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Fluid domain patterns in free-standing membranes captured on a solid support

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ABSTRACT

We devise a methodology to fixate and image dynamic fluid domain patterns of giant unilamellar vesicles (GUVs) at sub-optical length scales. Individual GUVs are rapidly transferred to a solid support forming planar bilayer patches. These are taken to represent a fixated state of the free standing membrane, where lateral domain structures are kinetically trapped. High-resolution images of domain patterns in the liquid-ordered (l_o) and liquid-disordered (l_d) co-existence region in the phase-diagram of ternary lipid mixtures are revealed by atomic force microscopy (AFM) scans of the patches. Macroscopic phase separation as known from fluorescence images is found, but with superimposed fluctuations in the form of nanoscale domains of the l_o and l_d phases. The size of the fluctuating domains increases as the composition approaches the critical point, but with the enhanced spatial resolution, such fluctuations are detected even deep in the coexistence region. Agreement between the area-fraction of domains in GUVs and the patches respectively, supports the assumption that the thermodynamic state of the membrane remains stable. The approach is not limited to specific lipid compositions, but could potentially help uncover lateral structures in highly complex membranes.

1. Introduction

The conundrum of cholesterol-rich membrane-domains as carriers of biological activity is facing a number of challenges related to the length and time-scale of such domains. The initially proposed *raft*-hypothesis [1] based on static sphingolipid–cholesterol domains as platforms for GPI-anchored proteins found a justification in membrane biophysics as a realization of thermodynamic phase separation between l_o and l_d phases of cholesterol-containing bilayers and monolayers [2,3]. However, while large macroscopic domains are not observed in the plasma membrane, the evidence for a crucial role of cholesterol and sphingolipids in multiple membrane functions is mounting [4,5]. If such lipid-mediated effects are associated with membrane domains, their size must be small, well below the optical diffraction limit of ~200 nm. Here spectroscopic techniques like NMR and ESR [6] provide the main source of information and thus indirect evidence about the lateral membrane structure. Recent experimental studies of *rafts*, using stimulated emission depletion (STED) far-field fluorescence microscopy, claim presence of 20 nm domains in the plasma membrane where proteins reside for 10–20 ms [7]. Such small-scale structures are local and short lived, which impose a challenge for their experimental observation, and only few tools for their imaging are available. For example, AFM and STED microscopy are promising techniques for imaging of nanoscale membrane structures. These high-resolution imaging methods require membrane fixation either by a solid support or by a chemical cross-linking. However, it is well known that solid-supported membranes are subject to perturbations from the substrate. Concrete manifestations of such effects may be a reduction in the lipid diffusion coefficient [8], an increased phase transition temperature [9], different domain-size distributions and slower coarsening dynamics in membranes with phase separation [10]. This may also influence the organization of membrane curvature active components [11] and protein organization mediated by membrane conformational fluctuations [12]. Therefore, results on membrane domains obtained on a supported membrane cannot implicitly be assumed to represent the equivalent free standing membrane [3, 13–15]. In fact, the very purpose of membrane fixation is to decrease membrane fluctuations, both in-plane and out of plane. The dilemma has been that available high-resolution imaging techniques are often only applicable to supported membranes while information on the free-standing analog is wanted. In this work we aim to resolve this dilemma by avoiding the solid substrate during sample preparation and instead explore the support as a tool to rapidly immobilize domain features in free-standing membranes

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of equilibrated GUVs. With this approach we characterize fluid domain patterns in collapsed GUVs using high resolution imaging with AFM.

An additional challenge for membrane biophysics is to establish a framework for explaining the prevalence of small-scale membrane domains. It has been proposed that l_o and l_d critical point fluctuations may provide such a framework [16]. But true critical fluctuations require finely tuned thermodynamic conditions, which are delicate to maintain in a biomembrane. The results presented here point to the existence of a previously undetected population of microscale and nanoscale domains in ternary membranes. If such small domains are also present in a free-standing membrane they may represent thermal fluctuations characterized by formation of small minority phase domains deep inside the coexistence region and far from the critical point. The observations are similar to the findings for the related *main* transition of one and two-component PC-lipid bilayer systems [17–19], where lateral density and compositional fluctuations give rise to dynamic membrane heterogeneity in a wide range of system conditions around the phase transition/coexistence. The presence of a nearby l_o and l_d coexistence is a much less restrictive thermodynamic condition to fulfill than proximity to a critical point.

2. Materials and methods

1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC), cholesterol and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) were purchased from Corden-Pharma. The fluorescence probes, N-Lissamine rhodamine B 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (RhPE) and Napthopyrene (NaP) were purchased from Molecular Probes and Sigma respectively. Chloroform was of HPLC grade quality purchased from Rathburn (Micro-lab, Aarhus, Denmark). 1 mM stock solutions in chloroform of each lipid and each dye are prepared separately. Phosphorous analysis of the lipid samples was performed. Ternary mixtures with molar ratio of DOPC:DPPC:cholesterol (3:5:2), (3.5:3.5:3) and (4:2:4), corresponding to the compositions *I*, *II*, and *III* respectively, were prepared using the 1 mM stock solutions. The dye solutions were added to the ternary mixtures such that the dye/lipid molar fraction is 0.8%. We have tested for the possible influence of the probe on domain patterns by using a separate control with a probe concentration of 0.4%. No discernible differences were found in this case. The lipid dyes RhPE and NaP prefer respectively the l_d and l_o phases, as checked by independent Laurdan GP experiments. Glucose, sucrose and $MgCl_2$ were from Sigma. Ultra-pure MilliQ water (18.3 M Ohm cm) was used in all steps involving water. A mica sheet (Plano GmbH, Wetzlar, Germany) of size 1 cm × 1 cm was glued on a round glass coverslip of diameter 24 mm using a biocompatible silicon glue (NuSil Technology, Carpinteria CA, USA). These substrate samples were used for membrane fixation and in AFM and epi-fluorescence imaging. The osmolarity of solutions was checked using an osmometer (Osmomat 030, Gonotec GmbH, Berlin, Germany).

2.1. Preparation of giant unilamellar vesicles (GUVs)

Electroformation is a widely known method for the preparation of GUVs [20]. We use a home-made electroformation chamber consisting of two parallel Pt-wires, connected to an external AC power-supply. We coat each of the two Pt-wires with 2 μl of the lipid solution (1 mM, in chloroform) using a Hamilton syringe. The chamber is then stored overnight in vacuum. To form vesicles by electroformation, the chamber is filled with 200 mM sucrose (400 μl) and an AC-voltage is applied at 55 °C in the following sequence: 10 Hz (0.2 VPP (peak-to-peak voltage) for 5 min, 0.5 VPP for 10 min, 1 VPP for 20 min, 1.5 VPP for 20 min, 2 VPP for 30 min), 4 Hz (2 VPP for 30 min). Finally, the temperature of the chamber is decreased to 23 °C at a rate of 0.02 °C/min before experiments at room temperature.

2.2. Epi-fluorescence microscopy

A Nikon TE2000 inverted microscope with 40× long working distance objective (Nikon ELWD, Plan Fluor, NA = 0.6) was used for epi-fluorescence observations. All experiments were done at room temperature (22 °C). Fluorescence excitation of the RhPE probe was done at 540 nm using a Xenon lamp (Polychrome V, Till Photonics GmbH, Grafeling, Germany) and a G-2A filter cube (Nikon) was used for imaging. Fluorescence images were recorded with an em-CCD camera (Sensicam em, 1004 × 1002 pixels, PCO-imaging, Kelheim, Germany) operated with TILLvision software (Till Photonics GmbH). Epi-fluorescence images were analyzed with Matlab (Mathworks, Natick MA, USA) and ImageJ (National Institute of Health, Bethesda MD, USA).

2.3. AFM imaging

Atomic force microscopy was performed using a JPK Nanowizard AFM (JPK Instruments, Berlin, Germany) operated in intermittent contact mode in fluid. The AFM was mounted on the epi-fluorescence microscope described above. Silicon cantilevers for soft-tapping were used (PPP-NCST-50, Nanosensors, Neuchatel, Switzerland), having a spring constant of 1.2–29 mN/m and a resonance frequency of 76–263 kHz. During scanning, the sample was located in a fluid cell (BioCell, JPK Instruments), with freshly cleaved mica on round coverslips as the substrate. The same cell was also used for epi-fluorescence imaging. AFM images were processed and analyzed using the program SPIP (Image Metrology, Horsholm, Denmark).

2.4. Confocal microscopy

For confocal microscopy, vesicles were transferred to an eight-well microscopy chamber (Nunc Lab-Tek, Thermo Scientific, Waltham MA, USA) and observed by a Zeiss LSM 510 Meta confocal laser scanning fluorescence microscope (Carl Zeiss GmbH, Jena, Germany). Images were obtained with a 40×, C-Apochromat, water immersion objective with NA = 1.2. Two-channel image stacks were acquired using multi-track mode, using Argon lasers of wavelengths 458 nm and 543 nm, for NaP and RhPE excitation, respectively. The lasers were directed to the sample using two dichroic mirrors (HFT 458/514, HFT 488/543/633) for exciting NaP and RhPE respectively. Fluorescence emission was collected with photo-multiplier-tube (PMT) detectors. A beam splitter was used to eliminate remnant scattering from the laser sources (NFT 545) in a two-channel configuration. Additional filters were incorporated in front of the PMT detectors in the two different channels to measure the fluorescent intensity, i.e. a long-pass filter (>560 nm) for RhPE and a band-pass filter (500 ± 20 nm) for NaP. The acquired intensity images were checked to avoid PMT saturation and loss of offsets by adjusting the laser power, the detector gain and the detector offset. The image stacks were acquired above the Nyquist frequency. The raw confocal fluorescence image stacks were used for analysis without deconvolution. All confocal experiments were performed at room temperature.

3. Results

We examine ternary DOPC/DPPC/cholesterol membranes in the l_o and l_d coexistence regions and characterize the domain patterns in free-standing GUVs and in planar patches resulting from collapse of the individual GUVs. We have opted for three different lipid compositions: Two inside the liquid–liquid coexistence region (*I* and *II*) while the third composition (*III*) is located close to the critical point [21], as shown schematically, in the phase diagram of Fig. 1A. The area-fraction of the two membrane phases is a sensitive indicator of the thermodynamic state of the membrane. For this reason we are quantifying the domain area-fraction to test for changes in the membrane state

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