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Spreading and Segregation of Lipids in Air-Stable Lipid Microarrays

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Spatial segregation of lipids, together with receptors and other signaling molecules, is common in most mammalian cell membranes.[1,2] The resultant lipid microdomains, known as lipid rafts, are crucial to numerous biological processes, ranging from protein sorting, signal transduction, membrane trafficking, to viral infection and cell−cell communications.[3−8] Supported model lipid membranes[9,10] have been used to mimic these naturally occurring microdomains for protein sorting and molecular recognition.[11−14] In this communication, we describe the spreading and segregation of lipids within a microspot of air-stable lipid microarrays, based on the patterns of cholera toxin subunit B (CTx) and streptavidin binding to their corresponding ligands, monosialoganglioside (G_{M1}) and *N*-(biotin)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (biotin-DHPE), coexisted in the host fluid lipid dilauroylphosphatidylcholine (DLPC).

Our experiments were initiated after we frequently observed uncontrolled spreading of fluid lipid molecules beyond the pin contact area, when quill pin printing technology was used to fabricate lipid microarrays. We hypothesize that demixing of distinct lipid molecules within the same microspot could occur when a mixture of lipid molecules are used for array fabrication. As shown in Figure 1a, the lipid mixture consisting of DLPC/G_{M1}/biotin-DHPE spreads beyond the pin contact area (the inner area shown by the broken pink circle), after being arrayed onto the surface of γ-aminopropylsilane (GAPS)-coated glass substrate. The DLPC bilayers on GAPS exhibit somewhat suppressed long-range lateral mobility, and only 50% of lipids are mobile, as determined by fluorescence recovery after photobleaching.[15] Compared to the host DLPC lipid and biotin-DHPE, the G_{M1} in the fluid DLPC tends to segregate[11] and thus has further attenuated mobility. As a result, biotin-DHPE becomes randomly distributed across the whole microspot, whereas G_{M1} is enriched within the pin contact area.

To test this hypothesis, we fabricated air-stable lipid microarrays on GAPS. Lipid mixtures suspended in 20 mM phosphate buffer (pH 7.1) were sonicated to form small vesicles. After centrifugation, the vesicle solution was used for array fabrication using a quill-pin printer (Cartesian Technologies Model PS 5000). A single CMP3 quill pin was used for all array fabrication. After printing, the arrays were subjected to 1 h immobilization under a controlled humidity chamber (relative humidity ~80−90%), dried under desiccation, and stored at 4 °C. Before use, the arrays were brought back to room temperature and rehydrated in a humid chamber (~85% relative humidity).

The spreading and segregation of lipids within the microspots are evident in Figure 1b and c. After being assayed with Cy3-streptavidin, fluorescent microspots show an average size of 240 ± 20 μm (n = 50) in diameter, about 80% larger than those of antibodies or GPCR membranes (130 ± 20 μm, n = 100) also fabricated with the CMP3 pin,[16−18] suggesting that there is significant spreading of lipid molecules within the microspots during the array fabrication and post-fabrication processes. The fluorescent pattern of Cy3-streptavidin also indicates that biotin-DHPE is

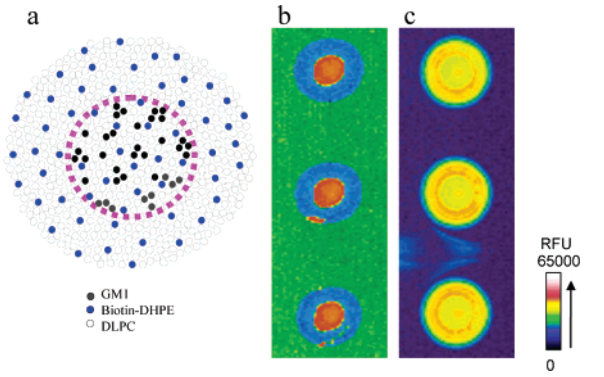

Figure 1. Lipid spreading and segregation within a microspot of air-stable lipid microarrays. (a) Schematic drawing of lipid segregation within a microspot. The pink broken line separates the inner G_{M1}-enriched area from the outer G_{M1}-poor area. (b−c) False-color fluorescence images of microarrays of DLPC/4 mol % of G_{M1}/1 mol % of biotin-DHPE after being assayed with 4 nM FITC−CTx (b) and 1 nM Cy3-streptavidin (c) in 50 mM phosphate buffer, pH 7.1, 0.1% BSA.

randomly and almost evenly distributed within each microspot. Conversely, the pattern of fluorescein−CTx (FTIC−CTx) binding to the same type of microarray dramatically differs. The binding of FITC−CTx is primarily located within the center area of the microspot. The average size of the FITC−CTx stains is 140 ± 10 μm (n = 50), similar to that expected using the CMP3 pin. However, the peripheral area of the FITC−CTx stains shows little signals, even lower than those further away from the microspot. This suggests the lack of G_{M1} in these peripheral areas because the DLPC/biotin-DHPE bilayer is resistant to the absorption of FITC−CTx. These results suggest that, during the array fabrication and post-fabrication processes, demixing of G_{M1} occurs, that is, the formation of both G_{M1}-enriched area within the pin contact area and G_{M1}-poor area in the rim of the microspots. On the other hand, there was still somewhat lipid spreading beyond the pin contact area, but no obvious G_{M1} segregation, when dipalmitoylphosphatidylcholine (DPPC), a gel phase lipid at room temperature, was used as the host lipid (see Supporting Information). One possible mechanism for lipid segregation is that heterogeneous mobility of distinct lipids within the fluid membrane bilayer plays important roles in the segregation. After a tiny volume (~0.5 nL) [18] of lipid mixture is deposited, lipid vesicles tend to rupture and spread in all directions until almost all lipid molecules are utilized to form a single bilayer, as shown by atomic force microscopy.[15] Since the segregation of membrane-bound molecules, including receptors, trisialoganglioside (G_{T1b}), and G_{M1}, was found to be related to lipid spreading (see Supporting Information), it is reasonable to speculate that during the spreading G_{M1} is transiently clustered, leading to alteration in mobility, which, in turn, enforces the segregation. Alternatively, since only ~50% of DLPC in the bilayer on GAPS surface is mobile, it is possible that the relative mobility of the individual leaflets in the bilayer and interaction between the upper

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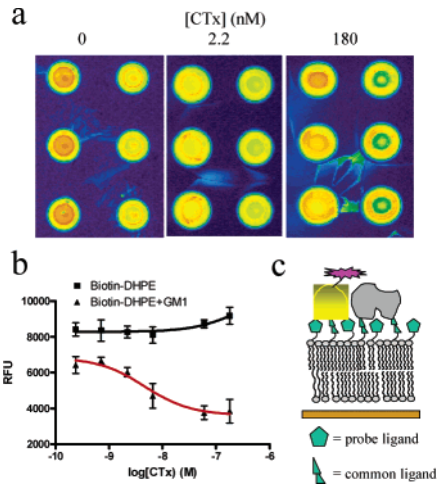

Figure 2. (a) False-color fluorescence images of lipid microarrays after being assayed with 1 nM Cy3-streptavidin in the presence of unlabeled CTx at different doses. In each image, there are two columns, each in triplicate, representing DLPC/4 mol % of biotin-DHPE (left) and DLPC/4 mol % of G_{M1}/4 mol % of biotin-DHPE (right). (b) The fluorescence intensity of the center area of DLPC/G_{M1}/biotin-DHPE microspots versus those of DLPC/biotin-DHPE microspots is plotted as a function of the concentration of unlabeled cholera toxin. (c) Schematic drawing shows the indirect competitive binding.

and lower leaflets also contribute to the clustering of the G_{M1} molecules into the inner regions.

The lipid spreading, however, results from the excess amount of lipids deposited onto the surface. The lipid spreading can be minimized by several approaches, such as by using appropriate concentration of lipids (~0.2 mg/mL for DLPC; data not shown), by controlling the volume of solution deposited with the preprinting of ~20−30 microspots on a spare slide in order to use up the solution adherent outside the quill of the pin, or by incorporating water-soluble protein (e.g., bovine serum albumin (BSA)) into the lipid solution before array fabrication. The BSA molecules tend to form packed layer surrounding the membrane microspot, thus limiting the lipid spreading.[17]

Since the glass substrate is substantially flat and sufficiently high numbers of both biotin-DHPE and G_{M1} are present within the center of each microspot, both streptavidin and CTx compete with each other for the same physical space, but different recognition sites. As shown in Figure 2, unlabeled CTx dose-dependently "displaces" the binding of Cy3-streptavidin within the center of each microspot but not in the rim of the same microspot. This indirect competition leads to an apparent IC_{50} of 4.9 ± 0.3 nM ($n = 3$), which is similar to those obtained through direct competition of unlabeled CTx against the binding of FITC−CTx using DLPC/G_{M1} microarrays.[16] The ability of unlabeled CTx to compete with Cy3-streptavidin, a pM binder to biotin, suggests the polyvalent interaction between CTx and G_{M1}. This is probably because G_{M1} is already clustered within the center of the microspots. Alternatively, CTx binding induces the rearrangement of G_{M1}.[19] Nonetheless, this suggests that it is possible to utilize a pair of common receptor−ligand interactions as a universal readout to examine the targets in a sample using two-dimensional microarrays. Here the common ligand coexists with all probe molecules in the microarray, while the common receptor, a universal readout, is capable of binding to the common ligand, but does not directly interfere with the binding of targets in a sample. The binding of the common receptor occupies the surface area of the microspot, thus providing a steric hindrance for the binding of targets to their corresponding probes. When a target in a sample binds to its probe(s), the target physically competes for the surface area with the binding of the common receptor, thus it can be quantified by measuring the signals of the common receptor.

In summary, we have fabricated lipid microarrays containing G_{M1} as a model for studying the spreading and segregation of lipid molecules in fluid membrane environment and demonstrated that spreading of distinct lipids within the same microspots is heterogeneous, which results in the segregation of lipids into distinct domains within the same microspots. The present study indicates an alternative means to precisely control molecular diffusion and separation based on the ability of lipids to heterogeneously spread once contacted with the surface of a substrate, besides conventional geometry-based approaches. [12,19]

Supporting Information Available: Details of array fabrication, assay protocols, and experimental design. This material is available free of charge via the Internet at http://pubs.acs.org.

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