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Coexistence of two liquid crystalline phases in dihydrosphingomyelin and dioleoylphosphatidylcholine binary mixtures

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ABSTRACT

Recently, DHSM, a minor constituent in naturally occurring SMs, was indicated to form a raft-like ordered phase more effectively than a naturally occurring form of SM because DHSM has greater potential to induce the intermolecular hydrogen bond. In order to examine the influence of the DHSM-induced hydrogen bond on the phase segregation, the thermal phase behavior of stearoyl-DHSM/DOPC binary bilayers was examined using calorimetry and fluorescence observation and compared with that of SSM/DOPC binary bilayers. Results revealed that the DHSM/DOPC bilayers undergo phase segregation between two L_α phases within a limited compositional range. On the other hand, apparent phase separation was not observed above main transition temperature in SSM/DOPC mixtures. Our monolayer measurements showed that the lipid packing of DHSM is less perturbed than that of SSM by the addition of small amount of DOPC, indicating a stronger hydrogen bond between DHSM molecules. Therefore, in DHSM/DOPC binary bilayers, DHSM molecules may locally accumulate to form a DHSM-rich domain due to a DHSM-induced hydrogen bond. On the other hand, excess accumulation of DHSM should be prevented because the difference in the curvature between DHSM and DOPC assemblies causes elastic constraint at the domain boundary between the DHSM-rich and DOPC-rich domains. Competition between the energetic advantages provided by formation of the hydrogen bond and the energetic disadvantage conferred by elastic constraints likely results in L_α/L_α phase separation within a limited compositional range.

1. Introduction

Since 1980s, it has been reported that specific domains with distinct lipid and protein compositions exist in biomembranes [1–3]. In addition, some scientists speculated that lipid distribution is not homogeneous [4] but specific lipids assemble to form distinct domains in live cells. For example, in 1982, Zalduondo et al. reported that a gel-like ordered domain exists in bovine luteal cell membranes and shows high contents of SM and chol [5]. Recently, this SM/chol-rich domain, called lipid rafts [6], has attracted scientific interest since the raft provides a platform for important biological events, including signal transduction [7–13].

The biophysical properties of the lipid rafts have been determined using model membrane systems. Previously, DSC measurements revealed that the addition of chol to SM bilayers leads to the chol-rich and chol-poor phase segregation [14] and SAXD measurements disclosed that the chol-rich domain possesses the intermediate lipid packing between the L_α and L_β phases [15]. Recently, further complex systems have been employed to examine the membrane properties of rafts. For example, Veach and Keller reported that SM/chol/DOPC ternary mixtures undergo phase separation with SM/chol-rich and DOPC-rich domains [16]. FCS measurements revealed that the diffusion coefficient of the SM/chol-rich region (0.1×10^{-8} cm^2/s to 0.8×10^{-8} cm^2/s) was significantly smaller than that of the DOPC-rich region (~4.9×10^{-8} cm^2/s to 5.1×10^{-8} cm^2/s), but much greater than that of the L_β (or P_β) phase in GUVs comprising pure SM, which is virtually immobile within the time scale of FCS [17]. The SM/chol-rich 'fluidizing' gel phase with an intermediate diffusion coefficient is defined as the L_o phase [18] and the L_o phase

Abbreviations: DHSM, dihydrosphingomyelin; SM, sphingomyelin; DOPC, 1,2-dioleoyl-*sn*-phosphatidylcholine; SSM, stearoyl-SM; L_α phase, fluid phase; chol, cholesterol; DSC, differential scanning calorimetry; SAXD, small angle x-ray diffraction; L_β phase, gel phase; FCS, fluorescent correlation spectroscopy; P_β phase, ripple phase; L_o phase, liquid ordered phase; L_d phase, liquid disordered phase; GUV, giant unilamellar vesicle; NMR, nuclear magnetic resonance; PE, phosphatidylethanolamine; bodipy-PC, 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-*sn*-glyceo-3-phosphocholine; L_c phase, subgel phase; TR-DPPE, Texas-red dipalmitoylphosphatidylethanolamine; DEPC, 1,2-dielaidoyl-*sn*-phosphatidylcholine; DPPE, 1,2-dipalmitoyl-*sn*-phosphatidylethanolamine

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is thought to physicochemically resemble the lipid raft because the L_o domain shows strong detergent resistance, which is characteristic of lipid rafts [19–21].

Why does the SM/chol mixture conduce to the phase segregation and raft phase formation? Previously, IR spectroscopy revealed intermolecular hydrogen bond between the amide group in SM and the 3-hydroxyl group in chol [22]. Moreover, NMR spectroscopy has indicated that SM forms intramolecular and intermolecular hydrogen bonds more effectively than glycerophosphatidylcholines do [23–25]. Therefore, the electrostatic interaction between SM and chol is likely a crucial factor for the stabilization of the raft-like L_o phase that phase-separated from the L_d (or L_α) phase. Despite many reports on the SM–chol hydrogen bond, contribution of the SM–SM hydrogen bond to the phase separation should be known.

In order to address this issue, some studies on miscibility of SM with unsaturated lipid have been reported. For example, Maulik et al. found that egg SM (consisting mainly of C16:0-SM) and egg PC (70% of all PCs consist of unsaturated lipids) are immiscible in fluid (L_α) phase bilayers, whereas bovine brain SM (consisting mainly of C18:0-SM) and egg yolk PC are miscible within a certain temperature range [15]. SAXD result demonstrated that binary mixtures of egg PC and egg SM show L_α–L_α immiscibility, while the mixtures of egg PC and brain SM apparently are miscible at all temperatures [26]. The MD simulations showed that phase separation occurred in SM/DOPC bilayers, which led to formation of only small clusters having cluster molecular lifetimes of less than 200 ns [27]. Anyway, so far, the subject is very complicated and, thus, influence of SM–SM hydrogen bond on the phase segregation remains unclear.

DHSM contains a saturated bond between the sphingosine C4 and C5 and accounts for 5–10% of all SMs in cultured cells, such as human skin fibroblasts and baby hamster kidney cells [28,29]. Several recent studies have indicated that the DHSM has a higher potential to provide the intermolecular hydrogen bond than the usual type of SM. For example, fluorescent quenching measurements showed that C16:0-DHSM/chol bilayers form more ordered domains than comparable C16:0-SM/chol bilayers [30]. Fluorescence microscopic observations also suggested that, in GUV consisting of C16:0-DHSM/egg PC/egg PE/chol in a 1:1:1:1 molar ratio, the L_β phase composed of C16:0-DHSM/chol appears at room temperature, which is more ordered than the L_o phase. In contrast, the replacement of C16:0-DHSM by egg-SM (mainly consisting of C16:0-SM) results in formation of the L_o phase [31]. Moreover, some studies on lipid packing in pure DHSM membranes have been reported. Nyholm et al. demonstrated that a fluorescent dye, prodan, partitions less toward the L_α phase in the order of C16:0-DHSM > DPPC > C16:0-SM, indicating the formation of more ordered bilayers by DHSM than by SM in the absence of chol [32]. These results suggest that DHSM provide hydrogen bond more effectively than SM. Thus, it is expected that the DHSM is useful to examine the contribution of SM–SM hydrogen bond to phase separation.

The present study examined the thermal phase behavior of DHSM/DOPC binary mixtures using DSC and confocal fluorescence microscopy. Results showed that DHSM/DOPC binary bilayers experience L_α/L_α phase separation within a limited compositional range at temperatures greater than the main transition temperature. Very few studies about the phase segregation between two L_α phases in binary phospholipid systems have been reported [33,34]; therefore, this raft-mimicking mixture that experiences phase segregation in the L_α phase is very interesting. In addition, a possible mechanism is proposed for the L_α/L_α phase separation in the DHSM/DOPC binary mixture.

2. Materials and methods

2.1. Materials

Porcine brain SM and DOPC were purchased from Avanti Polar Lipid (Alabaster AL), and SSM was extracted from brain SM using HPLC. DHSM was prepared from SSM by hydrogenation of SSM using palladium (Wako Pure Chemical Industries, Ltd., Osaka, Japan) as a catalyst [35]. The purity of DHSM and SSM was checked by thin layer chromatography (TLC), and showing a single spot. These SMs and DOPC were separately dissolved in chloroform/methanol (4:1 v/v) at a concentration of 1 mg/mL or 10 mg/mL and stored at −20 °C until use. A fluorescent probe, bodipy-PC, was purchased from Molecular Probe (Eugene, OR). This probe was dissolved in chloroform/methanol (4:1 v/v) at a concentration of 50 μg/mL and stored in the dark at −20 °C until use.

2.2. DSC

The phase behavior of SM/DOPC bilayers was examined by nano-differential scanning calorimeter (Calorimetry Science Corp., UT). Bilayer samples were prepared by a conventional method. Briefly, appropriate amounts of SM and DOPC dissolved in chloroform/methanol (4:1) were mixed in a glass vial. The solution was dried under a flow of nitrogen and then under reduced pressure for at least 24 h. The resulting lipid film was dispersed into distilled and deionized water (Simplicity UV, MerckMillipore, Billerica, MA) and incubated for approximately 30 min at 55 °C for SSM mixtures and at 65 °C for DHSM mixtures with intermittent vortexing. The final concentration of SM was 5.48 mM. Then, 330 μL of the sample were placed into the DSC immediately before measurements. A scanning rate of 0.5 °C/min was used for all DSC measurements.

2.3. Surface pressure vs. molecular area measurements and preparation of glass-supported monolayers

Monolayers of lipid mixtures were prepared on a computer-controlled Langmuir film balance (USI System, Fukuoka, Japan) calibrated using stearic acid (Sigma Aldrich, St. Louis, MO). The subphase, which consisted of distilled, freshly deionized water, was obtained using a Milli-Q System. The apparatus was covered with vinyl sheets, which prevented deposition of dust on the water surface. The sample solution was prepared by mixing the appropriate amount of SM and DOPC solution in a micro-vial. A total of 30 μL of lipid solution (1 mg/mL) was spread onto the aqueous subphase (100×290 mm^2) using a glass micropipette (Drummond Scientific Company, Pennsylvania, USA). After an initial delay period of 10 min for evaporation of the organic solvents, the monolayers were compressed at a rate of 20 mm^2/s. The subphase temperature and the ambient temperature were controlled to 25.0 ± 0.1 °C and 25 ± 1 °C, respectively. The measurements were repeated 3 to 5 times under the same conditions to obtain reliable results. These measurements provided the molecular area at a corresponding pressure within an error of ~± 1 Å^2. The influence of oxidation on the unsaturated chains in SSM and DOPC at the air–water interface was checked by intentionally exposing pure SSM and pure DOPC monolayers to air for 10–30 min before compression [36]. The change in the isotherm after prolonged exposure of SSM or DOPC monolayers to air was within the error described above.

For the preparation of the glass-supported monolayers, the bodipy-PC (0.1–0.2 mol% of total lipids) was added to the sample solution for subsequent fluorescent microscopic observations. The micro cover glass (thickness no. 1, Matsunami, Osaka, Japan) was dipped vertically into the water followed by compression of the sample at 20 mm^2/s to reach the appropriate surface pressure. After compression, the glass substrate was extracted from the water at a rate of 0.2 mm/s to form the glass-supported monolayer. The fluorescence observations were conducted immediately after the sample preparation using confocal laser scanning microscopy (FV1000-D IX81, Olympus Corp., Tokyo, Japan).