This is an Accepted Manuscript, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

*Accepted Manuscripts* are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the [Information for Authors](#).

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal’s standard [Terms & Conditions](#) and the [Ethical guidelines](#) still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

[www.rsc.org/softmatter](http://www.rsc.org/softmatter)
The effects of Globotriaosylceramide tail saturation level on bilayer phases

Weria Pezeshkian†‡, Vitaly V. Chaban§, Ludger Johannes §, Julian Shillcock †, John H. Ipsen †, Himanshu Khandelia †‡

Globotriaosylceramide (Gb3) is a glycosphingolipid present in the plasma membrane that is the natural receptor of the bacterial Shiga toxin. The unsaturation level of Gb3 acyl chains has a drastic impact on lipid bilayer properties and phase behaviour, and on many Gb3-related cellular processes. For example: the Shiga toxin B subunit forms tubular invaginations in the presence of Gb3 with an unsaturated acyl chain (U-Gb3) while in the presence of Gb3 with a saturated acyl chain (S-Gb3) such invagination does not occur. We have used all-atom molecular dynamics simulations to investigate the effects of the Gb3 concentration and its acyl chain saturation on the phase behaviour of a mixed bilayer of dioleoylphosphatidylcholine and Gb3. The simulation results show that: 1) The Gb3 acyl chains (longer tails) from one leaflet interdigitate into the opposing leaflet and lead to significant bilayer rigidification and immobilisation of the lipid tails. S-Gb3 can form a highly ordered, relatively immobile phase which is resistant to bending while these changes for U-Gb3 are not significant. 2) At low concentrations of Gb3, U-Gb3 and S-Gb3 have a similar impact on the bilayer reminiscent of the effect of sphingomyelin lipids and 3) At higher Gb3 concentrations, U-Gb3 mixes better with dioleoylphosphatidylcholine than S-Gb3. Our simulations also provide the first molecular level structural model of Gb3 in membranes.

INTRODUCTION

Glycosphingolipids (GSLs) are a class of lipids consisting of a ceramide linked to a carbohydrate moiety and are abundant in the outer leaflet of the plasma membrane. They are involved in many biological processes including macromolecular recognition, intracellular protein uptake and cell adhesion (reviewed in1). Globotriaosylceramide (Gb3) is an interesting GSL lipid which is required for the binding of Shiga toxin to the host cell membrane and the toxin’s subsequent internalization into the cell.2,3 Gb3 is over-expressed in metastatic colon cancer and its presence is sufficient for epithelial cells to be invasive.4 The high expression of Gb3 in invasive colon cancer cells suggests a possible route to target and detect these cells.4,5 The Gb3-binding B-subunit of Shiga toxin (STxB) has also been used to target defined antigens to dendritic cells for the induction of a therapeutic immune response against cancer or intracellular pathogens.6–8 STxB binds up to 15 Gb3 lipids on the surface of the host cell membrane9 and allows the intracellular transport of the toxin via the retrograde route.2 STxB can also bind to, ag-
(Gb3 with a saturated acyl chain with 22 carbon atoms) and Gb3-22:1 (Gb3 with an unsaturated acyl chain with 22 carbon atoms and a trans-double bound in C13 - C14). We refer to the saturated and unsaturated versions of Gb3 as S-Gb3 and U-Gb3 respectively. The simulations reveal two important features of the Gb3 structure which strongly affect lipid bilayer physical properties. First: Gb3 fatty acyl chain from one leaflet interdigitate into the opposite monolayer leading to a reduction in layer and result in a bilayer with higher bending resistance. Second: the degree of the Gb3 acyl chain saturation influences the phase behaviour of the bilayer where for fully saturated acyl chain an ordered phase was observed. Our simulations can explain several experimental observable processes, which we present in the DISCUSSION section of the report.

METHODS, FORCE FIELD AND SYSTEMS

Methods

We performed all-atom MD simulations of mixtures of DOPC and Gb3 lipids using GROMACS 17–20 software and the CHARMM36 force field (FF). 21,22 The Gb3 molecule had a C18 sphingosine tail and either a 22:0 acyl tail (S-Gb3) or a 22:1 acyl tail unsaturated at C13 (U-Gb3). For all systems, at least 100 water molecules (The TIP3P solvent model 23) were included. Na and Cl ions representing a biological concentration of 150 mmol were present. Electrostatic interactions were treated with particle-mesh Ewald (PME) with a short range cutoff 1.2 nm, and van der Waals interactions were switched off between 1.0 to 1.2 nm. The system temperature was kept constant at 37°C using Nose-Hoover temperature coupling. 25,26 Bonds containing hydrogen atoms were constrained using the LINCS algorithm. 27 Parrinello-Rahman barostat pressure coupling 28,29 was applied on all systems after equilibrating the systems with Berendsen pressure coupling. 30 The leap frog integrator was used with a timestep of 2 fs.

Force field parameters for Gb3

The Gb3 lipid contains three sugar moieties, αGal(1-4)βGal(1-4)βGlc (Blue segment of Fig.1A), which are connected to ceramide. The sugar moiety was described by the CHARMM36 FF for carbohydrates. 31–35 Point electrostatic charges needed for HO-CH2-CH-CH2-... (Green segment of Fig.1A) are not provided in the CHARMM36 FF. As point electrostatic charges are unknown for the HO-CH2-CH-CH2-... moiety of Gb3, we have performed electronic structure calculations of a set of similar systems to obtain information on the electron density distribution. All-electron Möller-Plesset second-order perturbation theory, MP2, was used. The wave function was constructed using the 6-311G basis set. Polarization and diffuse functions were added to this basis set. Additionally, polarization and diffuse functions were added to all hydrogen atoms. Strict self-consistent field convergence criterion of 10-9 Ha was applied. Geometries of the molecules were optimized at the same level of theory prior to computation of the electrostatic potential (ESP). The ESP, derived from electronic structure, was reproduced using a set of point charges centered on each atom, including hydrogen atoms. The atom spheres were defined according to the CHELPG scheme. 36 The calculations were performed in GAUSSIAN 09 program. 37 We considered 4 model compounds, 1) CH2=CH-CH2-OH, 2) CH3-CH2-CH2-OH, 3) CH3-CH=CH-CH3 and 4) CH3-CH=CH-CH2-OH. The inclusion of enumerated compounds into consideration was necessary to ensure reasonable transferability of the assigned charges. Please refer to the supplementary material (SI) for a more detailed description of development of the force field. The derived charges are summarized in Table 1. The HO-CH2-CH-NH-C=O moiety (Red segment of Fig.1A and B) was described using serine-serine peptide bond parameters in the CHARMM36 FF for proteins. Finally, the fatty acid chains were described by the CHARMM36 FF for lipids.

Table 1 Extracted point charges using quantum calculation for structure HO-CH-CH1-βCH2=γCH3.

<table>
<thead>
<tr>
<th>ATOMS</th>
<th>Charge</th>
</tr>
</thead>
<tbody>
<tr>
<td>OH</td>
<td>-0.70</td>
</tr>
<tr>
<td>HO</td>
<td>+0.40</td>
</tr>
<tr>
<td>αC</td>
<td>+0.55</td>
</tr>
<tr>
<td>H1</td>
<td>-0.05</td>
</tr>
<tr>
<td>βC</td>
<td>-0.30</td>
</tr>
<tr>
<td>H2</td>
<td>+0.10</td>
</tr>
<tr>
<td>γC</td>
<td>-0.10</td>
</tr>
<tr>
<td>H3</td>
<td>+0.10</td>
</tr>
</tbody>
</table>

Simulated Systems

Details of all simulated systems are presented in Table 2. Five different Gb3 concentrations in a DOPC bilayer: 0,12,25,50, 100 percent, were simulated. Two extra simulations to investigate the phase separation tendency of Gb3, were performed (Table 2). For systems 1-9, initial configurations were built by placing all DOPC lipids on a lattice and then a sufficient amount of the DOPC lipids were randomly replaced by Gb3 lipids to the desired concentration. The number of the Gb3 lipids in the both monolayers are equal. For Gb3 domain simulations (systems 10 and 11), 16 DOPC lipids were replaced by 16 Gb3 lipids in middle of upper mono-layer of the bilayer.
RESULTS

The last 200 ns of the simulation outputs were used for data analysis. We performed an initial check on our simulation methodology by measuring the structural properties of a pure DOPC bilayer, and found that they are in good agreement with previous results from experiments and other simulations. In particular, the area per lipid and bilayer thickness were 64.7 ± 1.2 Å² and 36.4 ± 0.6 Å, which are close to literature results 68.0 Å² and 36.6 Å extracted from simulations. Experimental values are 67.4 Å² for the area per lipids and 36.1, 36.7 and 37.6 at 45, 30, and 15 °C respectively for membrane thickness. The area and thickness are the two key mechanical properties that characterise a planar bilayer. The area per lipid responds sensitively to the lipid phase, and the thickness influences the membrane curvature modulus that governs its thermal fluctuations. Our results show that these mechanical properties strongly depend on the membrane composition and the structural details of the Gb₃ acyl chains.

The rest of the paper is organized as follows. First, we report on the area and thickness using several different techniques for accurate measurement of the area per molecule. We then explore the influence of the Gb₃ on the membrane’s dynamical properties, and measure the lipid tail order parameters, fatty acid tilt distributions, lipid diffusion, rotational correlation times and the tendency of Gb₃ to phase separate. All of these properties are found to be sensitive to the Gb₃ concentrations and degree of Gb₃ acyl chain saturation. In the discussion, we elaborate upon the main findings, and show how the simulations explain several previously unexplained experimental observations with regards to membrane invagination induced by Shiga toxins and the phase behaviour induced by Gb₃.

Area Per lipid and membrane thickness

We used three different methods to calculate the area per lipid.

Projected area per lipid \(a_p\), Calculated from box size.

For a one-component flat bilayer, the area per lipid is equal to \(a_p = 2L_xL_y/N\) where \(L_x\) and \(L_y\) are the simulation box dimensions in the plane of the bilayer and \(N\) is the number of the lipids in the system. Fig. 3 shows the \(a_p\) as a function of time for different systems. The time average of \(a_p\) is presented in the Table 3 column 2.

Projected area per lipid, Calculated by Voronoi tessellation \(a_v\).

To obtain the individual area per lipid values in a mixed bilayer, we used Voronoi tessellation. The APL@Voro software was used. The data for this method is presented in the Table 3 column 3.
Fig. 2 Last snapshot of the system for (A) 50%S-Gb$_3$ (B) 50%U-Gb$_3$ (C) 100%S-Gb$_3$ (D) 100%U-Gb$_3$. Yellow spheres are the first carbon atom of the lipids hydrophobic moiety. Gb$_3$ long chains (cyan color) interdigitate into the opposite monolayer. Also, the S-Gb$_3$ tails are highly ordered and tilted. These bilayers are not planer.

**Polynomial fitting.** For a curved bilayer, neither of the previous methods can give an accurate value of the area per lipid. Instead, a polynomial fit to the membrane surface gives a better estimate of the area. Additionally, this method can be used to calculate the curvature and membrane thickness profile. A functional form for the bilayer surface can be obtained by fitting a polynomial of degree \( s \) to the coordinates of a specific atoms, such as the phosphorus atom in DOPC lipids in one monolayer (Equation 1).

\[
z(x, y) = \sum_{n,m=0}^{m+n \leq s} a_{(m,n)} x^m y^n
\]  

(1)

Where \( m \) and \( n \) are integer numbers and taking all values in the interval zero to \( m + n \leq s \). \( a_{(m,n)} \) are polynomial coefficients that are extracted from fitting. The area is evaluated as:

\[
A = \int_0^{L_x} \int_0^{L_y} \left( 1 + \frac{\langle z(x,y) \rangle_x^2 + \langle z(x,y) \rangle_y^2}{\tau} \right)^{\frac{1}{2}} \, dxdy
\]  

(2)

Where \( \langle A(x,y) \rangle_\tau \) is a time average of the function \( A(x,y) \) in a specific time period, \( \tau \). (we have used \( \tau = 20ns \)) that minimizes the effects of membrane protrusions and shape fluctuations due to the thermal fluctuations. \( z(x,y)_x \) and \( z(x,y)_y \) are partial derivatives of the function \( z(x,y) \) with respect to \( x \) and \( y \) respectively. For most of the systems (except for pure U-Gb$_3$ or S-Gb$_3$) the polynomial method gives similar values for area per lipid as the previous methods (Table 3). However, for the pure systems, the polynomial method extract a higher value for the area per lipid, indicating that the pure Gb$_3$ bilayers are not planar (Fig. 2-C and D). In general, increase in Gb$_3$ concentration results in decreases in area per lipid and tighter bilayer packing (a similar result was observed experimentally). The decreases in the area per lipid comes from two sources: increase in the amount of the Gb$_3$ which has a smaller area per lipid or a change in membrane phase behaviour, from an disorder to an order phase. At low Gb$_3$ concentration, the first effect plays a major role, because the area per lipid \( a_v \) for Gb$_3$ remains constant while the \( a_v \) for DOPC changed because the number of the DOPC lipids in contact to the Gb$_3$ increased. However, at high S-Gb$_3$ concentration, the Gb$_3$ \( a_v \) is lowered significantly which indicates a change in the phase state of the bilayer. We will elaborate on this in the subsequent sections.

**Bilayer thickness.** We calculated membrane thickness as the distance between phosphorus atoms in the two monolayers \( L_m \) (Table 3 column 5). \( L_m \) does not change much for low Gb$_3$ concentrations, but for high S-Gb$_3$ concentration, this value increases significantly (a similar conclusion was made
using x-ray diffraction\textsuperscript{16}). We did not report any value for the 100% Gb3 since they are not a planer bilayer.

Table 3 Area per lipid and bilayer thickness: 1) \(a_p\) is bilayer projected area calculated from box dimensions, 2) \(a_v\) areal area estimated from polynomial fitting, 3) \(a_s\) projected area calculated using Voronoi tessellation and 4) bilayer thickness \(L_m\).

<table>
<thead>
<tr>
<th>System</th>
<th>(a_p(Å^2)/N)</th>
<th>(a_v(Å^2)/N)</th>
<th>(a_s/(\text{DOPC/Gb}_3))</th>
<th>(L_m(Å))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0% Gb3</td>
<td>64.7 ± 1.2</td>
<td>65.3 ± 0.9</td>
<td>64.4 ± 0.6</td>
<td>36.4 ± 0.6</td>
</tr>
<tr>
<td>12 S-Gb3</td>
<td>61.6 ± 0.8</td>
<td>61.9 ± 0.7</td>
<td>62.9/54.8</td>
<td>38.9 ± 0.5</td>
</tr>
<tr>
<td>12 U-Gb3</td>
<td>62.2 ± 0.8</td>
<td>62.9 ± 0.8</td>
<td>63.0/53.8</td>
<td>38.6 ± 0.5</td>
</tr>
<tr>
<td>25 S-Gb3</td>
<td>59.2 ± 1.0</td>
<td>60.4 ± 0.7</td>
<td>61.1/53.7</td>
<td>38.2 ± 0.6</td>
</tr>
<tr>
<td>25 U-Gb3</td>
<td>60.0 ± 1.0</td>
<td>61.7 ± 1.1</td>
<td>62.0/55.7</td>
<td>37.4 ± 0.5</td>
</tr>
<tr>
<td>50 S-Gb3</td>
<td>47.2 ± 0.4</td>
<td>50.8 ± 1.2</td>
<td>49.3/45.6</td>
<td>43.1 ± 0.3</td>
</tr>
<tr>
<td>50 U-Gb3</td>
<td>54.1 ± 1.0</td>
<td>55.0 ± 1.0</td>
<td>55.7/50.5</td>
<td>39.0 ± 0.5</td>
</tr>
<tr>
<td>100 U-Gb3</td>
<td>53.2 ± 0.5</td>
<td>58.0 ± 1.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>100 S-Gb3</td>
<td>52.6 ± 0.5</td>
<td>60.0 ± 1.0</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Chain Order parameter

The orientational order of lipid chains is described by a deuterium order parameter which is given as:

\[
S_{CD} = \frac{1}{2} \langle 3 \cos^2 \theta - 1 \rangle \tag{3}
\]

where \(\theta\) is the angle between the C-H vector and the bilayer normal.\textsuperscript{43,44} The reported order parameters in Fig. 4 show that Gb3 fatty acid chains are always more ordered compared to DOPC. Also, the Gb3 sphingosine chain is more ordered than the Gb3 acyl chain, which was also observed for sphingomyelin lipids in prior simulations.\textsuperscript{45} DOPC chains became more ordered with increasing Gb3 concentrations, because of unfavourable contacts of DOPC lipids with the Gb3 lipids, which results in decrease of the molecular volume of both DOPC and Gb3. This effect is much stronger for the S-Gb3 because lipids with unsaturated acyl chains dissolve more easily into each other.

The very high order parameter for 50% S-Gb3 indicate that this system is in an ordered phase state. The transition of this system from an initially disordered state to an ordered state can be observed from the distinct differences in the Voronoi diagrams at the beginning (Fig 9A in SI) and the end of the simulation (Fig. 9B in SI). In the ordered state, many lipids acquire a very low area per lipid, depicted by dark blue colors. It is important to note that S-Gb3 organizes the nearby DOPC acyl chains, whereas increasing the concentration of the U-Gb3 only has a small effect on the DOPC lipids, even up to 50% U-Gb3. The behaviour of the order parameter for the Sphingosine chain of the Gb3 in the low concentration regime is in a good agreement with previous simulation of sphingomyelin lipids.\textsuperscript{45}

Lipid Tilt

Tilt of the hydrophobic chains of lipid with respect to the bilayer normal is a characteristic parameter of the internal structure of the membrane in ordered phases. In the l\textsubscript{d} phase, average tilt is zero in the absence of constraints which is imposed by external objects (for example proteins). Changes in lipid tilt are associated with energy cost and coupled to the membrane bending energy.\textsuperscript{46}

The average orientation of the lipids chains is determined by the lipid tail director vector, \(\mathbf{n}\) (Figure5-A). Deviation of \(\mathbf{n}\) from the bilayer normal (\(\mathbf{N}\)) is quantified by the tilt vector (\(\mathbf{m}\)) which is given as:

\[
\mathbf{m} = \frac{\mathbf{n}}{\mathbf{n} \cdot \mathbf{N}} - \mathbf{N} \tag{4}
\]

Here we define lipid director as a unit vector pointing from lipid interface point to its corresponding tail point where the interface point is the carbon atom which connects both tails and the tail point is the midpoint between the last carbon atoms of the two chains of each lipid.

The probability of finding a lipid with tilt angle between \(\theta_1, \theta_2\) (in the l\textsubscript{d} phase, the tilt is independent of \(\phi\) because of the rotational symmetry) is given by:

\[
P(\theta_1, \theta_2) = \int_{\theta_1}^{\theta_2} \int_{\phi_1}^{\phi_2} p(\theta, \phi) \sin \theta d\theta d\phi \tag{5}
\]
Where $\theta$ is the angle between $n$ and $N$, $\phi$ is the azimuthal angle and $p(\theta, \phi)$ is density of the tilt probability. In the liquid phase, the membrane energy term corresponding to lipid tilt is (for more details see $^{46}$).

$$F_{\text{tilt}} = \frac{1}{2} \int dA \kappa_0 m^2 = \frac{1}{2} \int dA \kappa_0 \tan^2(\theta)$$

Where $\kappa_0$ is tilt modulus. $p(\theta)$ is expected to have a form like:

$$p(\theta) \propto \exp\left(-\frac{1}{2} a_p \kappa_0 \frac{\tan^2(\theta)}{k_B T}\right)$$

Where $a_p$ is projected area per lipid. Equation 7 shows, for small $\theta$, $p(\theta)$ has a Gaussian form with max value at $\theta = 0$. A deviation of the maximum from 0 indicates tilted lipids, and therefore an ordered phase.

Such a deviation, and thus an ordered phase is observed at high concentrations of S-Gb$_3$ (Fig. 5-B). Also, the higher peaks of the profiles containing high percentages of S-Gb$_3$ correspond to larger value for $\kappa_0$ and a more rigid bilayer. Thus, increase in Gb$_3$ concentration rigidify the bilayer, particularly for S-Gb$_3$. To further visualise the tilt in the systems, a 2D tilt distribution map of the director vector density ($\rho(T_x, T_y)$) was made. $\rho(T_x, T_y)$ was measured as the time average of the number of the lipids whose director vector projection on the membrane plane is equal to $T_x + T_y$. Fig. 5-C shows the 2D tilt distribution map for different systems. For 50% S-Gb$_3$, the maximum peak is a bit shifted from the center, implying an overall non-zero average tilt for the system.

**Diffusion coefficient**

Lipid diffusion constant for different systems were measured by evaluating the root mean-square deviation and using the Einstein relation. The values are given in Table 4. High concentrations of Gb$_3$ resulted in lower lateral diffusion coefficients of the lipid in the bilayer plane. At low concentrations, the values are close to the expected values for fluid lipid bilayers (around 8 $\mu$m$^2$/s for DOPC$^{47}$). Beyond 12.5% Gb$_3$, the diffusion of DOPC is significantly lowered by S-Gb$_3$ and to a lesser degree by U-Gb$_3$.

**Table 4 Diffusion constant for different systems.**

<table>
<thead>
<tr>
<th>System</th>
<th>DOPC $\mu$m$^2$/s</th>
<th>Gb$_3$ $\mu$m$^2$/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Gb$_3$</td>
<td>5.5 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>12 U-Gb$_3$</td>
<td>3.7 ± 0.4</td>
<td>3.3 ± 0.7</td>
</tr>
<tr>
<td>12 S-Gb$_3$</td>
<td>3.2 ± 0.5</td>
<td>3.0 ± 0.7</td>
</tr>
<tr>
<td>25 U-Gb$_3$</td>
<td>2.4 ± 0.1</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td>25 S-Gb$_3$</td>
<td>1.2 ± 0.2</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>50 U-Gb$_3$</td>
<td>1.8 ± 0.1</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>50 S-Gb$_3$</td>
<td>0.3 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>100 U-Gb$_3$</td>
<td>-</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>100 S-Gb$_3$</td>
<td>-</td>
<td>0.4 ± 0.3</td>
</tr>
</tbody>
</table>

**Effects of Gb$_3$ fatty acid chains length disparity**

The Gb$_3$ lipid has a big length mismatch in its two hydrocarbon tails. The sphingosine chain has 18 carbon atoms while the acyl chain contains 22 carbon atoms. Three different states for such chain mismatch have been suggested, which in some conditions could lead to interdigitation between the hydrocarbons of the two opposite monolayers and drive the system in a new phase state.$^{48}$ In the mixed systems, the shorter Gb$_3$ chain (sphingosine chain) is packed end to end with DOPC, while the Gb$_3$ acyl chain from the both leaflets penetrate the hydrocarbon region of the opposite monolayer, more so for S-Gb$_3$. (Fig. 2). The peak in the Gb$_3$ acyl chain density profile (Fig. 6) shows interdigitation between the acyl chain of the two opposing monolayers. The higher peak for S-Gb$_3$
**Fig. 5** A) Director \((\mathbf{n})\) is a unit vector along chains of a lipid and its deviation form \(\mathbf{N}\) is given by tilt vector \((\mathbf{m})\). The size of the tilt vector is equal to \(\frac{|b|}{|a|}\) and is in the same direction as \(\mathbf{b}\). B) Probability of lipid tilt. Top) \(p(\theta)\) for DOPC. Bottom) \(p(\theta)\) for Gb3. The cyan curve for 50% S-Gb3 does not peak at 0, indicating a net lipid tilt. The corresponding curve for DOPC (cyan curve, top panel) also has a non-zero maximum. C) 2D tilt distribution map. Left top) 50% S-Gb3. Right top) 25% S-Gb3. Left bottom) 50% U-Gb3. Right bottom) Pure DOPC.
Compared to that of U-Gb3 shows that it is more favourable for S-Gb3 to interdigitate into the other monolayer. Similar results are observed for lower Gb3 concentrations (data not shown).

**Fig. 6** Lipid fatty acid chain carbon density profile along the bilayer normal for the 50 % Gb3 systems (A) S-Gb3 (B) U-Gb3. Gb3 chain mismatch leads to interdigitation between the hydrocarbons of the two opposing monolayers. The higher peak for S-Gb3 shows that it is more favourable for S-Gb3 to interdigitate into the other monolayer.

**Lipid rotation**

Lipid rotation around the bilayer normal is one of the slower degrees of freedom of the lipids in a lipid bilayer. The two-time correlation function for lipid rotation is defined as

$$C_\theta(t_0) = \langle R_\theta(t)R_\theta(t+t_0) \rangle$$

(8)

Where $R_\theta(t)$ is the projection of a vector on the membrane plane, which points from the first carbon of the first chain to the first carbon of the second chain of each lipid. $\langle x \rangle$ is the ensemble average of the $x$. $C_\theta$ for Gb3 decays very slowly compared to DOPC lipids (Fig. 7), because the Gb3 lipid has a significant difference in its chain lengths that results in interdigitation. In this situation, the Gb3 lipid can rotate in two ways (1) around its principal axis: such a rotation is strongly restricted because one of the tails is interdigitated into the opposite leaflet. (2) around its longer chain: such rotation is also slowed down, because the axis of rotation is not along the principle axis, and the moment of inertia of the lipid increases. At high concentration of Gb3, DOPC lipids are in contact with many Gb3 lipids not only in the same leaflet, but also from the interdigitated C22 Gb3 tails from the opposing leaflet, and this can cause DOPC to freeze. Also similar to the other effects, the impact of S-Gb3 is stronger than the one for U-Gb3.

**Fig. 7** Rotational Correlation function: A) $C_\theta$ for DOPC B) $C_\theta$ for Gb3. Gb3 lipids rotate much more slowly than DOPC lipids, owing to the large mismatch in the two chain lengths of Gb3. The longer chains thus interdigitate into the opposing leaflet (see text).
Phase separation and mixing of the Gb₃ in a DOPC lipid bilayer

The time and length scales accessible to atomistic MD simulations are in the order of μs and 10s of nm using present soft/hardware. It is difficult to study the complete spontaneous phase separation of lipid mixtures without resorting to coarse-grained techniques. To deal with this, instead of waiting for the system to phase separate, a phase separated system was created and the number of mixed lipids was considered. The initial configuration contains 16 Gb₃ lipids (for both S-Gb₃ and U-Gb₃) in a patch embedded into one monolayer of a DOPC bilayer. The simulation was then run for 400 ns.

Two U-Gb₃ lipids mixed in the DOPC bilayer after 400 ns (Fig. 8B), while no mixing was observed for S-Gb₃ for the same simulation time (Fig. 8A). Thus, we can hypothesize from the data that U-Gb₃ has a higher affinity to mix with DOPC, compared to S-Gb₃. This effect could be because of the height mismatch between DOPC and S-Gb₃ (Fig. 8 C and D) and/or because of unfavourable contacts between the saturated acyl chain of the S-Gb₃ and the unsaturated DOPC chains.

The method above does not quantify phase behaviour accurately, but does provide a comparison between the mixing rates of S-Gb₃ and U-Gb₃.

DISCUSSION

The Gb₃ carbohydrate moiety, which is identical in all Gb₃ types, is essential for Shiga toxin binding to a cell membrane and its subsequent internalization into the host cell. However, different types of Gb₃ behave differently in the Gb₃ involving processes. The most notable example is the inability of STxB to induce tubular membrane invagination upon binding to S-Gb₃ on the surface of a DOPC model membrane. On the other hand, STxB bound to U-Gb₃ on the surface of DOPC model membrane induces tubular invaginations. Thus, STxB binding to the membrane is insufficient for the formation of membrane tubular invagination and the presence of a specific Gb₃ species is required.

We have used all-atom MD simulations to investigate the effects of varying Gb₃ concentration as well as the degree of unsaturation of its acyl chains on the physical properties of DOPC lipid bilayers. Our results reveal two important features of the Gb₃ structure that strongly affect the structure and dynamics of the lipid bilayers when it is present at high concentrations. 1) The Gb₃ chain length mismatch results in interdigitation of the longer chain into the opposite monolayer and in subsequent reduction in lipid fatty acid chains fluctuations, and ordering of all bilayer chains. The effect is stronger for S-Gb₃ compared to U-Gb₃. 2) The degree of Gb₃ acyl chain saturation influences the affinity of Gb₃ lipids to mix or demix in DOPC lipid bilayers.

Gb₃ concentration is low in the experiments (up to 10 %). However, considering that each STxB molecule can bind up to 15 Gb₃ lipids, it is reasonable to think that Gb₃ concentration is high (up to 40 %) under the protein. Combining this assumption with the fact that STxB proteins cluster on the surface of a membrane, a high concentration of Gb₃ is expected in the domains enriched by STxB that modifies the local bilayer structure. This argument and the simulation results provide the following insights into several experimental observations:

a. Binding of STxB to a bilayer containing S-Gb₃ and DOPC does not drive the membrane to invaginate. We hypothesize that invagination will not occur when STxB is bound to an ordered phase. A high energetic cost must be borne to bend such a rigid bilayer which can not be compensated by the system. Such an ordered and rigid bilayer is observed at a high concentration of S-Gb₃ in our simulations, as shown in Fig. 5. A similar Iₐ phase is present in a phase-separated lipid bilayer composed of high Gb₃ concentrations.
of DOPC, sphingomyelin, cholesterol and porcine Gb₃. Binding of STxB to the l₊ phase of this membrane does not induce tubular invagination. On the other hand, U-Gb₃ does not form a rigid bilayer, and thus membrane invagination can occur without incurring the high bending energy penalty. Experiments also show that tubular invagination is observed upon STxB binding to a membrane containing only DOPC and U-Gb₃, which does not exhibit the lₒ phase.  

b. For lipid bilayers containing DOPC, sphingomyelin, cholesterol and Gb₃-C24:0, which are phase separated at room temperature, no change in the area percentage of the lₒ phase was observed upon STxB binding experimentally: We have shown that S-Gb₃ tends to remain phase separated in such a DOPC bilayer. Thus, SGb₃ is probably already in a phase separated state in the mixed bilayer even before STxB binds. Thus, STxB binds to the lₒ phase, and no further changes occur in the system.

Table 3 shows that membrane thickness increases with increasing Gb₃ concentration. The change is particularly significant at high S-Gb₃ concentrations.

c. Increase in membrane height due to protein clustering: Table 3 shows that membrane thickness increases with increasing Gb₃ concentration. The change is particularly significant at high S-Gb₃ concentrations.

d. The condensation of phospholipid-Gb₃ monolayers at high Gb₃ concentrations: Increasing Gb₃ concentration induces progressively lower area per lipid and a dramatic reduction was observed at high S-Gb₃ concentrations (Table 3). In the experiments a similar impact for S-Gb₃ was observed.

Our results suggest that the STxB binding to a lipid bilayer indirectly influences the properties of the lipid bilayer by clustering and accumulating Gb₃ underneath the protein aggregate. Invagination is not induced when Gb₃ is saturated, because S-Gb₃ forms a rigid immobile phase resistant to bending whilst the unsaturated version does not. Our results provide several hypotheses which can resolve some of unexplained experimental observations with regards to the phenomenon of membrane invagination induced by Shiga toxins. Further investigation is needed to validate these hypotheses.

ACKNOWLEDGEMENT

The research leading to these results has received funding from the European Community’s Seventh Framework Programme (FP7/2007–2013) under grant agreement number TRANSPOL-264399. Some parts of the computations were carried out on the Horse-shoe clusters at the SDU node for the Danish Center for Scientific Computing (DCSC)

We acknowledge that the results of this research have been achieved using the PRACE-2IP project (FP7 RI-283493) resource ARCHER based in United Kingdom at https://www.archer.ac.uk/. HK is funded by a Lundbeckfonden Young Investigator Grant. The LJ group was supported by European Research Council advanced grant (project 340485), Agence Nationale pour la Recherche (ANR-09-BLAN-283 and ANR-11 BSV2 014 03), and Human Frontier Program organization (RGP0029/2014C101).

References

5 L. Johannes and D. Decadin, Gene Ther, 2005, 12, 1360–1368.
11 B. Windschiegl, A. Orth, W. Römer, L. Berland, B. Stech...
The effects of Globotriaosylceramide tail saturation level on bilayer phases

The globotriaosylceramide acyl chains from one leaflet interdigitate into the opposing leaflet and lead to significant bilayer rigidification and immobilisation of the lipid tails. Globotriaosylceramide with saturated acyl chain can form a highly ordered, relatively immobile phase which is resistant to bending.