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Sub-ten-nanometer heterogeneity of solid supported lipid membranes determined by solution atomic force microscopy

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ABSTRACT

Visually detecting nanoscopic structures in lipid membranes is important for elucidating lipid–lipid interactions, which are suggested to play a role in mediating membrane rafts. We use solution atomic force microscopy (AFM) to study lateral and normal organization in multicomponent lipid membranes supported by mica substrate. Nanoscopic heterogeneity is observed in a three-component system composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)/brain-sphingomyelin (bSM)/cholesterol (Chol). We find sub-ten-nanometer correlation lengths that are used to describe membrane lateral organization. In addition, we find that the correlation length is independent on cholesterol concentration, while the height fluctuation (variation) is not. To explore the mechanism that controls the size of membrane heterogeneity, we extend our study to a four-component system composed of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC)/POPC/bSM/Chol. By systematically adjusting the relative amount of DOPC and POPC, we obtain macroscopic-to-nanoscopic size transition of membrane heterogeneity. In contrast to the results from vesicle based fluorescence microscopy, we find that the structural transition is continuous both in the lateral and normal directions. We compare our nanoscopic structures to two theoretical models, and find that both the critical fluctuations and the nanodomain models are not sufficient to account for our solution AFM data. Finally, we propose a nanoheterogeneity model that could serve as the organization principle of the observed nanoscopic structures in multicomponent lipid membranes.

1. Introduction

Nonrandom lateral organization is a hallmark of the plasma membrane of eukaryotic cells [1]. Accumulating evidences point to the existence of nanoscopic spatial heterogeneity, or the so-called membrane rafts, in living cells. Since the conceptualization, membrane rafts have been proposed to play an array of biological functions, including signaling, membrane trafficking, viral entry and budding, and amyloid catalysis, to name a few. Most of the evidence supporting the existence of membrane rafts is based on indirect spectroscopy techniques. More recently, super-resolution microscopy revealed hindered diffusion [2] and transient trapping [3] of fluorophore-tagged molecules in living cells. Despite the compelling evidences, controversies remain pertaining to membrane rafts size, shape, and dynamics, or even their definitive existence has been challenged [4]. For example, a recent experiment employing super-resolution stimulated emission depletion microscopy and scanning fluorescence correlation spectroscopy (STED–FCS) showed that the diffusion behavior of fluorescent phospholipid and cholesterol analogues is homogeneous in living cells [5]. It is clear that further elucidation of membrane rafts and their exact nature requires direct visual evidence.

Simplified models employing a few lipid species have proven valuable to elucidate key properties associated with the heterogeneous organization in cell plasma membranes [6–8]. These models often contain a high-melting (high-Tm) lipid, a low melting (low-Tm) lipid, and cholesterol (Chol). Indeed, micron-scale liquid-ordered (Lo) and liquid-disordered (Ld) phase coexistence has been observed in a variety of ternary mixtures containing 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) as the low-Tm lipid, see Refs. [9,10]. The popularity of these mixtures is highlighted by the readily observable micron-scale domains using a light microscope. A more biologically relevant system is obtained by substituting DOPC with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) as the low-Tm lipid. Although optically homogeneous, mixtures containing POPC are often heterogeneous at the nanometer-scale as indicated by many spectroscopy measurements. To translate the nanoscopic heterogeneity inferred from model membranes to rafts in living cells, several models have been proposed to describe the nanoscopic structures present in lipid membranes. Among them the critical fluctuations and the nanodomain models have gained particular interest. Both models can successfully explain size transition from a few nanometers to microns. However, the critical fluctuations model cannot account for nanoscopic heterogeneity implied in a large compositional space of POPC containing membranes, while the nanodomain model suffers from the lack of visual evidence. In addition, it is known that nanomaterials often exhibit distinct properties

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from their bulk counterparts. This raises the question as to whether micron-sized domains can be faithfully translated into nanodomains.

Liquid compatible atomic force microscopy (AFM) is uniquely suited to explore lateral organization of planar lipid membranes [11–20]. Height contrast down to sub-angstrom enables AFM to detect structural features ranging from micron to nanometer sizes. In this paper we first use AFM to study composition dependent nanoscopic structures in a three-component system composed of POPC/brain-sphingomyelin (bSM)/Chol. Nanoscopic compositional heterogeneity is directly seen for the first time. Calculations of the height weighted pair distribution functions indicate that the heterogeneous structures have a correlation length of ~5–10 nm. To explore the mechanism of size transition in heterogeneous lipid membranes, we then investigate a four-component system composed of DOPC/POPC/bSM/Chol. Macroscopic-to-nanoscopic size transition is identified by gradually substituting DOPC with POPC. Based on the obtained nanoscopic structures in two multicomponent lipid systems, we propose a new model, the nanoheterogeneity model, to act as the organization principle of the nanoscopic structures in multicomponent lipid membranes.

2. Materials and methods

All lipid compositions are presented in mole ratio, fraction, or percentage. POPC, DOPC, bSM, egg sphingomyelin (eSM), rhodamine-DPPE (1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine), and cholesterol were purchased as lyophilized powders from Avanti Polar Lipids (Alabaster, AL).

2.1. Atomic force microscopy

Stock solutions were prepared by dissolving lipids in organic solvents (chloroform for POPC, DOPC, and cholesterol, and chloroform/methanol 3:1 for bSM and eSM). Lipid mixtures were prepared by mixing appropriate ratios of stock solutions in 13 ml glass test tubes. Organic solvents were removed by a gentle stream of argon gas using a 12-position N-EVAP evaporator (Organomation Associates, Inc.), and then vacuumed for >2 h. Lipid dry films were hydrated in ultrapure water and ultrasonicated using a Sonic Dismembrator at 40 W for 12 min. The obtained small unilamellar vesicles (SUVs) were centrifuged for 15 min at 13,000 rpm. Dynamic light scattering measurement using a Dynapro Nanostar (Wyatt Technology, Santa Barbara, CA) indicates that the SUVs have an average diameter of ~30 nm.

Solution AFM height images (at room temperature) were acquired using a Multimode 8 AFM (Bruker, Santa Barbara, CA) coupled with a Nanoscope V controller. A bungee cord supported platform sitting on a vibration isolated optical table was used to enhance system resolution (signal-to-noise ratio). After incubating SUVs (~0.2 mg/ml of total lipids) in the AFM liquid cell for >30 min, either at room temperature or at 50 °C using a heating accessory (Bruker model: MMHC-A60), a solid supported planar bilayer was formed by vesicle fusion onto a freshly cleaved mica substrate [21–23]. A unique mode referred to as the PeakForce quantitative nanomechanics (QNM) in liquid was used for data collection. A special Si_3N_4 cantilever designed to work with the PeakForce QNM mode (Bruker model: ScanAsyst-Fluid +) was used for bilayer scanning. The sensitivity of the cantilever (i.e., deflection versus applied voltage) was determined by deflection measurement on a mica film; the spring constant of the cantilever was determined using thermal oscillation (the method is built into the AFM software). Multiple square images (≥3) at different locations of the bilayer surface were acquired at a scan rate of 0.5–1.0 Hz. For control, we also scanned samples prepared at different days (e.g., POPC/bSM 3:2 + 20% Chol). The resulting bilayer structures are very similar for the same lipid composition. The peak force of each scan was set at ~300–600 pN depending on bilayer stiffness. (Note that the peak force does not affect bilayer topology.) AFM height images were leveled by subtracting a linear or second order polynomial background. The leveled images were used to calculate the height weighted pair distribution function [24,25]:

$$G(R) = \langle h(r) \times h(r+R) \rangle - \langle h(r) \rangle \langle h(r+R) \rangle \quad (1)$$

where brackets denote ensemble average over the radial distance r. Correlation length ξ corresponds to the exponential decay length of G(R).

2.2. Fluorescence microscopy

DOPC/eSM/Chol mixtures were prepared from stock solutions in the same manner as in AFM experiment. For fluorescence imaging, each lipid mixture contains 0.2% of the Ld phase marker, rhodamine-DPPE. Lipid mixtures were deposited onto ITO-coated glass slides and dried under vacuum for >2 h. Giant unilamellar vesicles (GUVs) in 100 mM sucrose were generated by swelling under an AC field of 10 Hz and 2.0 V (60 °C). Electroformed GUVs were dispersed in 100 mM glucose. After settling for >1 h, GUVs were transferred into a silicone gel well framed by a cover slip and a glass slide. Fluorescence images (at room temperature) were collected using an inverted microscope (Nikon Eclipse Ti-U), a CFI Super Fluor ELWD 60× objective, and an EM-CCD camera (Andor iXon 897).

3. Results and discussion

3.1. Three-component lipid membranes

Many indirect measurements have suggested the existence of nanoscopic structures in POPC/sphingomyelin (SM)/Chol. Domain sizes were proposed to range from hundreds to a few nanometers [26–31]. We use AFM to directly visualize solid supported planar bilayer structures composed of POPC/bSM/Chol. We first fix cholesterol concentration at 20% while varying the ratio of POPC/bSM. To span a large compositional space, eight ratios of POPC/bSM are chosen (Supporting Information, Fig. S1); the corresponding AFM topographic images are shown in Fig. 1. Heterogeneous structures with enhanced height variations (amplitude of ~0.2 nm) are observed when the ratio of POPC/bSM is near 1:1. Bilayers become exceedingly smooth at POPC/bSM ratios of 5:1 and 1:3.

To quantitatively characterize the structural transition as a function of POPC/bSM ratio, we calculate height probability distributions (Supporting Information, Fig. S2). By fitting to Gaussian functions, we find that the full width half maximum (FWHM) is 0.09, 0.10, 0.11, 0.16, 0.16, 0.09, 0.08, and 0.08 nm for POPC/bSM ratio at 5:1, 3:1, 2:1, 4:3, 1:1, 3:4, 1:2, and 1:3, respectively. The obtained FWHM values are consistent with our qualitative description of bilayer heterogeneity based on the height images and profiles (Fig. 1). In addition, the sub-angstrom values of FWHM highlight the good resolution of our experimental setup in the normal direction. Overall, our AFM data indicate that bilayers are more heterogeneous when the composition of POPC/bSM + 20% Chol is near the center of the Gibbs triangle phase diagram. Moving toward binary axes of POPC/Chol or bSM/Chol results in bilayers with less heterogeneity. Such a trend is compatible with several of the reported phase diagrams [31–33], and is against the prediction of phase coexistence near the POPC/Chol binary axis [34,35].

We next explore bilayer structures of POPC/bSM 1:1 with different cholesterol concentrations. The results are shown in Fig. 2. Dispersed macroscopic domains – presumably corresponding to the solid phase [9] – with irregular boundaries are observed at 0% Chol. Addition of 5% Chol disintegrates the solid domains into smaller sizes with smeared boundaries. Domain sizes remain similar at 8% Chol. However, the area fraction of the solid domains becomes noticeably larger. The bilayers at 10 and 12% Chol exhibit a remarkable structural feature with no distinct domains and surround. It seems that the transition