Abstract: The receptor lipid Gb₃ is responsible for the specific internalization of Shiga toxin (STx) into cells. The head group of Gb₃ defines the specificity of STx binding, and the backbone with different fatty acids is expected to influence its localization within membranes impacting membrane organization and protein internalization. To investigate this influence, a set of Gb₃ glycosphingolipids labeled with a BODIPY fluorophore attached to the head group was synthesized. C₂₄ fatty acids, saturated, unsaturated, α-hydroxylated derivatives, and a combination thereof, were attached to the sphingosine backbone. The synthetic Gb₃ glycosphingolipids were reconstituted into coexisting liquid-ordered (lₒ)/liquid-disordered (l_d) giant unilamellar vesicles (GUVs), and STx binding was verified by fluorescence microscopy. Gb₃ with the C₂₄:₀ fatty acid partitioned mostly in the lₒ phase, while the unsaturated C₂₄:₁ fatty acid distributes more into the l_d phase. The α-hydroxylation does not influence its partitioning.

Introduction

The eukaryotic plasma membrane of animals is a heterogeneous structure with a plethora of different lipids. The main lipid components are glycerophospholipids, sterols, and sphingolipids. Among them, glycosphingolipids serve a particular role. They are found in the outer leaflet of the plasma membrane and are discussed to reside preferentially in so-called raft domains, which are enriched in sphingomyelin (SM) and cholesterol (Chol). Their size, chemical composition, and physical characteristics are tightly associated with their signal processing capabilities. Raft domains are supposed to have diameters of 10–200 nm and are highly dynamic structures. This combination of smallness and dynamics bears the major challenge in visualizing raft domains in cellular membranes. Hence, two approaches have been pursued within the last decades to shed some light on the structure and function of these domains. On the one hand, detergent-resistant membranes were extracted from cells and their composition analyzed, however they turned out to be prone to artefacts. On the other hand, artificial membranes with lipid compositions resembling the outer leaflet of the plasma membrane were reconstituted, which separate into a liquid-disordered (l_d) and a liquid-ordered (lₒ) phase. Typical lipid compositions comprise a low-melting glycerophospholipid, a high-melting glycerophospholipid or SM, and Chol. The lₒ phase has loose lateral lipid packing, acyl chains with γγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγγgamma kinks, and fast lateral diffusion. In contrast, the l_d phase is characterized by a tighter lipid packing and a higher degree of order, but still rather fast lateral diffusion. However, the size and physical properties of l_d domains formed in artificial membranes are very different from those found in the plasma membrane. This difference becomes obvious if comparing, for example, the physicochemical properties of coexisting l_d/lₒ phase-separated GUVs with those of phase-separated cell-derived membranes termed giant plasma membrane vesicles (GPMVs). Despite this difference between the natural and artificial membrane systems, artificial coexisting l_d/lₒ membranes have been frequently used to analyze the partitioning of receptor lipids and proteins, such as bacterial toxins, in the different phases.

Bacterial toxins are known to bind to specific glycosphingolipids embedded in the outer leaflet of the plasma membrane. Cholera toxin (CTX) produced by Vibrio cholerae and Shiga toxin (STx) produced by Shigella dysenteriae and by enterohemorrhagic strains of Escherichia coli, both belonging to the class of Aβ toxins, bind specifically to monosialotetrahexosylganglioside (G₃M₃) (14) and globotriaosyl ceramide (Gb₃). (15,16) respectively. While the head groups of the glycosphingolipids indeed define the specificity of protein binding, not much attention has been drawn to the variability of the ceramide backbone harboring different fatty acids. In various cell types (human colon Caco-2, HCT-8 epithelial cells, human endothelial cell lines, primary human umbilical vein endothelial cells, primary human endothelial cells of the
brain and the kidney,[18] and references therein), a conserved repertoire of Gb₃ species was found carrying saturated C₁₆:₀–C₂₂:₀, or C₂₄:₀ fatty acids as well as the unsaturated C₂₄:₁ fatty acid. Results of Lingwood and co-workers[19] suggest that the pathogenic outcome of Shiga toxin producing E. coli (STEC) infections is related to the different Gb₃ species. To gather more molecular information, artificial membranes doped with Gb₃ were employed. In coexisting l/d supported lipid membranes, Gb₃ species differing in their fatty acid gave rise to a different phase behavior before and after binding of the B subunits of STx (STxB) as well as differences in the protein organization on the membrane surface.[20,21] In giant unilamellar vesicles (GUVs), Gb₃ species with an unsaturated acyl chain caused the formation of tubular invaginations upon STxB binding, in contrast to Gb₃ with a saturated acyl chain.[22] In all these studies, it became evident that STxB binds exclusively to the l phase, which also implies that the receptor Gb₃ is localized in the l phase after protein binding. However, it remains unclear how Gb₃ is distributed in coexisting l/d membranes prior protein binding.

To get access to this information, an approach based on fluorescently labeled Gb₃ molecules can be pursued. However, it turned out that, if a fluorescent label is attached to the fatty acid position to ensure that the STxB interaction with the head group is not influenced by the fluorophore, binding of STxB is greatly altered.[23] If a fatty acid labeled Gb₃ is reconstituted into l/d phase-separated GUVs, the protein binds to the l phase and not to the l phase as known from membranes containing naturally occurring Gb₃.

To date, only a few examples are found in the literature where synthetic routes towards glycosphingolipids with labeled head groups have been described.[24] Here, we decided on a new strategy in line with approaches pursued for G₃M and G₃M.[25] and focused on head group labeled Gb₃. The idea is to develop fluorescently labeled Gb₃ glycosphingolipids without altering its binding properties to STxB. We attached a fluorophore via an oligoethylene glycol spacer to the 2'-OH group of the middle galactose of the Gb₃ head group, which is not involved in STxB binding as deduced from crystal structure analysis[17] and binding studies of different trisaccharides.[26] This approach in turn allows us to alter the fatty acid of the Gb₃ molecules.

**Results and Discussion**

We synthesized a set of Gb₃ sphingolipids as depicted in Scheme 1. Altogether eight different glycosphingolipids were synthesized and they consist of the globotriaose head group with two different oligoethylene glycol (PEG) linkers, to which a BODIPY fluorophore was attached and the sphingosine. Saturated, unsaturated, α-hydroxylated derivatives, and a combination thereof were prepared, all based on a C₂₄ fatty acid. C₂₄ fatty acids were chosen as they are the major constituent (> 50%) found in natural Gb₃ mixtures such as toxin insensitive erythrocytes,[27] HeLa-cells[28] and HEP-2 cells.[29]

To access the head group labeled Gb₃ derivatives with different fatty acids and PEG linker lengths we designed a modular convergent synthesis in which a variation of the fatty acid and the fluorophore is possible with minimal synthetic effort (Scheme 1). In contrast to semisynthetic approaches, a convergent total synthesis ensures the highly defined nature of the obtained material, which was crucial for our biophysical experiments. The retrosynthetic analysis of the desired structures 1–8 led to four different components. The commercially available BODIPY dye 9 should be attached to the carbohydrate head group in the last step of the synthesis by a Huisgen cycloaddition (click chemistry). The sphingosine core should be introduced as the azido sphingosine 10. The azide serves as a masked amine which undergoes amidic coupling with the four selected fatty acids (11–14) with a C₂₄ backbone. Assembling the globotriaose building blocks 15 and 16, in which the 2-hydroxy group of the middle galactose was modified with the PEG linker and the reducing end was activated for the glycosylation reaction with 10, would be the most challenging endeavor during this synthesis. Monosaccharide building blocks with carefully chosen patterns of temporary and permanent protecting groups had to be synthesized starting from the simple monosaccharides α-glucose and α-galactose.

Naturally occurring Gb₃ molecules carry 24 carbon long fatty acids, either saturated or monounsaturated.[30] The galactosyl trichloroacetimidate 17, galactosyl phosphate 18, and glucoside 19 were identified as suitable precursors to build up the trisaccharide (Scheme 2). They were prepared according to literature procedures.[31] The union of 18 and 19 under Lewis-acidic conditions utilizing TMSCOCl as a promoter afforded the respective (1→4)-linked disaccharide. Perfect β-selectivity was observed because of the neighboring-group
participation of the Fmoc group of 18. During the course of the reaction the para-methoxybenzyl group of the galactose was cleaved,[32] yielding the lactose acceptor for the second glycosylation step with 17 under Lewis-acidic conditions without any additional deprotection step. The desired α-configured product was isolated as the main product when diethyl ether was used as a cosolvent. Subsequent removal of the Fmoc protecting group with piperidine led to the trisaccharide 20.

The trisaccharide 20 was then equipped with a pentenyl chain in the position where the fluorophore needs to be attached (Scheme 3). In the next step the substrate was subjected to Birch conditions to remove all benzyl protecting groups. Despite the strongly reducing conditions, the anomic CH₂CH₂TMS group and the pentenyl handle stayed intact. Deprotection was followed by DMAP-mediated benzoylation. In contrast to benzyl groups, benzoyl esters have the advantage that they can be easily removed at the end of the synthetic route without affecting the double bond in the lipid part of the glycosphingolipid.[21] To attach the PEG linker, the double bond of the pentenyl handle was first transformed into a thioester with tioacetic acid under radical conditions. This species was hydrolyzed under basic conditions and the emerging highly nucleophilic thiol was subsequently reacted with the PEG bromides 21 (13 ethylene glycol units) and 22 (3 ethylene glycol units). To ensure a full protection of all hydroxy groups, the benzoylation step was repeated. Finally, the anomic protecting group was removed with trifluoroacetic acid and the reducing end was converted into the corresponding trichloroacetimidates 15 and 16.

To build up the glycolipid, the trichloroacetimidates were reacted with the protected azidosphingosine 10,[33] which was synthesized starting from the chiral pool compound L-serine (for detailed information see the Supporting Information), in a glycosylation reaction utilizing TMSOTf as the Lewis acid to afford 23 and 24 in moderate yields (Scheme 4). Comparing experiments with globotriaosyl trichloroacetimidates devoid of the PEG modification indicated that the Lewis-basic linker might hamper this very sensitive glycosylation step. Staudinger reduction of the azides and direct coupling with the fatty acids 11–14,[21,34] without isolating the intermediary amines, afforded the PEG-modified glycosphingolipids 25–32. Global deprotection under Zemplén conditions set the stage

Scheme 2. Assembly of the C₈ trisaccharide.

Scheme 3. Synthesis of the trichloroacetimidates 15 and 16 with two different PEG linkers.

Scheme 4. C₈ glycosphingolipid assembly.
for the final step of the synthesis. The commercially available BODIPY dye 9 was introduced into the glycosphingolipids by coupling its azide unit with the alkyne moiety of the PEG linker under mild copper(I)-catalyzed conditions (Scheme 5).

In total, eight different fluorescently labeled glycosphingolipids (1–8), varying in the PEG linker length and the acyl chain of the fatty acid, were obtained. The linker length (n) is either 3 or 13 oligoethylene glycol groups. The fatty acid (Cm: D) is either saturated (C24:0) or unsaturated (C24:1). Hydroxylation at the α-position is indicated by OH, and non-hydroxylation is indicated by H.

Starting with the saturated C24 fatty acid and a PEG spacer composed of 13 oligoethylene glycol units, we prepared GUVs composed of the well-known raft mixture 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC)/SM-porc/Chol labeled with 5 mol% 1 and 0.25 mol% Texas Red-DHPE (39.75/35/20/5/0.25) to address the question of whether STxB is indeed still capable of binding to the head group modified Gb3 and whether it binds to the liquid phase as expected. Figure 1 shows representative confocal images of a GUV composed of DOPC/SM-porc/Chol/1/Texas Red-DHPE (39.75/35/20/5/0.25) in an aqueous solution of STxB-Cy5 (500 nM, monomer). A) Texas Red-DHPE fluorescence (red). B) STxB-Cy5 fluorescence (green). The yellow lines indicate the position of the fluorescence intensity profiles shown below the images.

To quantitatively compare the phase partitioning among the different Gb3 species, we used the fluorophore Dy731-DOPE as a δ marker[23] to guarantee that the fluorescence of the BODIPY labeled Gb3 does not spectrally overlap with the absorption of the δ marker. Moreover, the concentration of Gb3 was reduced from 5 to 1 mol% to ensure that self-quenching of the BODIPY fluorophore is minimized (see Figure S1 in the Supporting Information). GUVs composed of DOPC/SM-porc/Chol/Gb3/Dy731 (39/39/20/1/1) were prepared. As it is known that the composition of GUVs obtained by electroformation is rather heterogeneous,[35] at least two independent GUV preparations with about 30 individual GUVs each were analyzed. Confocal z-stack images were measured for each GUV and line profiles were taken from each slice, where phase separation was visible. An example of fluorescence images of a liquid coexisting GUV together with the line profile is shown in Figure 2. The fluorophore Dy731-DOPE indicates the δ phase (Figure 2A),[23] From the BODIPY fluorescence intensity (Figure 2B), the preferential localization of 1 is visible. To quantify the partition of 1, the BODIPY intensity of the δ phase (Iδ) and of the liquid phase (Iliq) as obtained from the corresponding line profile was determined and the liquid distribution (%liq) was calculated [Eq. (1)]:[23]

\[
\%_{\text{liq}} = \frac{I_{\text{liq}}}{I_{\text{liq}} + I_{\delta}}
\] (1)

Several tens of line profiles were taken from each GUV. All %liq values were cast into a histogram (Figure 2C). Data obtained in this manner are presented as violin plots throughout the manuscript.

There is increasing evidence that the size of the linker attached to the head group of a lipid alters the phase behavior.
To investigate whether distributions (%OH) membranes is required to favor their partitioning in GUVs composed of DOPC species in GUVs composed of DOPC/SM-porc/Chol/Dy731-DOPE (39/39/20/1/1). A) Dy731-DOPE fluorescence (red). B) 1 fluorescence (green). The yellow lines indicate molecules differing in composition as in (A/B). The red solid line indicates the median value, the red star the mean value.

The mean values are summarized in Table 1. The differences in the mean values dependent on the functional group.

| No. | Gb | %N (N) |
|-----|----|--------|
| 1   | Gb,PEG13,C24,H  | 0.74 ± 0.07 (2525) |
| 2   | Gb,PEG13,C24,H  | 0.41 ± 0.07 (2516) |
| 3   | Gb,PEG13,C24,OH  | 0.71 ± 0.05 (3064) |
| 4   | Gb,PEG13,C24,OH  | 0.42 ± 0.08 (2273) |
| 5   | Gb,PEG13,C24,H  | 0.47 ± 0.15 (1654) |
| 6   | Gb,PEG13,C24,OH  | 0.32 ± 0.07 (2351) |
| 7   | Gb,PEG13,C24,OH  | 0.50 ± 0.08 (2377) |
| 8   | Gb,PEG13,C24,OH  | 0.27 ± 0.06 (2701) |

The errors are the standard deviation of the mean. N = number of line profiles.

The mean values are summarized in Table 1. The differences in the mean values dependent on the functional group.

| Group | ΔPEG | ΔC24 | ΔOH |
|-------|------|------|------|
| 1-2   | 0.33 ± 0.14 | 1-5: 0.27 ± 0.22 | 1-3: 0.03 ± 0.12 |
| 3-4   | 0.29 ± 0.13 | 3-7: 0.21 ± 0.13 | 5-7: 0.03 ± 0.23 |
| 5-6   | 0.15 ± 0.22 | 2-6: 0.09 ± 0.14 | 2-4: -0.01 ± 0.15 |
| 7-8   | 0.23 ± 0.14 | 4-8: 0.15 ± 0.14 | 6-8: 0.05 ± 0.13 |

ΔPEG = %N(PEG13) - %N(PORC); ΔC24 = %N(C24:1) - %N(C18:0); ΔOH = %N(OH) - %N(H).
attached by a short linker, the size of the lipid’s head group is expanded and the lipid is more conically shaped, favoring the \( l_o \) phase.\[39\] For the slightly hydrophobic but small BODIPY fluorophore used in our study, a hydrophobic PEG spacer of suitable length is required to mitigate interactions with the membrane. Momin et al.\[38\] found a linker with 10 ethylene glycol units to be sufficient to decouple the fluorophore from the membrane.\[39\] In our study, a 13-unit long linker decoupled the fluorophore from the membrane interface with the result that \( l_o \) is predicted to at least preferentially partition into the \( l_o \) phase, indeed has a \( l_o \) distribution of almost 0.75. From these results, we conclude that the \( G_b \) species with PEG\(_{13}\) are better suited to report on the natural partition of \( G_b \) than those with PEG\(_{5}\). Thus, the experiments in which we compare the influence of unsaturation and hydroxylation of the fatty acid of \( G_b \) are all performed with the PEG\(_{13}\) species. The corresponding results with the PEG\(_{3}\) linker can be found in the Supporting Information (see Figures S2 and S3).

We investigated the influence of the fatty acid saturation on the partition behavior of \( G_b \) (Figure 4). The results show that introducing a fatty acid with a cis-double bond redistributes the \( G_b \) sphingolipid in the \( l_o \) phase, and can be rationalized by the increased space requirement of the \( G_b \) species with the C\(_{24:1}\) fatty acid. The differences between the \( l_o \) distribution of 1/5 and 3/7 harboring the PEG\(_{13}\) linker are significant and range between 0.21 and 0.27 (Table 2, \( \Delta C24 \)).

![Figure 4](image)

**Figure 4.** \( l_o \) distribution of different \( G_b \) species with a PEG\(_{13}\) linker in GUVs composed of DOPC/SRnc-porc/Chol/Gb\(_{Dy731}\) (39/39/20/1/1). The partition of the \( G_b \) species harboring a saturated fatty acid (C\(_{24:0}\)) were compared with that with an attached unsaturated fatty acid (C\(_{24:1}\)). The mean values are given as red stars, while the red lines show the median value (Table 1).

Björkqvist et al.\[40\] investigated different glycosphingolipids as well as sphingomyelins and found by differential scanning calorimetry (DSC) that the phase-transition temperature was decreased by about 20 K for all sphingolipids harboring the C\(_{24:1}\) fatty acid compared to the corresponding C\(_{24:0}\) sphingolipids, demonstrating their different packing behavior. The ability to pack tightly with ordered acyl chains in case of a saturated fatty acid\[40\] is a requirement for membrane lipids to partition into \( l_o \) domains and they concluded that the C\(_{24:1}\) sphingolipids are less likely to partition into the \( l_o \) phase. Fluorescence quenching experiments revealed that sphingolipids with a C\(_{24:0}\) fatty acid form \( l_o \) domains in multicomponent membranes composed of either the sphingolipid or mixed with palmitoyl sphingomyelin.\[40\] This behavior was also found by Mate et al.,\[42\] who reported that sphingomyelin with the C\(_{24:0}\) fatty acid constituted into a DOPC/Chol membrane leads to visible phase separation into an \( l_o \) and \( l_i \) phase, while the sphingomyelin with the C\(_{24:1}\) fatty results only in one lipid phase.

These results support our notion that the packing of the unsaturated \( G_b \) species disfavors its partition in the \( l_o \) phase. Similar to our in vitro results, Legros et al.\[43\] found in primary human blood brain endothelial cells that Gb, with C\(_{24:1}\) fatty acids resides more strongly in non-detergent-resistant membranes compared to Gb with C\(_{24:0}\) fatty acids.

In nature, about 50% of the \( G_b \) sphingolipids are decorated with an OH group in the \( \alpha \)-position of the fatty acid, raising the question, whether this OH group alters the \( G_b \) partition. The results (Figure 5) clearly indicate that the OH group in the \( \alpha \)-position does not influence its distribution. The differences of \%\( l_o \) for \text{1/3} and \text{5/7} are in the range of −0.03–0.03 and are not significant (Table 2, \( \Delta O H \)).

Monolayer experiments on galactosyl ceramide (GalCer), harboring either an \( \alpha \)-hydroxylated or nonhydroxylated C\(_{24:0}\) fatty acid on a Langmuir trough, suggest that the \( \alpha \)-hydroxylation does not change the area per lipid at 30 mN m\(^{-1}\).\[44\] a surface pressure that reflects the packing density of bilayers.\[45\] Using \( ^{2}H \) NMR spectroscopy, Morrow and co-workers\[41,46\] also demonstrated that the order parameter of the fatty acids of GalCer embedded in a POPC/Chol membrane and the orientation of the head group does not change considerably.

This report is in line with our observation that the OH group does not significantly alter the partitioning of the \( G_b \) species in phase-separated GUVs. However, in a previous study, we found that the 2-OH group influences the fraction of \( l_o \) phase in phase-separated supported lipid bilayers.\[25\] In the case of the hydroxylated C\(_{24:0}\) fatty acid, the \( l_o \) fraction was smaller than that of the nonhydroxylated species. Stölte and co-workers\[27\] showed that the 2-OH group increases the hydration in the membrane interface and decreases the affinity of a sphingolipid for steroids. The same was found by Lingwood et al.\[48\] and Yahi et al.\[49\] and implies that the
Hence, it has been observed. This trend was not found and agrees with the idea that the interaction of Gb₃ with Chol owing to its better solubility in SM membranes than in PC membranes.[55–57] Interdigitation was also reported for glycosphingolipids carrying a C₂₀ fatty acid.[51,54] Hence, it is likely that the Gb₃ species under investigation preferentially partition into the lₒ phase if SM interdigitates. Interdigitation of SM in the liquid-crystalline phase occurs for C₂₀ fatty acids and longer, in agreement with our observation that the partition in the lₒ phase is increased for SM species with C₂₀ fatty acids or longer.

However, the lₒ phase consists not only of SM but also of Chol owing to its better solubility in SM membranes than in PC membranes.[55–57] Chol is best soluble in SM C₁₆:0.[56,57] If the solubility of Chol in the lₒ phase greatly influenced the Gb₃ distribution in the lₒ phase, the opposite trend would have been observed. This trend was not found and agrees with the idea that the interaction of Gb₃ with Chol is less important than the one with SM.

### Table 3: Mean values of the lₒ distributions (%(lₒ)) for the different Gb₃ sphingolipids with the PEG₃ linker in GUVs composed of DOPC/SM/Chol/Gb₃/Dy731 (39/39/20/1/1) varying in the SM species.

| Gb₃     | %(lₒ)(SM C₁₆:0) (N) | %(lₒ)(SM C₁₈:0) (N) | %(lₒ)(SM C₂₀:0) (N) | %(lₒ)(SM C₂₂:0) (N) | %(lₒ)(SM C₂₄:0) (N) |
|---------|---------------------|---------------------|---------------------|---------------------|---------------------|
| 1       | 0.41 ± 0.11 (2392)  | 0.55 ± 0.17 (2986)  | 0.74 ± 0.05 (2397)  | 0.74 ± 0.09 (2077)  | 0.75 ± 0.09 (1707)  |
| 3       | 0.45 ± 0.10 (3232)  | 0.59 ± 0.14 (3396)  | 0.71 ± 0.05 (2414)  | 0.72 ± 0.07 (2893)  | 0.77 ± 0.07 (2509)  |
| 5       | 0.24 ± 0.10 (2035)  | 0.28 ± 0.11 (1845)  | 0.54 ± 0.11 (2482)  | 0.45 ± 0.15 (2759)  | 0.57 ± 0.12 (1730)  |
| 7       | 0.35 ± 0.06 (1814)  | 0.34 ± 0.08 (2465)  | 0.46 ± 0.08 (2648)  | 0.51 ± 0.09 (2266)  | 0.50 ± 0.10 (2491)  |

SM C₁₆:0 (palmitoyl SM), SM C₁₈:0 (stearoyl SM), SM C₂₀:0 (arachidoyl SM), SM C₂₂:0 (behenoyl SM), and SM C₂₄:0 (lignoceroyl SM). The errors are the standard deviation of the mean. N = number of line profiles.

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**Figure 6.** lₒ distribution of different Gb₃ species with a PEG₃ linker composed of DOPC/SM/Chol/Gb₃/Dy731 (39/39/20/1/1). The partition of four different Gb₃ sphingolipids in lₒ/lᵣ phase-separated GUVs with different sphingomyelin species are shown: SM C₁₆:0 (palmitoyl SM), SM C₁₈:0 (stearoyl SM), SM C₂₀:0 (arachidoyl SM), SM C₂₂:0 (behenoyl SM) and SM C₂₄:0 (lignoceroyl SM). The mean values are given as red stars and the solid red lines represent the median value (Table 3).
Conclusion

Gb<sub>d</sub> and Gb<sub>3</sub> detection by fluorescently labeled Cholera toxin B subunits (CTXB) and Shiga toxin B subunits (STXB), respectively, is a well-established tool for monitoring <i>I</i><sub>c</sub> membrane domains<sup>18</sup> and implies that these glycosphingolipids are localized in the <i>I</i> phase. However, as each CTXB and STXB pentamer can recruit a maximum of 5 (CTX) or 15 (STX) receptor lipids, the glycosphingolipid partitioning in coexisting <i>I</i><sub>c</sub> membranes after protein binding does not necessarily reflect the situation prior protein binding. Hence, to be able to quantify the partitioning of Gb<sub>d</sub> in phase coexisting <i>I</i>/<i>I</i><sub>c</sub> membranes by means of fluorescence readout, chemical access to fluorescently labeled pure Gb<sub>d</sub> molecules is required. The approach of synthesizing head group labeled glycosphingolipids enables one to address the question how the fatty acid of a glycosphingolipid influences its distribution in <i>I</i>/<i>I</i><sub>c</sub> phase-separated membranes, a question that has been hardly addressed because most of the glycosphingolipids are not available in chemically pure form. Our results clearly demonstrate that the fatty acid (un)saturation significantly shifts the Gb<sub>d</sub> molecules from the <i>I</i><sub>c</sub> phase (<i>C</i><sub>44:0</sub>) to the <i>I</i> phase (<i>C</i><sub>36:0</sub>). As STXB exclusively binds to Gb<sub>d</sub> in the <i>I</i> phase, the amount of redistributed Gb<sub>d</sub> and probably also other <i>I</i><sub>c</sub> phase lipids thus depends on the fatty acid of Gb<sub>d</sub>. However, the α-hydroxylation does not alter the partition of Gb<sub>d</sub>, even though it has been shown that the OH group of Chol can form a hydrogen bond only to the nonhydroxylated fatty acid. Instead, the length match of the fatty acids of SM and Gb<sub>d</sub> appear to play a more decisive role in determining where the Gb<sub>d</sub> glycosphingolipids are preferentially localized. As the combination of the attached fatty acids of SM and Gb<sub>d</sub> considerably impacts the distribution of the Gb, glycosphingolipids, it is conceivable that the overall recruitment of lipids and thus the Shiga toxin induced membrane reorganization that eventually leads to the invagination of the protein into the host cell, is strongly influenced by the fatty acid composition of Gb<sub>d</sub>.

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Conflict of interest

The authors declare no conflict of interest.

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