluorescence and single molecule images were analyzed in ImageJ (U.S. National Institutes of Health, Bethesda, MD) and MATLAB (Mathworks, Natick, MA), respectively, as discussed elsewhere.[31]

In this work, we report the measured tilt angles of the individual BODIPY-PC molecules doped into supported monolayers containing DPPC, GM1, and/or DPPS. Each monolayer condition was studied at multiple areas of at least 3 separate films, and N represents the total number of single molecule orientations measured at each monolayer condition. The following monolayer conditions are included in this study: pure DPPC monolayers deposited at 7 mN/m (N= 324), 23 mN/m (N= 363), and 40 mN/m (N= 418); pure GM1 monolayers deposited at 7 mN/m (N= 258), 23 mN/m (N= 375), and 40 mN/m (N= 328); mixed monolayers of DPPC/GM1 deposited at 23 mN/m containing 0 (N= 363), 0.05 (N= 594), 0.1 (N= 865), 0.5 (N= 1013), 1 (N= 512), 5 (N= 980), 10 (N= 1312), 15 (N= 960), 20 (N= 653), 33 (N= 926), 50 (N= 605) and 100 (N= 375) mol% GM1; and mixed monolayers of DPPC/DPPS deposited at 23 mN/m containing 0 (N= 363), 0.05 (N= 603), 0.1 (N= 623), 1 (N= 638), 5 (N= 386), 20 (N= 764) mol% DPPS.

RESULTS AND DISCUSSION

The macroscopic membrane structure of DPPC monolayers doped with increasing amounts of GM1 was characterized using fluorescence microscopy with the results shown in Figure 2. The DPPC/GM1 monolayers were doped with 0.5 mol% of the fluorescent lipid analog BODIPY-PC and transferred onto glass substrates at a surface pressure of 23 mN/m using the LB technique. This surface pressure has previously been found to mimic the effective surface pressure of bilayers, although higher surface pressures have also been reported.[32] The bright areas of the monolayers shown in Figure 2 denote liquid expanded (LE) phase regions of the membrane that incorporate the BODIPY-PC probe while the dark areas reflect liquid condensed (LC) domains. In agreement with previous studies, the series of images

shown in Figure 2 reveal a striking evolution in monolayer structure as the GM1 concentration is increased from 0 to 60 mol%.[14]

At low GM1 levels (<5 mol%), the dark LC domains are relatively irregular in shape and lack boundary definition. At approximately 5 mol% GM1, the LC domains take on a distinctive shape and the LC and LE phases become clearly defined, with sharp borders marking the transitions between the phases. As seen previously, as the mol% of GM1 is increased the percent LC area of the monolayer steadily increases up to approximately 20 mol% GM1. Above this concentration, additional GM1 leads to the steady reduction in LC domain size and increase in the expanded regions of the membrane. The trends observed in Figure 2 are quantified in Figure 3 which plots the percent area of the LC phase as a function of GM1 concentration.

As shown in Figure 3, the percent area coverage of LC phase in the DPPC/GM1 mixture increases up to ~20 mol% GM1, after which the percent area steadily decreases as more GM1 is added. Previous studies have found similar trends and suggest that GM1 initially partitions into the condensed regions of membranes at low mole percentages.[18] Molecular area measurements suggest DPPC and GM1 mix stoichiometrically in these condensed domains, forming complexes with reduced area compared to the sum of the individual components.[14] Increasing GM1 concentration beyond the optimal packing ratio leads to GM1 appearing in the LE phase leading to increasingly expanded films above ~20 mol% GM1. The results shown in Figures 2 and 3, therefore, are consistent with previously observed trends.

As illustrated in Figure 2, fluorescence microscopy is useful in visualizing and characterizing macroscopic phase separation in supported lipid monolayers of DPPC and GM1 when GM1 concentrations are above ~5 mol%. These approaches, therefore, can add insight into the role of GM1 in biological systems such as neuronal membranes where the GM1 component is highly enriched and can reach levels as high as ~2 to 10 mol%.[23] For the majority of biological membranes, however, gangliosides represent only a minor fraction of the membrane composition which raises intriguing questions about their role in modifying membrane structure at low abundance.[24] As shown in Figure 2, fluorescence microscopy measurements become less informative at GM1 concentrations less than ~5 mol% as the macroscopic phase structure and effects of GM1 on film properties become less well defined.

Previously, we have shown that single molecule fluorescence measurements can provide new insight into membrane properties.[31–34] These measurements track changes in the orientation of fluorescent lipid analogs doped into films at trace levels and have been shown to be sensitive to changes in film surface pressure and the presence of additives.[31–34] These single molecule fluorescence measurements use defocused polarized total internal fluorescence microscopy (PTIRF-M) to excite and image fluorescent probes doped into lipid membranes at ~10^{-8} mol%. As an example, Figure 4 compares defocused single molecule PTIRF-M images of BODIPY-PC doped into pure monolayers of DPPC and GM1. Monolayers transferred onto glass substrates at low (π = 7 mN/m) and high (π = 40 mN/m) surface pressures using the LB technique are compared.

As discussed elsewhere, the single molecule emission patterns observed in the defocused PTIRF-M images reflect the three-dimensional orientation of the probe emission dipole in the lipid matrix.[31] Emission dipoles oriented normal to the membrane plane, for example, lead to donut-like emission features while dipoles oriented in the membrane plane appear as elliptical features with wings. In Figure 4, for instance, an evolution in the single molecule emission patterns from predominantly elliptical with wings to doughnut-like is observed for

DPPC in going from low to high pressure, respectively. The BOPIDY-PC probe shown in Figure 1 has an acyl-linked fluorophore and anisotropy studies have shown that the emission dipole lies approximately along the long axis of the conjugated ring system.[37] The location of the fluorophore in the tail region makes its orientation sensitive to the structure of the surrounding lipid matrix. As the surface pressure of DPPC is increased, therefore, more doughnut-like emission features are observed as BODIPY-PC tails adopt an elongated conformation.

The results for DPPC can be contrasted with single molecule orientation measurements of BODIPY-PC doped into monolayers of GM1. The fluorescence images shown in Figure 4 reveal little change in the single molecule emission patterns with surface pressure for BODIPY-PC doped into GM1 monolayers. Qualitatively, the lack of donut-like emission features suggests that most emission dipoles lie in the membrane plane. The similarity between the images collected at low and high surface pressure indicates that surface pressure has little effect on the BODIPY-PC probe orientation in GM1 films. This is consistent with previous measurements showing GM1 films remain expanded over the surface pressures studied.

The emission patterns, such as those shown in Figure 4, can be analyzed to extract both the polar (ϕ) and azimuthal (θ) angles for each emission dipole in the image. This orientation information is used to populate histograms of the polar (ϕ) or tilt angle of the emission dipole with respect to the membrane normal. Previous studies have shown that the tilt angle of BODIPY-PC within supported lipid monolayers is a sensitive probe of the surrounding membrane environment. Tilt angle histograms for BODIPY-PC in monolayers of pure DPPC and pure GM1 at the surface pressures studied in Figure 4 are compared in Figure 5.

For BODIPY-PC in DPPC, Figure 5 reveals a bimodal distribution of probes predominantly oriented either normal ($\phi \leq 10°$) or parallel ($\phi \geq 81°$) to the membrane plane, with only a small fraction occupying intermediate tilt angles. Increasing the surface pressure leads to a shift in population from parallel to normal oriented probes, with little change in the intermediate tilt population. The reduced area per lipid at higher surface pressures in DPPC favors an elongated acyl chain configuration leading to normal oriented emission features. By tracking the proportion of BODIPY-PC probes oriented normal ($\phi \leq 10°$) to the membrane plane, known henceforth as the *ordered abundance*, changes in the molecular level structure of the monolayer can be characterized for DPPC. The ordered abundance plotted in Figure 5 shows an increasing trend with surface pressure. This approach has been used previously to probe DPPC membranes as a function of relative humidity, the addition of additives such as cholesterol, and membrane surface pressure.[31–34]

The results for GM1 show markedly different results. The tilt angle histograms show that the vast majority of the BODIPY-PC probes are oriented with their emission dipole lying in the plane of the film. Moreover, the tilt angle histograms measured at the surface pressures for GM1 are statistically indistinguishable, indicating that changes in monolayer surface pressure have little effect on BODIPY-PC orientation. As mentioned earlier, these measurements are consistent with bulk studies that have shown that GM1 films remain expanded at all surface pressures.[30]

The results summarized in Figures 4 and 5 show that single molecule measurements of membrane order lead to dramatically different trends for films of DPPC and GM1 as a function of surface pressure. To probe the effects of GM1 on DPPC, similar measurements were carried out in mixed monolayers doped with ~10^{-8} mol% BODIPY-PC. Single molecule defocused P-TIRF-M studies of DPPC/GM1 monolayers as a function of GM1 concentration were measured on monolayers transferred at 23 mN/m. At each GM1

concentration, single molecule emission patterns were measured and modeled to extract the tilt angle of the BODIPY-PC probes. As shown in Figure 4, the ordered abundance or proportion of probes oriented normal ($\phi \leq 10°$) to the membrane plane provides a useful metric for tracking changes in film structure. Therefore, Figure 6 plots the ordered abundance extracted from tilt angle histograms of BODIPY-PC in DPPC/GM1 monolayers as a function of GM1 concentration. Each point in Figure 6 represents the ordered abundance extracted from the measured tilt angle of at least 350 individual molecules from least 3 separate films.

Figure 6 reveals a complicated trend in BODIPY-PC orientation with increasing GM1 levels. A sharp decrease in the single molecule ordered abundance is observed immediately upon the addition of GM1 to DPPC. The ordered abundance drops from ~30% in pure DPPC to just over 10% with the addition of 0.1 mol% GM1. As GM1 levels increase, however, this trend reverses. As shown in Figure 6, the ordered abundance increases to 24% with the addition of 0.5 mol% GM1 and continues to rise back to ~30% at 15 mol% GM1. This value is approximately the same as that measured in pure DPPC containing no GM1 at this surface pressure. As GM1 levels increase above 15 mol%, however, the single molecule ordered abundance again decreases towards a final value of 3% observed in pure GM1 monolayers transferred at 23 mN/m.

Comparing the trends observed in Figures. 3 and 6 reveals close agreement between the single molecule orientation measurements and bulk fluorescence measurements of film structure when GM1 levels are greater than 5 mol%. Both trends reflect increasing film order as GM1 levels rise from 5 to 15 mol% GM1. The single molecule measurements and ensemble fluorescence studies also sense the reversal in film order above approximately 20 mol% GM1, as increasing GM1 levels expand the film. At low GM1 levels, however, the single molecule orientation measurements reflect changes in film structure that are not apparent in the bulk fluorescence measurements. This distinction is significant since GM1 is usually present at low levels in most biological membranes.

Figure 6 reveals the profound effect the addition of GM1 has on the molecular orientation of BODIPY-PC in DPPC films even when added at trace amounts. While bulk fluorescence measurements reveal no significant change in macroscopic membrane structure at low mol% GM1, single molecule orientation measurements reveal substantial changes occur in the molecular level structure of the film upon the addition of trace amounts of GM1. As shown in Figure 6, the addition of just 0.05 mol% GM1 causes a 10% drop in the single molecule ordered abundance. This drops another 10% as the GM1 content is increased to 0.1 mol%. These results suggest that even small additions of GM1 to DPPC can lead to significant structural changes in the film which are reflected in the single molecule orientation measurements, but hidden in bulk fluorescence measurements.

Previous single orientation studies have shown that BODIPY-PC orientation in lipid monolayers is influenced by the surrounding lipid acyl chain ordering and electrostatic interactions between the fluorophore and the lipid headgroups.[34] GM1 is a glycosphingolipid, containing neutral sugars and a single negatively charged sialic acid residue in the headgroup. This charge contributes to the electrostatic potential of the membrane and can influence the orientation of nearby BODIPY-PC probe molecules through electrostatic interaction.[38] To confirm that nearby charged headgroups can alter BODIPY-PC orientation, studies in mixed monolayers of DPPC and negatively charged DPPS were conducted. As shown in Figure 1, DPPS contains a negatively charged serine group but is otherwise structurally similar to DPPC.

Single molecule orientation measurements of mixed monolayers of DPPC and DPPS were carried out as a function of DPPS concentration. As in the GM1 studies, the monolayers were doped with 10^{-8} mol% BODIPY-PC and transferred at 23 mN/m. As before, the single molecule emission patterns were imaged and analyzed to create tilt angle histograms, from which the ordered abundance was extracted and plotted as shown in Figure 7. As seen in Figure 7, the single molecule ordered abundance immediately drops upon the addition of small mole percentages of DPPS to the DPPC film. The ordered abundance drops from ~30% in pure DPPC to ~10% upon the addition of just 0.1 mol% DPPS, similar to trends observed with the addition of GM1. Unlike the GM1 results, however, the ordered abundance levels off and remains roughly constant as the concentration of DPPS is increased further.

The results shown in Figure 7 suggest that the negatively charged headgroups of DPPS can influence the orientation of nearby BODIPY-PC probes in the mixed monolayer, leading to decreased abundance of normal oriented probes. The addition of either DPPS or GM1 to DPPC causes a similarly sharp decrease in ordered abundance at low concentrations, suggesting a general mechanism based on favorable electrostatic interactions between the BODIPY-PC fluorophore and the negatively charged headgroups. The sharp decrease may also reflect a disruption in acyl chain packing. However, given the similarity in response to the addition of DPPS and GM1 at low concentrations, an electrostatic mechanism is favored. Given the propensity of BODIPY-PC to partition into the expanded phase and proximity required for GM1 to influence BODIPY-PC orientation, these results further suggest that GM1 partitions into expanded membrane regions at low concentrations.

At higher concentrations, a significant divergence is observed when comparing the effects that GM1 and DPPS have on the BODIPY-PC probe. Figure 7 shows that the ordered abundance reaches a minimum at ~0.1 mol% DPPS and remains level as more DPPS is added to DPPC. Previous reports have shown that DPPC and DPPS are miscible in the absence of calcium ions.[39] The leveling of ordered abundance with increasing DPPS concentration reflects a saturation of its effect on the BODIPY-PC probe. The results for GM1 shown in Figure 5, however, reveal a more complicated evolution in probe orientation.

As GM1 concentration is increased from ~0.1 to ~15 mol%, the single molecule ordered abundance increases to a maximum value equal to pure DPPC films at this surface pressure. This is consistent with a mechanism where GM1 moves from the expanded phase to condensed domains where it packs tightly with condensed phase DPPC. This segregates GM1 from the expanded phase where the BODIPY-PC probe resides, thus leading to the recovery in normal oriented probes. As GM1 concentration is increased above ~20 mol%, the single molecule ordered abundance again decreases as GM1 begins partitioning back into the expanded regions of the film, where it can interact with the BODIPY-PC probe. This is consistent with previous fluorescence studies and results such as those shown in Figures. 2 and 3.[14]

The single molecule measurements presented here add new insight into the influence that GM1 has on membrane structure at the low GM1 concentrations found in most biological membranes. The significant influence that GM1 has on the BODIPY-PC probe orientation at low GM1 concentration suggests there is a close proximity between the two, placing GM1 in the expanded regions of the membrane. Given previous observations of stoichiometric interactions between GM1 and DPPC, this may reflect the formation of small GM1/DPPC condensed domains in the expanded membrane regions of the film.[14] It is interesting to note that AFM studies have also found evidence for GM1 rich domains in the expanded domains of model membranes.[15, 18] While not conforming to the working definition of a lipid raft due to the lack of cholesterol, these structures may nonetheless provide organizational or

signaling platforms in cellular membranes. These measurements also help illustrate the utility of single molecule orientation measurements for providing new complementary views into membrane structure.

CONCLUSION

Single molecule fluorescence measurements of the fluorescent lipid analog BODIPY-PC are used to probe the effects of GM1 in monolayers of DPPC. Defocused TIRF-M measurements lead to distinct single molecule fluorescence emission patterns which reflect the three-dimensional orientation of each BODIPY-PC probe molecule doped into the film. As shown previously, extracting the tilt angle of the emission dipole with respect to the membrane normal provides a sensitive marker for structural changes taking place in the lipid film. Using this approach, we track the effects of GM1 on LB monolayers of DPPC from 0.05 mol% to 100 mol% GM1. Above 5 mol% GM1, the single molecule measurements closely track trends seen in bulk measurements of membrane structure. At low GM1 levels, however, the single molecule measurements reveal dramatic changes in film properties that are hidden in bulk fluorescence measurements. Large changes are observed in the single molecule tilt histograms with the addition of just 0.05 mol% GM1 to the DPPC monolayer. Control studies using DPPS suggest that electrostatic interactions between the charged headgroup of GM1 and BODIPY-PC probe play an important role in influencing the measured tilt of the emission dipole. These measurements suggest that GM1 initially partitions into the expanded phase, in close proximity to the BODIPY-PC probe where it influences the tilt angle distribution. As GM1 levels increase, GM1 partitions into condensed domains leading to a recovery in the BODIPY-PC tilt angle distribution as GM1 is segregated away from the probe in the membrane. Finally at high GM1 levels, GM1 appears in the expanded phase again and eventually leads to homogenously fluid membranes. The significant influence that GM1 exerts at low levels is interesting since it is only a minor component in most biological membranes. The formation of small, GM1 rich domains in the expanded phase is also intriguing given the evolving view of the importance of small membrane domains in cellular organization and signaling. Single molecule orientation measurements, therefore, provide a promising new tool for probing these enigmatic structures.

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References

1. Singer SJ, Nicolson GL. The fluid mosaic model of the structure of cell membranes. Science. 1972; 175:720–731. [PubMed: 4333397]
2. Simons K, Ikonen E. Functional rafts in cell membranes. Nature. 1997; 387:569–572. [PubMed: 9177342]
3. Dietrich C, Bagatolli LA, Volovyk ZN, Thompson NL, Levi M, Jacobson K, Gratton E. Lipid rafts reconstituted in model membranes. Biophys J. 2001; 80:1417–1428. [PubMed: 11222302]
4. Pike LJ. Rafts defined: a report on the Keystone Symposium on Lipid Rafts and Cell Function. J Lipid Res. 2006; 47:1597–1598. [PubMed: 16645198]
5. Simons K, Gerl MJ. Revitalizing membrane rafts: new tools and insights. Nat Rev Mol Cell Bio. 2010; 11:688–699. [PubMed: 20861879]
6. Shaw AS. Lipid rafts: now you see them, now you don’t. Nat Immunol. 2006; 7:1139–1142. [PubMed: 17053798]
7. Munro S. Lipid rafts: Elusive or illusive? Cell. 2003; 115:377–388. [PubMed: 14622593]

8. McConnell HM. Structures and Transitions in Lipid Monolayers at the Air-Water-Interface. Ann Rev Phys Chem. 1991; 42:171–195.

9. Kaganer VM, Mohwald H, Dutta P. Structure and phase transitions in Langmuir monolayers. Rev Mod Phys. 1999; 71:779–819.

10. Silvius JR. Role of cholesterol in lipid raft formation: lessons from lipid model systems. Biochim Biophys Acta-Biomembranes. 2003; 1610:174–183.

11. Brown DA, London E. Structure and Origin of Ordered Lipid Domains in Biological Membranes. J Membr Biol. 1998; 164:103–114. [PubMed: 9662555]

12. Simons K, Ikonen E. Cell biology - How cells handle cholesterol. Science. 2000; 290:1721–1726. [PubMed: 11099405]

13. Samsonov AV, Mihalyov I, Cohen FS. Characterization of cholesterol-sphingomyelin domains and their dynamics in bilayer membranes. Biophys J. 2001; 81:1486–1500. [PubMed: 11509362]

14. Frey SL, Chi EY, Arratia C, Majewski J, Kjaer K, Lee KYC. Condensing and fluidizing effects of ganglioside G(M1) on phospholipid films. Biophys J. 2008; 94:3047–3064. [PubMed: 18192361]

15. Bao R, Li L, Qiu F, Yang YL. Atomic Force Microscopy Study of Ganglioside GM1 Concentration Effect on Lateral Phase Separation of Sphingomyelin/Dioleoylphosphatidylcholine/ Cholesterol Bilayers. J Phys Chem B. 2011; 115:5923–5929. [PubMed: 21526782]

16. Burns AR. Domain structure in model membrane bilayers investigated by simultaneous atomic force microscopy and fluorescence imaging. Langmuir. 2003; 19:8358–8363.

17. Burns AR, Frankel DJ, Buranda T. Local mobility in lipid domains of supported bilayers characterized by atomic force microscopy and fluorescence correlation spectroscopy. Biophys J. 2005; 89:1081–1093. [PubMed: 15879469]

18. Vie V, Van Mau N, Lesniewska E, Goudonnet JP, Heitz F, Le Grimellec C. Distribution of ganglioside G(M1) between two-component, two-phase phosphatidylcholine monolayers. Langmuir. 1998; 14:4574–4583.

19. Yuan CB, Johnston LJ. Distribution of ganglioside GM1 in L-alpha-dipalmitoylphosphatidylcholine/cholesterol monolayers: A model for lipid rafts. Biophys J. 2000; 79:2768–2781. [PubMed: 11053150]

20. Ohta Y, Yokoyama S, Sakai H, Abe M. Membrane properties of binary and ternary systems of ganglioside GM1/dipalmitoylphosphatidylcholine/dioleoylphosphatidylcholine. Colloids Surf B Biointerfaces. 2004; 34:147–153. [PubMed: 15261067]

21. Coban O, Burger M, Laliberte M, Ianoul A, Johnston LJ. Ganglioside partitioning and aggregation in phase-separated monolayers characterized by bodipy GM1 monomer/dimer emission. Langmuir. 2007; 23:6704–6711. [PubMed: 17477552]

22. Yu RK, Tsai YT, Ariga T, Yanagisawa M. Structures, biosynthesis, and functions of gangliosides-an overview. J Oleo Sci. 2011; 60:537–544. [PubMed: 21937853]

23. Derry DM, Wolfe LS. Gangliosides in isolated neurons and glial cells. Science. 1967; 158:1450–1452. [PubMed: 4862383]

24. Sonnino S, Mauri L, Chigorno V, Prinetti A. Gangliosides as components of lipid membrane domains. Glycobiology. 2007; 17:1R–13R.

25. Cheresh DA, Harper JR, Schulz G, Reisfeld RA. Localization of the gangliosides GD2 and GD3 in adhesion plaques and on the surface of human melanoma cells. Proc Natl Acad Sci USA. 1984; 81:5767–5771. [PubMed: 6385004]

26. Burns GF, Lucas CM, Krissansen GW, Werkmeister JA, Scanlon DB, Simpson RJ, Vadas MA. Synergism between membrane gangliosides and Arg-Gly-Asp-directed glycoprotein receptors in attachment to matrix proteins by melanoma cells. J Cell Biol. 1988; 107:1225–1230. [PubMed: 2458363]

27. Hakomori S. Structure and function of sphingoglycolipids in transmembrane signalling and cell-cell interactions. Biochem Soc Trans. 1993; 21:583–595. [PubMed: 8224473]

28. Reed RA, Mattai J, Shipley GG. Interaction of cholera toxin with ganglioside GM1 receptors in supported lipid monolayers. Biochemistry. 1987; 26:824–832. [PubMed: 3567148]

29. Chinnapen DJ, Hsieh WT, te Welscher YM, Saslowsky DE, Kaoutzani L, Brandsma E, D'Auria L, Park H, Wagner JS, Drake, et al. Lipid sorting by ceramide structure from plasma membrane to

ER for the cholera toxin receptor ganglioside GM1. Dev Cell. 2012; 23:573–586. [PubMed: 22975326]

30. Maggio B. The Surface Behavior of Glycosphingolipids in Biomembranes - a New Frontier of Molecular Ecology. Prog Biophys Mol Biol. 1994; 62:55–117. [PubMed: 8085016]
31. Livanec PW, Dunn RC. Single-molecule probes of lipid membrane structure. Langmuir. 2008; 24:14066–14073. [PubMed: 19053664]
32. Livanec PW, Huckabay HA, Dunn RC. Exploring the effects of sterols in model lipid membranes using single-molecule orientations. J Phys Chem B. 2009; 113:10240–10248. [PubMed: 19572622]
33. Huckabay HA, Dunn RC. Hydration Effects on Membrane Structure Probed by Single Molecule Orientations. Langmuir. 2011; 27:2658–2666.
34. Armendariz KP, Huckabay HA, Livanec PW, Dunn RC. Single molecule probes of membrane structure: Orientation of BODIPY probes in DPPC as a function of probe structure. Analyst. 2012; 137:1402–1408. [PubMed: 22322157]
35. Patra D, Gregor I, Enderlein J. Image analysis of defocused single-molecule images for three-dimensional molecule orientation studies. J Phys Chem A. 2004; 108:6836–6841.
36. Toprak E, Enderlein J, Syed S, McKinney SA, Petschek RG, Ha T, Goldman YE, Selvin PR. Defocused orientation and position imaging (DOPI) of myosin V. Proc Natl Acad Sci. 2006; 103:6495–6499. [PubMed: 16614073]
37. Karolin J, Johansson LBA, Strandberg L, Ny T. Fluorescence and Absorption Spectroscopic Properties of Dipyrrometheneboron Difluoride (Bodipy) Derivatives in Liquids, Lipid-Membranes, and Proteins. J Am Chem Soc. 1994; 116:7801–7806.
38. Patel RY, Balaji PV. Characterization of the conformational and orientational dynamics of ganglioside GM1 in a dipalmitoylphosphatidylcholine bilayer by molecular dynamics simulations. Biochim Biophys Acta. 2007; 1768:1628–1640. [PubMed: 17408589]
39. Ross M, Steinem C, Galla HJ, Janshoff A. Visualization of chemical and physical properties of calcium-induced domains in DPPC/DPPS Langmuir-Blodgett layers. Langmuir. 2001; 17:2437–2445.

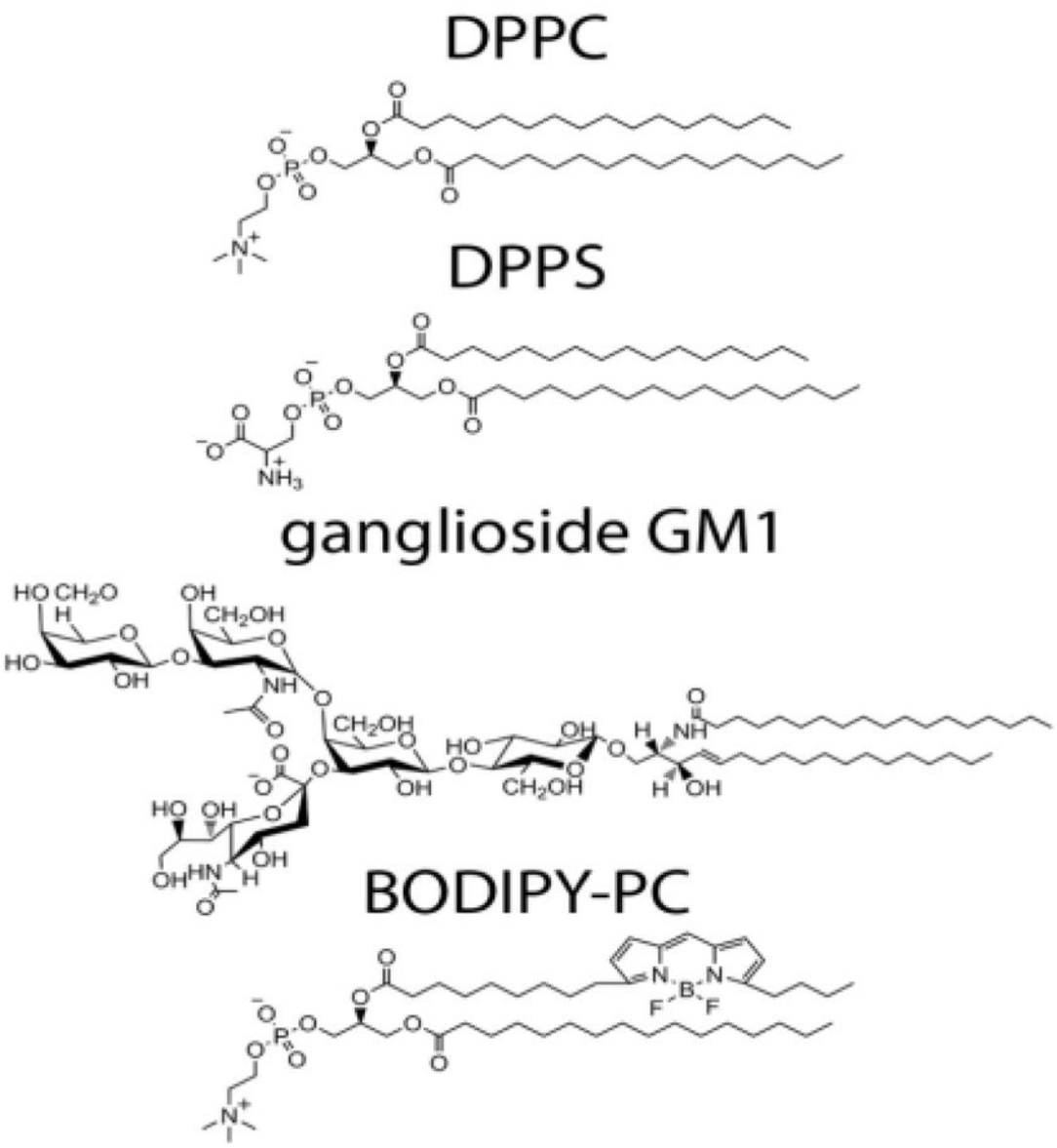

Figure 1.
Chemical structures for DPPC, DPPS, ganglioside GM1, and the fluorescent lipid analog, BODIPY-PC.

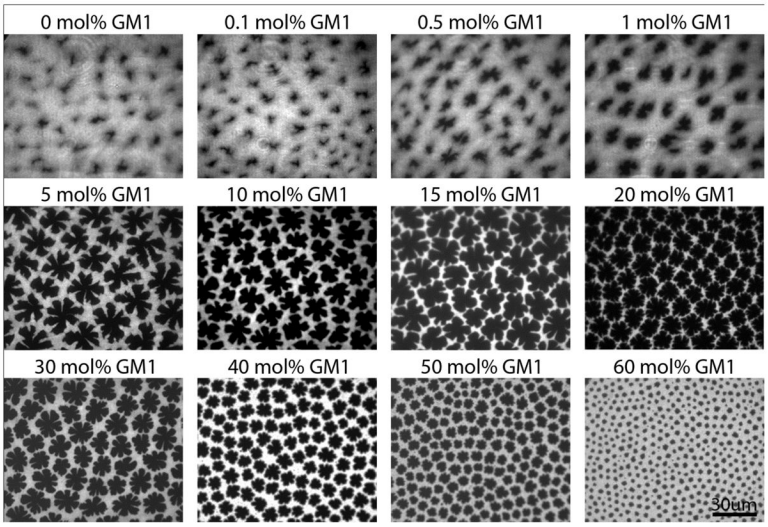

Figure 2.

Fluorescence images of DPPC monolayers containing the indicated concentration of GM1. Monolayers were doped with 0.5 mol% of the fluorescent lipid analog BODIPY-PC and transferred onto a glass substrate at a surface pressure of 23 mN/m using the Langmuir-Blodgett technique. The liquid condensed (LC) phases exclude the BODIPY-PC probe and appear dark, while the liquid expanded (LE) phases incorporate the dye and appear bright. The scale bar is 30 μm.

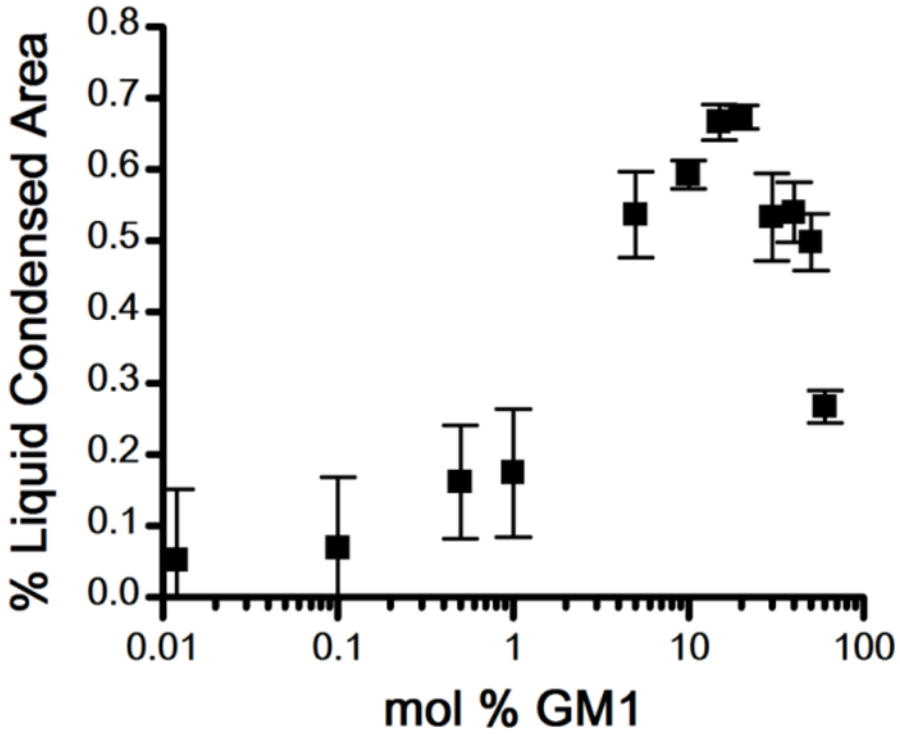

Figure 3.
Plot of the percent coverage of liquid condensed (LC) phase as a function of GM1 concentration, extracted from the fluorescence images shown in Fig. 2. The data points represent 0, 0.1, 0.5, 1, 5, 10, 15, 20, 30, 40, 50, and 60 mol% GM1 in DPPC.

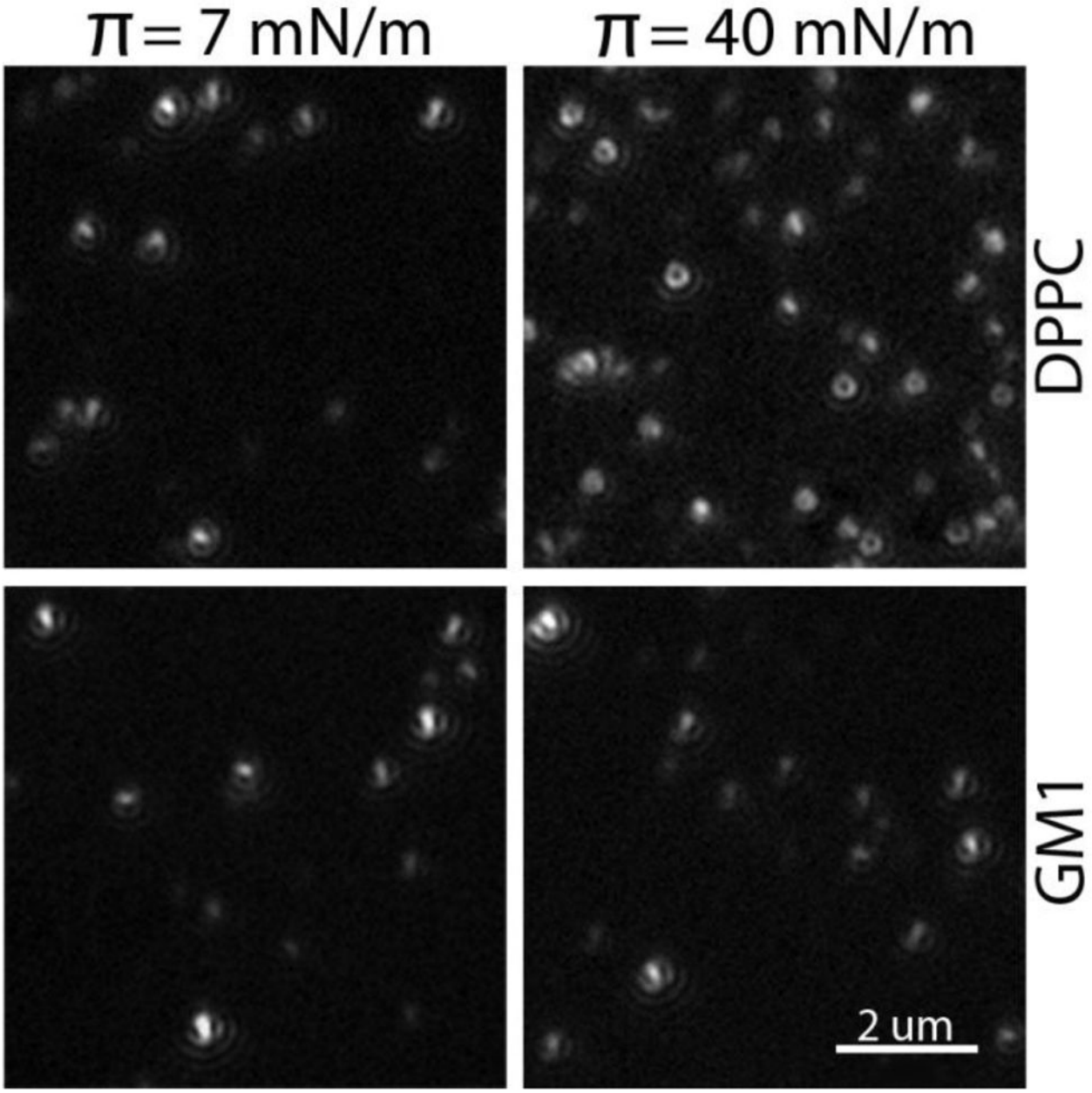

Figure 4.

Representative defocused TIRF-M single molecule fluorescence images of pure DPPC and GM1 LB monolayers doped with ~10^{-8} mol% BODIPY-PC and transferred at 7 mN/m and 40 mN/m. Each bright feature represents the fluorescence from a single BODIPY-PC dye molecule and has a distinctive emission pattern reflective of its orientation within the film. Fluorescent probes with emission dipoles lying in the plane of the film appear as elliptical structures with wings while those oriented perpendicular to the membrane plane exhibit doughnut like structures. For DPPC, a significant change in BODIPY-PC orientation with surface pressure is observed while orientations within GM1 films remain qualitatively the same with surface pressure. The scale bar is 2 μm.

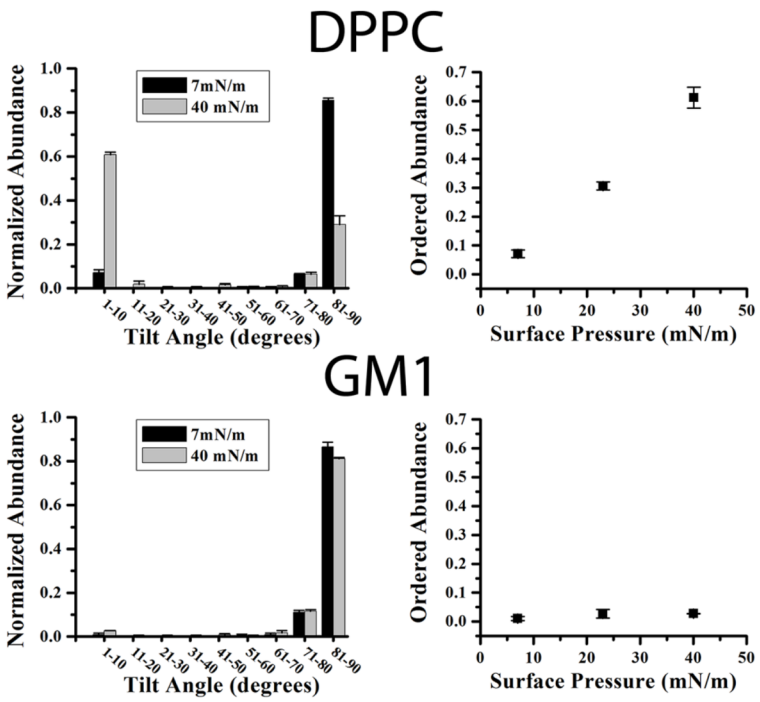

Figure 5.

Tilt angle histograms for BODIPY-PC doped into monolayers of DPPC and GM1 transferred at 7 mN/m and 40 mN/m. Single molecule emission patterns, such as those shown in Fig. 4, are modeled to extract the BODIPY-PC emission dipole tilt angle with respect to the membrane normal to populate each histogram. At each surface pressure studied, more than 250 individual molecules were measured from at least 3 separate monolayers. The ordered abundance or population oriented normal to the surface normal ($\phi \leq$ 10°) at each surface pressure is extracted and plotted as a function of surface pressure. The ordered abundance in DPPC increases with surface pressure while ordered abundance in GM1 remains low at all surface pressures, reflecting its expanded state.

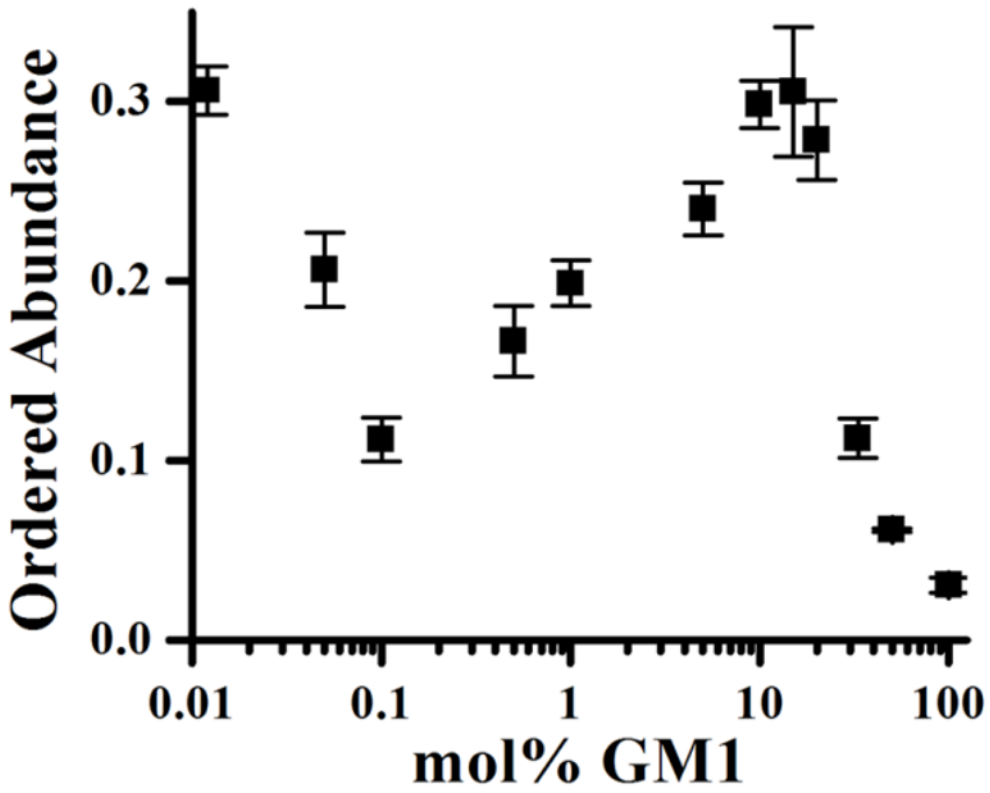

Figure 6.
Plot of the BODIPY-PC single molecule ordered abundance versus mol% GM1 in DPPC monolayers transferred at 23 mN/m. The ordered abundance was quantified using the procedure outlined in Figs. 4 and 5. The data points represent 0, 0.05, 0.1, 0.5, 1, 5, 10, 15, 20, 33, 50, and 100 mol% GM1 in DPPC and are plotted on a log scale for clarity in the low mole percent region. Each point was extracted from tilt angle histograms including at least 350 individual molecules, measured from multiple areas of at least 3 films to ensure representative measurements.

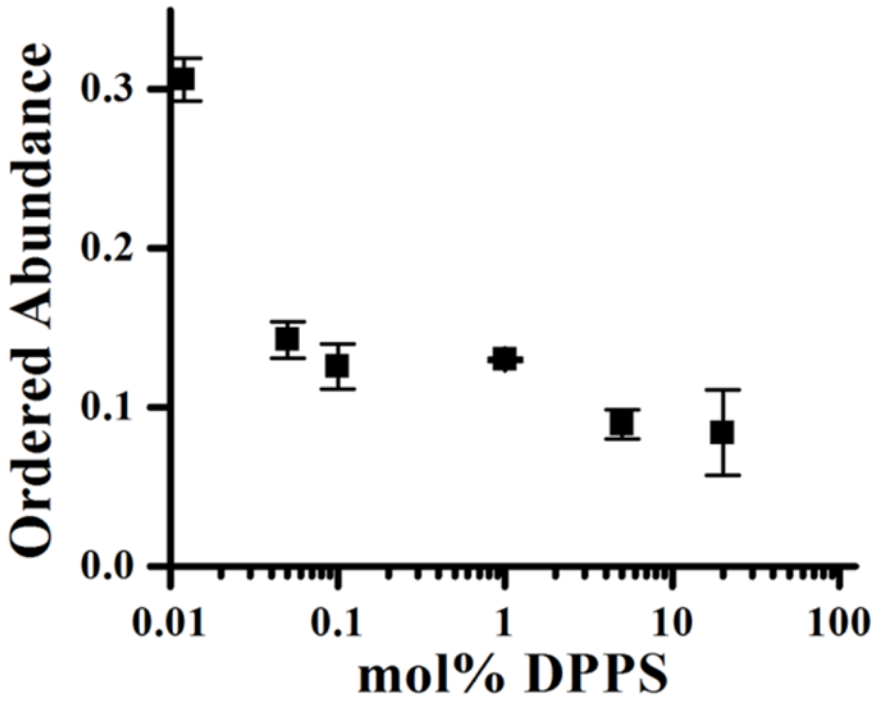

Figure 7.
Plot of the BODIPY-PC single molecule ordered abundance versus mol% DPPS in DPPC monolayers. All monolayers were transferred at 23 mN/m and the data points represent 0, 0.05, 0.1, 1, 5, and 20 mol% DPPS in DPPC. Each point was extracted from tilt angle histograms including at least 350 individual molecules, measured from multiple areas of at least 3 films to ensure representative measurements.