Molecular structures of GM1 and SM in lipid bilayer of $\text{GM1}_x/\text{SM}_{32-x}/\text{CHOL}_{32}$ on mica surface, for $x=16$ (a) and 24 (b) after 10-ns simulation.

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**Clustering effects of GM1 and formation mechanisms of
interdigitated liquid disordered domains in
GM1/SM/CHOL-supported planar bilayers on mica surfaces**

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Received 20 March 2010; in final form 11 July 2010

ABSTRACT

We have observed by atomic force microscopy that an interdigitated liquid disordered domain (ILDD) is formed in the ganglioside (GM1)/sphingomyelin (SM)/cholesterol (CHOL) bilayers on a mica surface and accelerates the formation of fibrillar A β agglomerates. By studies of the mechanisms using molecular dynamics simulations, we conclude that the ILDD structure is formed as a result of the phase separation to SM- and GM1-rich domains on the mica surface induced by the effects of GM1 clustering and the interaction between the GM1 head group and the water layer adsorbed in the ditrigonal cavity on the mica surface.

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Key words; lipid bilayer, sphingomyelin, cholesterol, ganglioside GM1, molecular dynamics, phase separation

1. Introduction

Glycosphingolipids have been implicated in trophic effects, cell-cell interactions, and differentiation [1-3]. In addition, they serve as receptors for toxins and viruses [4]. Ganglioside GM1 (GM1) has been extensively studied because of its role as a cell surface receptor for cholera toxin [1,4]. It has been suggested that GM1 is involved in modulating the effects of growth factors on cell adhesion and in neurite formation by neuronal cells [4]. Due to the importance of GM1, there have been many reports on its properties using artificial membranes, supported planar bilayer (SPB) systems [4-7], for which one can obtain precise information on the structure by applying advanced surface-analyzing techniques such as atomic force microscopy (AFM) [4-7], fluorescence microscopy (FM) [5], and secondary ion mass spectroscopy (SIMS) [8]. As for the structural analysis with lipid bilayers, nuclear magnetic resonance (NMR) [9], neutron scattering [10] and molecular dynamics (MD) computer simulation [11-13] have also made important contributions.

GM1 forms clusters through the hydrogen bonding between neighboring head groups [4]. Cholera toxin B (CTX-B) is combined with a maximum of five GM1 molecules and is often used for quantitative estimation of the density of GM1 molecules [1,4]. Shi et al. have investigated the dependence of the cluster size on GM1 concentrations in GM1/1-palmitoyl-2-oleoyl-phosphatidyl-choline (POPC) SPB systems [4]. They have also clarified that the apparent dissociation constant, K_d , for CTX-B-GM1 agglomerates increases from 0.11 to 0.86 nM with increasing GM1 concentrations from 0.02 to 10.0 mol%. GM1 is known to mediate amyloid beta ($A\beta$) aggregation, which is the hallmark of Alzheimer's disease [14-19]. It has also been reported that $A\beta$ agglomerates are formed on the surface of the GM1 cluster domain [20,21].

We have recently studied the interaction between $A\beta$ 40 and GM1/sphingomyelin (SM)/cholesterol (CHOL) SPBs on mica and SiO_2 substrates [22]. At a GM1 concentration of 20 mol%, unique triangular regions were formed on the mica surface, and formations of $A\beta$ fibrillar agglomerates were observed only outside these triangular regions. The speed of $A\beta$ 40 aggregation and the shape of the agglomerates depended on the molecular conformation of GM1. We have identified the conformation of the SPB domain, which significantly accelerates $A\beta$ 40 aggregation, as the interdigitated liquid

disordered domain (ILDD). However, no detailed mechanism of the phase separation forming unique ILDD has yet been understood.

In the present work, we have investigated the formation mechanism of the ILDD in GM1/SM/CHOL ternary SPB systems in detail. Our studies using MD and AFM have led us to the conclusion that phase separation of the SM- and GM1-rich domains is induced by both the GM1 clustering effects and the interaction between the GM1 head groups and the water layer adsorbed in the ditrigonal cavity on the mica surface.

2. Materials and methods

2.1. Preparations of SPB

SM (porcine brain, Avanti), CHOL (Sigma), and GM1 (bovine brain, Sigma) were used as delivered. A β -Protein (human, 1-40) was purchased from Peptide Institute Inc. All solvents and chemicals used in the experiments were analytical grade. A SiO₂ surface was formed by wet oxidation of a mirror-polished Si(100) wafer. These wafers were cleaned by successive treatments with the following solutions: concentrated H₂SO₄ + H₂O₂ (30%) (4:1, v/v), NH₄OH + H₂O₂ (30%) + H₂O (1:1:3), HF (5%), and concentrated HCl + H₂O₂ (30%) + H₂O (1:1:4). The cleaned surface was covered with a chemically oxidized SiO₂ layer with surface roughness of 0.14 ± 0.02 nm.

The A β 40 protein was dissolved in 0.02% ammonia solution. After being centrifuged at 100,000 rpm for 3 h (Beckman Instruments) to remove undissolved peptide aggregates that might act as nucleating factors, the supernatant (final concentration of 500 μ M) was collected and stored in aliquots at -20 °C until use.

The detail of the preparation of SPBs is reported in Ref. [22]. Lipids were dissolved in chloroform/methanol (1:1), mixed at predetermined ratios, and then spread by N₂ blowing. Finally, they were dried in a rotary evaporator followed by overnight storage under high vacuum. The lipid film formed by solvent evaporation was rehydrated to the final concentration of 0.12 mg/mL using the buffer solution (150 mM NaCl, 10 mM HEPES, 2 mM CaCl₂, pH 7.4), freeze-thawed, and sonicated at 60 °C to obtain small unilamellar vesicles (SUVs). The SUVs were first allowed to fuse on the substrate surface at room temperature for 30 min and then heated to 70 °C for 15 min. AFM was

performed in the buffer solution using a system of SPI3800 scanning probe microscopy (Seiko Instruments Inc.) in the tapping mode using a Si_3N_4 cantilever. The spring constant and the resonance frequency of the cantilevers were 1.6 N/m and 26 kHz, respectively, and all bilayers were imaged at room temperature.

2.2. Molecular dynamics simulation

The molecular structure of SM was created according to the topology file downloaded from the official NAMD (version 2.5) page. The molecular structures of CHOL and N-acetylneuraminic (Neu5Ac) acid were constructed with Gaussian 98 after calculations at the levels of B3LYP/6-311+G** and B3LYP/6-31+G**, respectively. The force field parameters of Neu5Ac were transferred from the force fields of organic molecules by using CHARMM [23], and CHOL was directly adopted from the force field parameters of alkanes and alkenes by using CHARMM27 [24]. The lipid bilayer model structure was constructed with PyMOL and VMD software [25]. In the MD simulations, unfavorable contacts between molecules were first removed by energy minimization. Then the lipid bilayer model structure was heated to 37 °C, and runs were executed to equilibrate the system at a constant temperature of 37 °C and a constant pressure of 1 atm. Water boxes of 5.0-nm thickness were added to both upper and lower sides of the starting bilayer model. The bilayer thickness is defined as the average distance between the N atoms in the upper and lower leaflets of SPBs. In SPBs containing GM1, the N atoms used for defining the thickness were those in N-acetylneuraminic acid (Neu5Ac α). To investigate the substrate surface effects, a fixed water layer was set under the lipid bilayer model. The fixed water layer adsorbed in the ditrigonal cavity on the mica surface was created according to the report by Park et al. [26]. The distance of each oxygen atom in the fixed water layer was 0.52 nm, and all oxygen atoms were set in the same level. The randomly fixed water layer on the SiO_2 surface was created using visual basic.

3. Results

3.1. Calculation of stable SPB conformations for several GM1 concentrations

We have shown the induction of unique ILDD by the surface effects of mica and significant acceleration of the formation of fibrillar A β agglomerate [22]. To clarify this

mechanism, we have analyzed the dependence of the clustering effects on the GM1 concentration and the formation of the ILDD using MD.

The stable structure of the SPBs affected by the ditrigonal water layer on the mica surface was calculated for GM1_{*x*}/SM_{32-*x*}/CHOL₃₂: the number of molecules $x = 2, 4, 6, 8, 16, 24$, and 32 , as shown in Figs. 1A and B, and the calculated time course of the SPB thickness is shown in Fig. 1C for a 10-ns simulation. A fixed water layer adsorbed in the ditrigonal cavity on the mica surface was set under the starting lipid bilayer model. The starting models in these calculations, i.e., the GM1 head-group-standing bilayer models for $x = 2$ to 16 and the ILDD models for $x = 24$ and 32 , were constructed using VMD and PyMOL software. Stable structures were obtained within 5-ns simulations in these models, as shown in Fig. 1C, except in those for the GM1 head-group-standing bilayer models with $x = 24$ and 32 , where no stable structures were attained within 10-ns simulations.

The composition dependence of the lipid bilayer thickness averaged in the simulation from 5 to 10-ns is shown in Fig. 2. The thickness of SPBs consisting of GM1 cluster varies with the number of GM1 molecules.

The clustering of GM1 molecules was clearly observed, and the fluctuation of the thickness due to the bending motion of the GM1 head group was larger for the lower density of GM1, i.e., in a smaller cluster. The detailed molecular conformations of these GM1 clusters, shown in Fig. 3, indicate that the hydrogen bonding and the hydrophobic interactions among GM1, SM, and CHOL stabilize the domain structures and reduce thickness fluctuations (Fig. 1C). Notably, the ILDD structure is stabilized for $x \geq 20$, as shown in Figs. 1 and 2.

To investigate the effects of hydrogen bonding in the OH groups on the SiO₂ surface, we have estimated the stability of the ILDD structure with GM1₂₄/SM₈/CHOL₃₂ on the randomly distributed water layer on the SiO₂ surface, as shown in Fig. 4A, by generation of random numbers. The starting model used for Fig. 1A(f) was used. The fixed water layer was set under the ILDD starting model with a lower leaflet GM1 head group of 0.25 nm from the water layer, as shown in Fig. 4, on both mica and SiO₂ surfaces. We found that the ILDD conformation was also stable on the randomly

distributed water layer on the SiO₂ surface, as shown in Fig. 4A. In experiments using AFM, however, no ILDD structures appeared on the SiO₂ surfaces at any concentrations of GM1 examined [22]. Therefore, a more careful consideration is needed to understand the phase separation induced by the effects of GM1 clustering and the substrate surface.

3.2. Phase separation on mica surface using AFM

Triangular regions often appeared only in the 20:40:40 mol% GM1/SM/CHOL SPBs, and the fibrillar A β agglomerates formed only outside these regions, where the lipid bilayer was found to take on ILDD conformations [22]. On the other hand, no fibrillar A β agglomerates were formed on either SiO₂ or mica substrates on the SPB surfaces, where GM1 molecules had the head-group-standing conformations. These observations led us to assume that fibrillar A β formation was due to the existence of the ILDD formed by phase separation.

To study the phase separation of GM1/SM/CHOL SPBs on the mica surface in more detail, we worked on the formation of fibrillar A β agglomerate using AFM under several conditions (all at 37 °C): (1) addition of A β followed by incubation for 36 h just after the formation of a 20:40:40 mol% GM1/SM/CHOL SPB, (2) addition of A β followed by incubation for 12 h after incubation of SPB for 36 h and appearance of triangular regions, (3) addition of A β followed by incubation for 12 h after incubation of SPB for 36 h (the same condition as (2) but no generation of triangular regions), and (4) addition of A β followed by incubation for 12 h on the surface of 50:50 mol% GM1/CHOL SPB after the SPB was incubated for 36 h. The time courses of the AFM images were observed for conditions (1) [Figs. 5 a, b], (2) [Figs. 5 c, d], (3) [Figs. 5 e, f] and (4) [Figs. 6 a, c].

4. Discussion

4.1 Details of molecular structures and phase separation

Figures 3b and 3c show that clustering of GM1 occurs through the hydrogen bonding interaction between the GM1 head groups. The MD calculation also shows that GM1 molecules move quickly in the 10-ns time scale and form clusters on the SiO₂ surfaces (data not shown). As for the formation of ILDD, however, AFM measurements show no evidence for formation of fibrillar A β agglomerates, i.e., the mark of the existence of

ILDD on the SiO₂ surfaces. This indicates that the clustering of GM1 is necessary but not sufficient for the phase separation leading to the formation of ILDD. The detailed molecular structures shown in Fig. 3 reveal that the SM head groups support the GM1 head group by hydrogen bonding and/or hydrophobic interactions, so that the GM1 head groups are not bent. Therefore, the results in Fig. 1A can be explained by the reduction of the SM concentration and the increase in the GM1 concentration. The SM molecules, which support the GM1 head group, disappear at some compositions between $x = 8$ and 16, and the ILDD structure appears at $x = 16$ and 32. We can conclude from these data that the ILDD structure is formed due to the deficiency of SM introduced by the phase separation on the mica surface. Furthermore, the sialic acid group, which is a sugar component of the pentasaccharide that forms the GM1 head group and is known to enhance the aggregation of A β [27], is exposed to the lipid bilayer surface in the ILDD structures (Fig. 3 d). On the other hand, it is stored in the forest of the GM1 head group moieties in the head-group-standing structures, as shown in Fig. 3c for example. Thus, it is reasonable to assume that formation of fibrillar A β agglomerate due to a high reaction rate indicates the existence of ILDD.

4.2 Morphology of SPB surfaces and dynamics of phase separations

Figures 5a and b show that fibrillar A β agglomerates are generated, but the triangular region is not observed clearly when A β is added just after SPB preparation (condition (1) in Sec. 3.2). This region often appears after incubation for 36 h at 37 °C, as shown in Fig. 5c, for 20:40:40 mol% GM1/SM/CHOL SPBs. In some cases, however, it does not appear even after incubation in the same condition, as shown in Fig. 5e. This seemingly mysterious phenomenon can be explained by the generation of this triangular region by coalescence, i.e., by the slow and long-distance movement of the microdomains, and this formation process is easily affected by a slight uncontrollable change in the conditions [22]. If A β is added after the appearance of the triangular region (Fig. 5c), formation of fibrillar A β agglomerates is observed outside the triangular regions (Fig. 5d), which is the phenomenon reported in Ref. [22]. If A β is added on the SPB surface (Fig. 5e) after incubation for 36 h at 37 °C but with no triangular regions appearing, note that both fibrillar A β agglomerates and triangular regions are formed after additional incubation for 12 h at 37 °C, and fibrillar agglomerates can also be observed only outside the triangular regions, as shown in Fig. 5f.

The data in Figs. 5a and b show that the formation of the ILDD, i.e., the phase separation of SM- and GM1-rich (SM deficient) microdomains starts just after preparation of the LPB on the mica surface. The size of the GM1-rich domain increases due to the coalescence of the microdomains. This coalescence process proceeds in parallel with the growth of A β fibrillar agglomerates. Since the size of the GM1-rich domain is small just after the formation of SPB and that of A β fibrillar agglomerates hinders the growth of the GM1-rich domain due to coalescence, no visible-size triangular region can be generated. In the process shown in Figs. 5e and f, on the other hand, the growth of fibrillar A β agglomerates does not hinder the coalescence of the phase-separated domains, because A β is added after the GM1- and SM-rich domains have grown large enough after 36 h of incubation, and wide triangular regions appear as well as the fibrillar A β agglomerates outside the triangular regions, as shown in Fig. 5f.

For 50:50 mol% GM1/CHOL SPBs, a stable ILDD forms as predicted using MD (Fig. 1A(f)). Interestingly, triangular or round triangular patterns are observed just after formation (Fig. 6a). The observed height of this region, 3.7 ± 0.7 nm (Fig. 6b), indicates that they are the second bilayers formed on the mica substrate with ILDD structures (point g in Fig. 2). In this case, fibrillar A β is formed by addition of A β followed by incubation for 12 h at 37 °C, as shown in Fig. 6c. Since A β aggregation proceeds also on these second bilayers, the patterns become indistinct after formation of full-coverage fibrillar A β agglomerates.

The ILDD structure is stable both on the mica and SiO₂ surfaces, as shown in Fig. 4. However, the experimental results show that no ILDD structure can be formed on the SiO₂ surface with any significant compositions. This indicates that no phase separation of SM- and GM1-rich microdomains is induced on the SiO₂ surface, and that the interactions between the GM1 head group and the water layer adsorbed in the ditrigonal cavity of the mica surface play an important role in the induction of phase separation in addition to the GM1 clustering effects.

4.3. Phase separation mechanism on the mica surface

To investigate the mechanism of phase separation on mica surfaces, we have compared the results of MD simulation for 10 ns with the models of GM1₁₆/SM₁₆/CHOL₃₂ head group standing between the SiO₂ and mica surfaces. As for

the bilayer thicknesses, no clear difference is observed between these surfaces (Fig. S1, Supplementary material). For the ILDD models, the MD simulation also showed that they were stable on both surfaces of SiO₂ and mica (Fig. 4C), whereas the corresponding experimental results were significantly different. First, the head-group standing model with GM1 /SM /CHOL was stable only on the SiO₂ surface, and phase separation occurred on the mica surface [22]. In the 50:50 mol% GM1/CHOL SPB, the ILDD structure was observed only on the mica surface, and no stable lipid bilayer was observed on the SiO₂ surface at this composition (Fig. 6). From these results, we conclude that (1) 10 ns MD simulation is not sufficient in the calculation time to explain the observed phenomena such as phase separation which progresses in a much longer time scale, and (2) the ILDD structure which appears at SM-deficient composition (Figs. 1 f and g) is formed as a result of the phase separation induced only on the mica surface, while experiments show that it is unstable on the SiO₂ surface.

4.4. Estimation of diffusion constants on SiO₂ and mica surfaces

For a more detailed discussion on the phase separation on the mica surface, we have calculated the diffusion constant D of GM1 for the head-group standing models with GM1₁₆/SM₁₆/CHOL₃₂ SPBs on the SiO₂ and mica surfaces using the MD simulation results and the Einstein relation [28],

$$D = \lim_{t \rightarrow \infty} \frac{1}{4tN} \sum_{i=1}^N \left\langle \left| \vec{r}_i(t) - \vec{r}_i(0) \right|^2 \right\rangle, \quad (1)$$

where $\vec{r}_i(t)$ is the center of mass position of molecule i and at time t , N is the total number of molecules, and the sum is taken over all molecules of a given species. The D values averaged between $t = 1.5$ to 2 ns are 59.5 ± 10.2 and $92.5 \pm 15.5 \mu\text{m}^2/\text{s}$ on the mica and SiO₂ surfaces, respectively. While these absolute values appear larger than those reported for GM1 [29], the difference between the diffusion constants of the mica and SiO₂ surfaces is quite evident.

The significant delay in the diffusion speed on the mica surface can be interpreted as that the intermolecular spaces among the GM1 molecules are limited by the effects of the GM1 head-group clustering and by the strong interaction between the GM1 head

groups and the water molecules adsorbed in the ditrigonal cavity of the mica surface. Since molecular compositions and conformations depend sensitively on these intermolecular spaces, it is likely that the molecular motion or diffusion is restricted and the SM molecules are excluded from the limited intermolecular spaces of GM1's, resulting in the observed phase separation.

5. Conclusions

We have investigated the formation mechanisms of the ILDD in GM1/SM/CHOL SPBs on a mica surface using MD and AFM. The MD simulation shows that the SM deficiency induced by phase separation causes the formation of ILDD and suggests that the sialic acid groups exposed to the SPB surface in the ILDD structures cause the formation of fibrillar A β agglomerates. On the SiO₂ surface, however, the sialic acid is hidden in the forest of the standing GM1 head groups in SPBs. This supports the assumption that the formation of fibrillar A β agglomerates is related to the existence of ILDD. We have also investigated the surface morphologies of 20:40:40 mol% GM1/SM/CHOL SPBs on a mica surface using AFM and found that, when A β is added after 36 h incubation at 37 °C, triangular regions appear and fibrillar A β agglomerates are selectively formed outside the triangular regions, regardless of the presence of the triangular regions at the time of A β addition. Furthermore, if A β is added just after the formation of SPB, fibrillar A β agglomerates, but not triangular regions, are formed after incubation. These observations suggest that the phase separation to SM- and GM1-rich domains, i.e. ILDD, starts just after SPB formation, and each domain size increases during the incubation through coalescence. Since the coalescence process is sensitive to a slight uncontrollable change in the conditions, triangular domains appear in some cases but not in others. The ILDD is formed as a result of the phase separation induced by both effects of GM1 clustering and the interaction between the GM1 head groups and the water layers adsorbed in the ditrigonal cavity of the mica surface.

Acknowledgements

This work has been partly supported by Grants-in-Aid for Scientific Research, Basic research (A) promoted by the Ministry of Education, Culture, Sports, Science and

Technology Japan, the JSPS Asian Core Program, the NINS Cooperation Project “Biomolecular Sensors”, IMS special research projects, and JST-CREST.

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Figure Captions

Fig. 1. (A) Side-views of lipid bilayers composed of GM1_x/SM_{32-x}/CHOL₃₂ and water layer adsorbed in a ditrigonal cavity on mica surface: GM1 in green, CHOL in red, and SM in blue, and (B) top-views of GM1 molecules (with their head groups in different colors) in lipid bilayers of (A) after 10-ns simulation and (C) simulation time dependence of lipid bilayer thickness of each model. $x=2$ (a), 4 (b), 6 (c), 8 (d), 16 (e), 24 (f), and 32 (g).

Fig. 2. Thickness of lipid bilayers composed of GM1_x/SM_{32-x}/CHOL₃₂. Number of molecules $x = 2$ (a), 4 (b), 6 (c), 8 (d), 16 (e), 24 (f), and 32 (g), averaged during simulation from 5 to 10 ns.

Fig. 3. Molecular structures of GM1 and SM in lipid bilayer of GM1_x/SM_{32-x}/CHOL₃₂, $x=2$ (a), 6 (b), 16 (c), and 24 (d) after 10-ns simulation. Sialic acid groups are indicated in (c) and (d).

Fig. 4. Side-views of lipid bilayers composed of GM1₂₄/SM₈/CHOL₃₂, on (A) SiO₂ and (B) mica surfaces. (C) Simulation time dependence of lipid bilayer thickness in models (A) and (B).

Fig. 5. AFM images of 20:40:40 mol% GM1/SM/CHOL SPBs on mica substrates. (a) SPB formed on freshly cleaved mica by incubation under vesicle suspension at RT for 30 min and then at 70 °C for 15 min, (b) A β was added to (a) and then incubated at 37 °C for 36 h, (c) SPB in (a) following incubation at 37 °C for 36 h, (d) A β was added to (c) and then incubated at 37 °C for 12 h, (e) the same condition as (c), but no triangular regions was formed, and (f) A β was added to (e) and then incubated at 37 °C for 12 h.

Fig. 6. (a) AFM image obtained just after formation of 50:50 mol% GM1/CHOL SPB on mica substrate, (b) line profile of SPB shown in (a), (c) A β aggregation on GM1/CHOL (50:50) SPB on mica substrate after addition of 2 μ M A β solution on (a) followed by 12 h incubation at 37 °C, (d) AFM image for the 50:50 mol% GM1/CHOL film formed by the same process as (a) on the SiO₂ surface, and (e) after addition of 2 μ M A β solution on (d) followed by 12 h incubation at 37 °C. Each scale bar is 500 nm.

Fig. 7. Time dependence of the diffusion constants of GM1 molecules on the (a) SiO₂ and (b) mica surfaces in the GM1₁₆/SM₁₆/CHOL₃₂ SPBs. These were calculated for 8

GM1 molecules in the upper half leaflets of the SPBs using the Einstein relation, Eq. (1).

Figure1

Figure2

Figure3

Figure4

Figure5

Figure6

Figure7