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Phase separation in lipid bilayer membranes induced by intermixing at a boundary of two phases with different components

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ABSTRACT

We have demonstrated that dynamic phase separation is induced by coalescence of two self-spreading supported lipid bilayers (SLBs) with different components. Coalescence between a phosphocholine/sphingolipid SLB and a phosphocholine/cholesterol one forms raft-like liquid ordered (L_o) domains, which can be observed by fluorescence microscopy at the boundary of two phases. This phase separation process indicates that lipid molecules, such as sphingolipid and cholesterol, are intermixed. When saturated phospholipid is used instead of sphingolipid, small L_o domains are formed. Cholesterol is harder to incorporate with domains of saturated phospholipid than that of sphingolipid. This technique is very useful for observation of lipid–lipid interactions.

1. Introduction

In living cell membranes, co-operative functionality of lipids and proteins are closely-involved in biological activities (Anderson and Jacobson, 2002; Maxfield and Tabas, 2005; Simons and Ikonen, 1997; Lingwood and Simons, 2010). Therefore, it is important for elucidation of cell membrane functions to observe molecular interactions, such as lipid–lipid, lipid–protein and protein–protein in a lipid membrane. Lipid rafts, nano-domains consisting of sphingolipids and cholesterol (Chol) on cell membranes, play a significant role in biological activities, such as protein sorting and signal transduction in cells (Simons and Ikonen, 1997; Lingwood and Simons, 2010). Artificial two- or three-component lipid membranes, such as vesicles (liposomes) and supported lipid bilayers (SLBs), have been used to reproduce properties of lipid rafts as model systems of living cell membranes (Dietrich et al., 2001; Veatch and Keller, 2002; Rinia et al., 2001). In a two component lipid system consisting of unsaturated phospholipid and saturated one (or sphingolipid), a fluid phase and a gel one spatially coexist (Veatch and Keller, 2002; Rinia et al., 2001). Chol, another element of rafts, has an important role to modulate the physicochemical properties of cell membranes (Rekilä et al., 2002). When Chol is added to a two-component lipid system, its phase separation type is transformed to another type: a liquid disordered (L_d) phase that is enriched with unsaturated phospholipids and a liquid ordered (L_o) one that corresponds to a lipid raft structure in cell membranes enriched with saturated phospholipids (or sphingolipids) and Chol (Dietrich et al., 2001; Veatch and Keller, 2002; Rinia et al., 2001).

SLBs, which are lipid bilayer membranes expanded on a solid substrate for support, are generally prepared by the vesicle fusion method (Kalb et al., 1992), the Langmuir–Blodgett technique (Tamm and McConnell, 1985), and the self-spreading method (Rädler et al., 1995; Nissen et al., 1999; Isono et al., 2010; Yokota et al., 2014; Furukawa et al., 2008). In the self-spreading technique, SLBs spontaneously form on the substrate surface only by the deposition of a lipid lump on the solid substrate followed by incubation in a buffer solution, where an energy gain accompanied with an increase in the contact area between the lipid bilayer membrane and a hydrophilic surface of the solid substrate is utilized. In our previous works, we have reported behaviors of the self-spreading SLBs influenced by the atomic structure on the substrate (Isono et al., 2010) and two-dimensional fluidity of stacked structures of ternary lipid membranes fabricated by the self-spreading technique (Yokota et al., 2014). When two SLBs are formed by the self-spreading technique at near areas to each other on an identical substrate, both SLBs coalesce and fuse into a continuous membrane (Nissen et al., 1999; Furukawa et al., 2008). Moreover, if two self-spreading SLBs including different species of lipid molecules coalesce, these molecules are intermixed owing to diffusion of the molecules from one side of the SLBs to the other side. This technique enables us to observe dynamics of molecular interactions that are occurring in a

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lipid membrane. In fact, using this technique, resonant energy transfer in fluorescence has been observed as an example of the molecular interactions, in which lipids labeled with different fluorescent molecules were used (Furukawa et al., 2008).

In this study, we observed formation process of L_o domains, which correspond to lipid raft in a living cell, by coalescence of two lipid membranes. One of the lipid membranes contains sphingolipids and the other Chol, and the self-spreading technique combined with fluorescence microscopy was used, as shown in Fig 1. This technique enables us to insert Chol into a lipid membrane after its formation on the substrate. Therefore, we can clearly observe dynamic effects of Chol incorporation on the phase separation behavior. We also observed the L_o domains formed from sphingolipid or saturated phospholipid using the self-spreading technique, and demonstrated that lipid-Chol interaction depends on lipid-type. Previously, difference in the phase separation fashion in ternary lipid systems including sphingolipid or saturated phospholipid was studied with fluorescence microscopy (Veatch and Keller, 2005) and atomic force microscopy (AFM) (van Duyl et al., 2003). Veatch and Keller (2005) reported from fluorescence microscopy study that phase diagram of unsaturated-phospholipid/sphingolipid/Chol system and unsaturated-phospholipid/saturated-phospholipid/Chol one are similar. Using AFM, van Duyl et al. (2003) observed differences in L_o domain formation between unsaturated-phospholipid/sphingolipid/Chol and unsaturated-phospholipid/saturated-phospholipid/Chol SLBs. In this study, we have clearly observed difference between the ternary lipids including sphingolipid or saturated phospholipid in formation of the gel-phase domains and the L_o ones by fluorescence microscopy.

2. Materials and methods

2.1. Materials

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), *N*-palmitoyl-*d*-erythro-sphingosyl-phosphorylcholine (SM) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) were used as unsaturated phospholipid, sphingolipid and saturated phospholipid, respectively. The molecular structure of DPPC closely resembles palmitoyl SM in their chain length (16:0) and headgroup structure (choline). The transition temperatures of both palmitoyl SM and DPPC are 41 °C (Sripada et al., 1987; Curatolo, 1985). Fluorescent-labeled 1-myristoyl-2-[12-{(7-nitro-2-1,3-benzoxadiazol-4-yl) amino}dodecanoyl]-*sn*-glycero-3-phosphocholine (NBD-PC) was used as a probe of lipid membranes with fluorescence microscopy. These lipids were purchased from Avanti Polar Lipids. Cholesterol (Chol) was purchased from Sigma–Aldrich. All lipids were dissolved in a chloroform solution and stored at −28 °C before use. Chloroform and sodium chloride were purchased from Kanto Chemical. Buffer solution of *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) was purchased from Nacalai Tesque. All of the materials were used without further purification. Deionized water (>18 MΩcm^{-1}, Millipore) was used throughout the experiments in this work.

2.2. Methods

DOPC/SM, DOPC/DPPC and DOPC/Chol of a mole ratio of 1:1 or 3:1 were mixed in chloroform. 5 mol% NBD-PC was added to the chloroform solutions of the lipid mixtures where the mole ratio of the fluorescent-labeled lipid was selected to observe fluorescence for a long time. The chloroform solutions of the lipid mixtures were dried in vacuum more than 6 h, and then the obtained dry lipid films were used as lipid sources in the self-spreading of lipid membranes. The dry lipid films were stored in N_2 gas at −28 °C and used within a week.

The buffer solution of pH 7.4 was prepared from deionized water, 150 mM KCl, 1.0 mM $CaCl_2$ and 10 mM HEPES/NaOH and filtered through a 0.2 μm filter. The buffer solution was stored at 4 °C and used within a week.

Coalescence of the self-spreading lipid membranes was carried out on SiO_2/Si substrates whose surface was cleaned with a mixture of H_2SO_4 (98%) and H_2O_2 (33%) in a volume ratio of 3:1 at 80 °C for 10 min to remove organic contaminants and sonicated in deionized water for 5 min. The substrates were used immediately after cleaning.

Lipid membranes were prepared according to the previously reported method (Yokota et al., 2014). Two small lumps of dry lipids with different compositions, DOPC/SM (or DOPC/DPPC) and DOPC/Chol, were deposited on an identical SiO_2/Si substrate as the lipid sources, and then a buffer solution was added. To facilitate lipid membrane formation, the buffer solution was kept at 50 °C for 30 min. After the incubation, the buffer solution was cooled down to room temperature (20 ± 1 °C) to induce the phase separation of the formed membranes into the fluid and gel-phase domains.

To observe the coalescence processes of the self-spreading lipid membranes, we used fluorescence microscopy (Olympus BX51) with ×10 or ×40 objective lens usable in the buffer solution at room temperature. We observed the coalescence between DOPC/SM (3:1) and DOPC/Chol (3:1), DOPC/SM (1:1) and DOPC/Chol (1:1), and DOPC/DPPC (1:1) and DOPC/Chol (1:1).

3. Results and discussion

3.1. Coalescence between DOPC/SM (3:1) and DOPC/Chol (3:1) membranes

Fig. 2 shows time-lapse fluorescence microscopy images of the coalescence between two self-spreading lipid membranes; the left

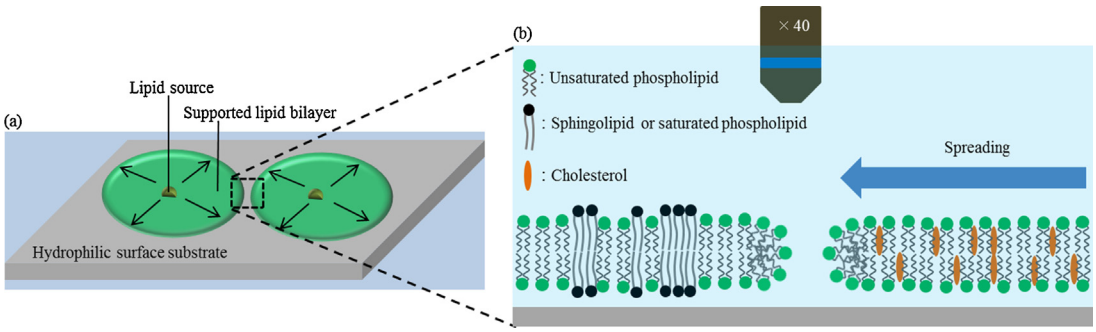

Fig. 1. Schematic illustrations of coalescence of two lipid membranes by utilizing the self-spreading method: (a) overview and (b) cross-sectional view. The left side membrane consists of lipids in gel- and fluid-phases, and the right side one lipids in fluid phase and Cholesterol (Chol). The lipid membrane on the right side spreads and coalesces into the left side lipid membrane, and then Chol is incorporated into the left side membrane.