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Published in:
Biochimica et Biophysica Acta - Biomembranes

DOI:
10.1016/j.bbamem.2016.09.001

Publication date:
2016

Document version
Peer reviewed version

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Review

Exploring the raft-hypothesis by probing planar bilayer patches of free-standing giant vesicles at nanoscale resolution, with and without Na,K-ATPase

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ABSTRACT

The structure of functional lipid domains (rafts) in biological membranes has for long time been unresolved due to their small length scales and transient nature. These cooperative properties of the lipid bilayer matrix are modelled by free-standing giant unilamellar vesicles (GUVs) with well-characterized lipid composition. We review a series of recent advances in preparation and analysis of GUVs, which allows for characterization of small domains by high-resolution imaging techniques. These includes a new GUV preparation method with a desired overall lipid composition achieved by mixing small unilamellar vesicles (SUVs), test of the lipids compositional uniformity in GUVs and swift adsorption of GUVs to solid support by kinetically arresting the lateral structure of membrane prior to collapse for subsequent imaging. The techniques are applied to the analysis of membrane domains in GUVs formed from mixtures of DOPC/DPPC/cholesterol with and without Na,K-ATPase (NKA), a transmembrane protein known to be associated with rafts. Two mechanisms of domain formation are revealed: 1) close to lo/ld phase coexistence, domains in size up to 100 nm appear as thermally induced droplet fluctuations, 2) NKA shows interfacial activity and cluster in lo/ld micro-emulsion droplets. Some perspectives for the application of the techniques and the understanding of the nature of raft domains are outlined.

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Keywords:
Membrane lateral structure
Na,K-ATPase
lo/ld domains
Membrane rafts
Giant unilamellar vesicles

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http://dx.doi.org/10.1016/j.bbamem.2016.09.001
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Please cite this article as: T. Bhatia, et al., Exploring the raft-hypothesis by probing planar bilayer patches of free-standing giant vesicles at nanoscale resolution, with and ..., Biochim. Biophys. Acta (2016), http://dx.doi.org/10.1016/j.bbamem.2016.09.001
1. Introduction

The discoveries at the end of 1960s that single component lipid bilayers pose cooperative phase transitions [1] opened up for a new avenue in biomembrane research concerning the lateral organization of the membrane facilitated by the lipids and the lipid-protein interactions. In particular the finding that the lipid chain melting transition has the potential to promote phase segregation of complex membranes into spatially separated regions with distinct lipid composition and physico-chemical characteristics [2,3] has received utmost importance. However, the main transition takes the membrane from a lipid-chain conformationally and positionally ordered state, the solid-ordered phase ($s_o$) to the melted lipid-chain conformationally disordered phase, the liquid-disordered phase ($l_d$), while it was commonly accepted that biological membranes are mostly fluid [4]. This dilemma was partly resolved in 1980s with the finding that cholesterol, a major component in mammalian cell membranes, has the capacity to separate the melting of the lipid chain and positional degrees of freedoms and facilitate a new phase transition between two liquid phases from the $l_d$ phase to the new liquid-ordered phase ($l_{lo}$) [5,6]. These results provided the membrane biophysical basis for the membrane raft hypothesis which in cell biology context suggests how the lateral compartmentalization of the membrane organizes into “platforms” for bioactivities [7]. It became soon clear that in general, few-component model membranes exhibit macroscopic $l_d/l_{lo}$ phase separation which is not observed in biological membranes and therefore a simple interpretation of the membrane rafts (sub-optical domains) of complex membranes was not possible [8]. Hence the evidences for roles of cholesterol-dependent small-scale lipid domains in multiple membrane functions are mounting [9-11]. The mechanism behind such small domains have been puzzling biophysicists, e.g. it has been suggested that the coupling to the cytoskeleton may organize the $l_d/l_{lo}$ domains into smaller compartments [12], the active processes in the membrane may generate small domains [13] and the critical fluctuations close to the $l_{lo}$ critical point [14] or pre-transitional fluctuations [15] could be the cause for absence of macroscopic domains. A difficulty in the experimental characterization of the small membrane domains is their length scale well below the optical diffraction limit and time scale too short to capture by the current super-resolution imaging techniques.

In this mini-review we have described a new approach to this problem based on “GUVs collapse technique” that is used to prepare planar bilayer patches of free-standing GUVs which represent a “kinetically arrested” state of GUVs prior to its collapse such that features within patches at sub-optical length scales are captured in native state [16]. Fluorescence microscopy experiments showing GUVs collapse at ~10 ms time scale has been reported previously [17]. The studies in [17] and our own observations suggests that the adsorption of GUVs on a solid support starts with a single contact and quickly spread out until the whole vesicle is collapsed, accompanying instant immobilization of the lipid structures within the planar bilayer patch. The average speed of this adhesion front will be ~1 μm/ms/10 ms = 1 mm/s for a GUV of size ~10 μm. Thus the minimum size of the features in membrane which do not have time to rearrange during the event of collapse and therefore can be arrested in the planar bilayer patches in native state becomes ~1 μm²/s/1 mm/s = 1 nm. High resolution images of the planar bilayer patches reveal the elongation of the domains towards the edge of the GUV patch showing that the domains are not created under the fixation, but formed before and sheared in the process of adsorption of the GUV to the surface.

Planar bilayer patches are prepared by fast GUVs collapse that occurs upon adding an overall minimal concentration of the Mg^{2+} -ions (~0.27 μM). Support (mica) acts as a tool to immobilize the patch in a “kinetically arrested” state of GUVs. Previous studies of supported lipid bilayers (SLBs) prepared on mica show that support stabilizes the ordered lipid phases giving rise to significant shifts in phase equilibria for SLBs [18]. We have quantified membrane phase before and after the GUVs collapse to assure that membrane lateral structure is not influenced by the support. However, it is important to mention that if we use higher than overall minimal concentration of the Mg^{2+} -ions to collapse GUVs then membrane phase is influenced by the support and patches found to contain holes and cracks [16].

In this review, first we show that we can exploit $l_d/l_{lo}$ phase coexistence to demonstrate that GUV preparations actually produce vesicles which individually represent the desired lipid composition. Then a protocol for GUVs collapse on a solid support is described to capture the lateral membrane structure, which subsequently are imaged by high-resolution atomic force microscopy. This approach has been applied to two problems related to sub-optical domains in ternary lipid mixtures, so called model raft mixtures. We have found that 1) the $l_d$ and the $l_{lo}$ phases are accompanied by small-scale domain fluctuations close to phase coexistence, even at lipid compositions far from the critical conditions, 2) reconstituted Na-K-ATPase seems to have the capacity to emulsify the $l_d/l_{lo}$ coexistence.

2. Lateral membrane domains in GUVs

2.1. Lipid compositional uniformity among GUVs

Giant unilamellar vesicles (GUVs) have become standard experimental system for studies of the properties of free-standing model membranes [19], since their large size ~10 μm permits observations by light microscopy techniques of the individual vesicles and make them accessible to micro-manipulation techniques. Since 2001, GUV techniques became very popular in the quest for raft domains, since it was shown that $l_d/l_{lo}$ phase coexistence can be imaged by fluorescence microscopy [20]. Previously, the phase equilibria were mainly inferred from interpreting data from calorimetry and spectroscopy techniques [6]. This opened up for a wealth of new possibilities for studies of membrane domain properties of free-standing membranes at system conditions. In particular confocal fluorescence microscopy makes it possible to make global analysis of the lateral membrane structure [21,22]. The most popular model membrane systems are three or four component lipid mixtures involving high and low melting lipids and cholesterol, since they posses a deep $l_d/l_{lo}$ coexistence region, as shown in Fig. 1a for DOPC/DPPC/cholesterol lipid mixture at two different temperatures [23]. However, it was first recently shown that it is possible to make preparations of GUVs which are compositionally uniform between vesicles, so the findings from single vesicle observations actually can be considered as representative for the lipid composition [22]. This was done by utilizing the $l_d/l_{lo}$ phase coexistence, where the ratio between global $l_d/l_{lo}$ areas for a vesicle is highly sensitive to lipid compositional variations [21]. The quantification method is validated by evaluating the area-fraction of the $l_d/l_{lo}$ domains in GUVs for many different compositions. The possibility for such a quality check of the preparations furthermore gives the opportunity to systematically increase the complexity of the membrane [24], exemplified in Section 3, and to evaluate the effect of support on lateral structure of the planar bilayer patches (cf. Section 2.2).

Despite many advantages of GUVs as biomimetic membrane model systems they have not provided an answer to the existence of rafts as sub-optical domains, since standard optical microscopy is limited to length scales above the optical diffraction limit. Attempts to analyze free-standing GUVs at shorter length scales by super-resolution microscopy techniques are hampered by the configurational flexibility and dynamics of the membrane. One possibility to overcome this problem is to fixate the membrane in a way that preserves the lateral membrane structure of the GUV and thus create a snapshot, which can be imaged by high-resolution imaging techniques, as described in the section below.

2.2. Preparations of GUVs and their collapse on a solid support

GUVs are prepared by conventional electrooswelling method: dissolving lipids DOPC/DPPC/cholesterol in chloroform with an overall
desired lipid compositions I–III shown in Fig. 1a. The colored region in the coexistence region represents values of the area-fraction of the \( \text{lo} \) domains \( A_{\text{lo}}(l)/A \) varying between 0 and 1 indicated by a color bar. We have added 0.4 mol% of the fluorescent probes RhdPE and NaP to lipid mixtures for fluorescence observations. The lipid solution is coated onto two Pt-wires (Fig. 1b) and the chamber is stored in vacuum overnight (max 12 h) at low pressure for removing chloroform. GUVs are prepared by hydrating the dried lipids with 200 mM sucrose in a fluid observation chamber (mica bottom) followed by application of alternating electric field, as described in [16]. We have acquired confocal xyz stacks of GUVs and have collected the fluorescence intensities of the two fluorescence probes NaP and RhdPE into two separate channels. NaP and RhdPE prefer to partition into the \( \text{lo} \) and the \( \text{ld} \) domains respectively, as described in [21, 22]. GUVs are modeled as a sphere with surface as a triangulated mesh. Each pixel in the mesh represents the projection of corresponding voxel (volume pixel). We count fluorescence intensities in each voxel (from 3D confocal stacks) and project it onto the surface of the sphere to obtain two-dimensional histograms for fluorescence intensities counts. The counts are in the units of surface area (normalized to the unit sphere) per intensity unit squared, such that the integral of a region of the histogram gives the total area of triangles on the mesh with intensities in that region. For estimating the area-fraction of \( \text{lo}/\text{ld} \) domains in GUVs we have used histograms of fluorescence intensities counts of the two probes NaP and RhdPE on the surface of GUVs, which includes an overall contribution from sub-optical as well macroscopic domains.

To prepare planar bilayer patches of GUVs, we have transferred 50 μl of GUVs suspension in a fluid observation chamber (mica bottom) filled with 950 μl of the equi-osmolar glucose solution, as shown in Fig. 1c where the \( \text{lo} \) and the \( \text{ld} \) domains appear as bright and dark regions respectively. We have added 1 μl of 0.274 mM of MgCl₂ [16] at the center of the fluid chamber upon which GUVs in contact with mica collapse spontaneously to form planar bilayer patches, as shown in Fig. 1d (a movie is provided in the supplementary materials to show GUVs collapse). We found that the outer leaflet of GUV’s membrane remains in contact with support and the inner leaflet gets exposed to the bulk solvent upon rupture and collapse. The macroscopic \( \text{lo}/\text{ld} \) domains of GUVs are found to be retained in planar bilayer patches post vesicle collapse (numbers 1–4 in Fig. 1d indicate same GUVs before and after collapse). Previous studies reporting GUVs collapse [17] showed that the details in the shape of the patch depend on the position of the membrane pore of a critical size set by membrane tension and pore’s edge energy [25] that leads to GUVs rupture and collapse. We have imaged the planar bilayer patches at high spatial resolution using atomic force microscopy (AFM) as described in the Section 2.3 below.

### 2.3. Imaging of sub-optical and macroscopic membrane domains in GUVs

Fig. 2a–c show epi-fluorescence images of planar bilayer patches of lipid composition I–III displaying macroscopic \( \text{lo}/\text{ld} \) membrane phases where bright and dark regions are the \( \text{lo} \) and the \( \text{ld} \) domains respectively. For imaging small domains we have scanned a region of the patch (not the entire patch) at low scan speed and high spatial resolution. Fig. 2d shows topography scan of a region of the patch that is marked in Fig. 2a by small black dashed square. Small domains of the thicker phase (\( \text{lo} \)) in the macroscopic \( \text{ld} \) domain are found. The z-height profile along a line (red color) in Fig. 2d shows a step height difference of ~1 nm, as expected between the \( \text{lo} \) and the \( \text{ld} \) membrane phases. The lateral width of small \( \text{lo} \) domains is an overestimate because AFM lateral resolution is limited by tip’s size. Fig. 2e,f show topography images of the patches shown in Fig. 2a,b respectively that reveal the elongation of the domains towards the edge of the GUV patch showing that the domains are not created under the fixation, but are formed before and sheared in the process of adsorption of the GUV to the surface. We have found that the size of the small domains increases from lipid composition I to II. Fig. 2g shows z-height profile along the line shown in Fig. 2f displaying \( \text{lo}/\text{ld} \) membrane phases with a step height difference of ~0.8 nm and the bilayer thickness of ~3 nm. Fig. 2h shows cartoon of patch shown in Fig. 2f displaying the \( \text{lo} \) (green) and the \( \text{ld} \) (red) domains. Fig. 2i shows topography image of a patch shown in Fig. 2c of
lipid composition III. The bilayer thickness is ~3 nm (as shown in the inset) and we do not observe domains for composition III. Fig. 2j shows a small region in the patches (marked in Fig. 2e,f by dashed white rectangle) highlighting membrane domains of thinner (l_d) and thicker (l_o) phase with two colors: red for the l_d phase and yellow for the l_o phase. It is evident that patches when viewed in epi-fluorescence microscope appears to have two macroscopic l_d/l_o domains and imaging of the same region at high spatial resolution AFM reveals a population of small domains of the l_d phase within the macroscopic l_o phase and vice-versa.

To measure the area-fraction of the l_d/l_o domains in planar bilayer patches, we have obtained AFM topography image of entire patches by scanning at low speed and relatively low spatial resolution. The area-fraction of l_d/l_o domains in planar bilayer patches is calculated by counting the number of pixels that belongs to l_d/l_o membrane phases in topography images of entire patches. Lipid compositional homogeneity for a batch of GUVs and planar bilayer patches is quantified and compared, as shown in Fig. 2k. We have found that mean values of the area-fraction of the l_o (and l_d) membrane phase are consistent with the values as predicted by equilibrium thermodynamics in GUVs assuring that support has no influence on the membrane phase, in contrast to the observations with supported lipid bilayers (SLBs) [18].

We have detected small domains of size up to 100 nm of the l_d phase in the macroscopic l_o phase. The macroscopic l_d/l_o phase coexistence region. They also showed that domain size for different ternary lipid mixtures is controlled by mismatch in the lipid bilayer thickness between l_d/l_o membrane phases. These sub-optimal domains represent fluxation of transient domains of size up to (k_BT/λ) ~ (200 – 10) nm. Using small-angle neutron scattering Heberle et al. has reported sub-optical domains of size around 15 nm [27] in l_d/l_o coexistence region. Such short-scale cooperative behavior will be present even if the macroscopic phase separation domains are disrupted by various perturbations, e.g. cytoskeleton or proteins as quenched obstacles in the membrane.

3. Lateral membrane domains in GUVs containing Na,K-ATPase (NKA)

3.1. Functional reconstitution of NKA in vesicles

We have increased the complexity of the model membranes in GUV preparations to mimic the biological membranes. Reconstitution of
membrane proteins into GUVs to study their effect on the lateral membrane structure is an important step in that direction. It has been argued that the interaction with the cytoskeleton and the immobile proteins (quenched) provide obstacles in the membrane which effectively compartmentalize the membrane into small domains [28,29]. In this section we demonstrate that even mobile proteins can facilitate sub-optical domains in the membrane. In 2012, we have succeeded in reconstitution of one of the major integral protein Na/K-ATPase (NKA) into GUVs [30] of single fluid-phase membrane at physiological buffer conditions. Recently, we have developed a preparation method that allows for reconstitution of NKA into membranes with lo/l'd phase separations composed of phospholipids and sphingomyelin [31,32]. We have identified the experimental conditions that are required for preparing GUVs of uniform lipid composition by mixing small unilamellar vesicles (SUVs) of heterogeneous lipid compositions and different populations, thus allowing flexibility to increase the complexity of lipid mixtures forming GUVs in a controlled manner [24].

3.2. Preparation and characterization of proteoliposomes

NKA is functionally reconstituted into small unilamellar vesicles namely proteoliposomes, as described in [33–36]. First, NKA is isolated from shark rectal gland by mechanical disruption of tissue followed by centrifugation to collect the membrane fractions. The membrane fragments and SUVs of DOPC and DOPC/cholesterol (0.6/0.4) are cosolubilized with detergent C12E8 to form ternary protein:lipid:detergent micelles. Polystyrene beads (biobeads SM2) are carefully washed and incubated with solubilized membrane preparations at 4 °C. With a 14C-labeled C12E8, it was demonstrated that biobeads adsorbs the detergent from the mixed micelles following a double exponential with time constants of 5 and 60 min having C12E8 concentration of 0.75 g/g protein and 0.25 g/g protein respectively (c.f. Fig. 3 in [34]) leading to the formation of proteoliposomes. The proteoliposomes are unilamellar with a diameter of about 220 nm, as determine from the freeze-fracture EM (Fig. 3a) [35] and quasi-elastic laser light scattering [36]. The protein content of the liposomes was determined according to Peterson’s modification [37] of the Lowry method [38] and the specific hydrolytic activity of the NKA by the method of Baginski [39]. Three possible orientations of NKA were suggested [40], inside-out (i–o), right-side-out (r–o) and the non-oriented (n–o), as shown in Fig. 3b. Among these r–o is the native orientation in the cell membrane and is opposite of i–o. NKA in n–o orientation have their cytoplasmic and exoplasmic parts exposed to the medium. Activity of NKA is stimulated by adding ATP that is required for transport of ions 3Na+→(in):2K+→(out) across the membrane as shown in Fig. 3b. Based on activity measurement tests it was shown that typically ~10–15% of the NKA are in i–o orientation, ~50% are in r–o orientation and rests are in n–o orientation [34]. The test medium for activity measurements contained 120 mM NaCl, 30 mM KCl, 4 mM MgCl2, 3 mM ATP and 30 mM histidine at pH 7.4.

3.3. Preparation and characterization of GUVs containing NKA

We have prepared GUVs containing NKA of one, two and three component lipids by mixing SUVs followed by electroswelling at physiological buffer and temperature conditions, as described in [24,30–32]. The lipid compositional homogeneity among GUVs containing NKA is quantified by measuring the area-fracti of the Ld/Ld domains. We have identified two critical requirements to produce uniform samples of GUVs at physiological temperature prior to mixing are: 1) liposomes are SUVs and 2) membrane of SUVs remains in the fluid state. As described in [24], the lipid compositional state of GUVs has large variations and mixing of SUVs is not uniform if SUVs are in solid or gel phase instead of fluid phase. We find that proteoliposomes that are stored at ~80 °C for prolonged use no longer remain SUVs and it is necessary to produce SUVs from larger vesicles by invoking freeze-thaw cycles [24]. We have prepared GUVs of ternary lipid composition containing NKA, by mixing SUVs of two different populations i.e.; DOPC proteoliposomes with DPPC/cholesterol (0.538/0.462) SUVs. We add DOPC proteoliposomes to SUVs of DPPC/cholesterol (0.538/0.462) in an eppendorf tube at 37 °C, as described in [31,32]. At the end of electroswelling, the temperature of the chamber is decreased at the rate of 0.02 °C/min from 37 °C to 23 °C. For preparing GUVs of single (DOPC) and two component lipid mixtures (DOPC 0.6/cholesterol 0.4) containing NKA, we have used proteoliposomes of DOPC and DOPC/chole (0.6/0.4) and have followed the same preparation protocol as described above.

Fig. 4a shows confocal image of a DOPC/chol GUV containing NKA, which is imaged by adding 4 mol% of the probe Laurdan in the observation chamber. NKA in the same GUV are imaged using antibodies (c.f. section 2.6 in [32] for immunolabeling details) shown in Fig. 4b, displaying a homogeneous distribution of NKA in the membrane. Fig. 4c shows ternary GUVs displaying lo/l’d domains for which we
have quantified the area-fraction, as described in [21,22]. For a batch of vesicles, measurement of the area fraction of the \( l_q \) domains (\( A_{l_q}/A \)) gives a value \( 0.57 \pm 0.03 \) (mean ± SEM, \( N = 16 \)) as shown in Fig. 4d, which is consistent with the previously found values (colored region as shown in Fig. 1a) for GUVs prepared by electroswelling of lipid mixtures dissolved in organic solvent.

We have measured the NKA content in GUVs by using the same test medium for activity measurements as used for proteoliposomes. The protein content in the DOPC, DOPC/cholesterol (0.6/0.4) and DOPC/DPPC/cholesterol (0.35/0.35/0.3) GUVs (without fluorescence probes) is found to be almost 40%, 40% and 30% relative to proteoliposomes. The specific hydrolytic activity DOPC and DOPC/cholesterol GUVs is consistent with NKA reconstitution from proteoliposomes in which is 110 ± 4 \( \mu \)mol/mg h and 147 ± 2 \( \mu \)mol/mg h respectively at 23 °C. For ternary GUVs, we found a barely measurable activity of 10 ± 2 \( \mu \)mol/mg h (mean ± SEM, \( n = 6 \)) at 23 °C.

3.4. Lateral structure of membrane containing NKA

Fig. 5 shows planar bilayer patches of DOPC, DOPC/cholesterol (0.6/0.4) and DOPC/DPPC/cholesterol (0.35/0.35/0.3) which are prepared by the GUVs collapse protocol as described in the Section 2.2. We found that an overall minimal amount of \( \text{Mg}^{2+} \) ions that is required to collapse GUVs containing NKA in buffer (without \( \text{MgCl}_2 \)) is \( \sim 2 \text{mM} \) [31,32]. The patches are scanned at high spatial resolution using an AFM at room temperature inside the fluid observation chamber. GUVs of DOPC are in the \( l_q \) phase and that of DOPC/cholesterol (0.6/0.4) are in the \( l_i \) phase (c.f. section 3.3 in [32]). Fig. 5a,b show an AFM topography image of a selected region (4 μm 4 μm) of a DOPC planar bilayer patch displaying many particles embedded in the membrane. In the XY-plane (Fig. 5a), the particles appear as bright spots (elevated features) distributed randomly in the topography image. A three dimensional view of the same region is shown in Fig. 5b that reveals that bright spots in Fig. 5a are particles protruding out from the lipid bilayer membrane. Fig. 5c shows histogram of z-height of particles relative to bilayer (\( z = 0 \)) displaying a major population of particles having vertical height between 2 and 5 nm. In the inset, we have plotted height and phase line profile for two particles as indicated in Fig. 5a (encircled by an ellipse), showing height around 3–4 nm, lateral width of ~50 nm and phase (−0.5) degree relative to the membrane. Fig. 5d shows a high resolution topography image of a selected region (1 μm 1 μm) in a DOPC/cholesterol (0.6/0.4) planar bilayer patch displaying particles (bright spots) embedded in the membrane distributed randomly. Fig. 5e is a three dimensional view of the two particles (encircled by an ellipse in Fig. 5d), where different colors represent different z-heights, as indicated by a color bar. We found that lateral width of the particles is around ~50 nm which is an overestimate (due to convolution of the features width with the AFM tip) but is consistent in patches of DOPC and DOPC/cholesterol. Fig. 5f shows histogram of z-height for 170 particles in DOPC/cholesterol planar bilayer patches indicating a major population of particles having z-height values around 2–6 nm. Fig. 5g shows an epi-fluorescence image and corresponding schematic of a DOPC/DPPC/cholesterol (0.35/0.35/0.3) planar bilayer patch containing NKA. The red and green colors represent the \( l_q \) and \( l_i \) domains respectively. We have selected a region in the macroscopic \( l_q \) domain of the patch as indicated by a white box in Fig. 5g, close to the \( l_i/l_q \) interface which is scanned by AFM at high spatial resolution. We have used z-height values stored in the topography image to distinguish between the \( l_q/l_i \)
**Fig. 5.** Planar bilayer patches of GUVs containing NKA. (a) AFM topography image of a region (4 μm × 4 μm) of DOPC patch. (b) 3 dimensional view of the same region of patch. (c) Histogram of z-height of particles in patches. In the inset, we plot height and phase profile for the two particles shown in the “a” encircled by a blue ellipse. (d) AFM topography image of a region (1 μm × 1 μm) of a DOPC/chol patch. (e) 3 dimensional view of the same region of patch. Different colors represent different values of height indicated by the color bar. (f) Histogram of z-height of 170 particles in patches. (g) Epi-fluorescence image of a DOPC/DPPC/chol patch. The scale bar is 20 μm. A schematic is drawn to indicate the L₀ (green) and the Ld (red) domains in the patch. The white box shows a selected region of size (~4 μm × 4 μm) in the macroscopic L₀ domain close to the domain boundaries. (h) AFM topography image of selected region (white box in “g”). (i) Vertical height profile along the two lines shown in “h” displaying L₀/Ld membrane phases with a z-height difference of ~1 nm. (j) For a particle in “h” marked by blue arrow, z-height values in its neighborhood are extracted from topography image and are highlighted with different colors. Z at (0, − 1, 1 and 4) nm are labeled. R is an equivalent circular radius of the particle from its center. (k) Histogram of z-height for 103 Vertical height particles embedded in ternary patches.

**Fig. 6.** Voronoi data analysis. Panels a–c are for DOPC/chol patches and panels d–f are for DOPC/DPPC/chol patch. (a) Voronoi diagram for particles in a patch. (b) N is the number density of particles in the DOPC/cholesterol (0.6/0.4) patches. (c) Cumulative probability of the normalized Voronoi cells area t = A / Ā where A and Ā are the area and mean-area of Voronoi cells for the particles. The solid black line f(t) is the fit for random distribution. (d) Voronoi diagram of particles in a DOPC/DPPC/chol patch. (e) Histogram of the nearest neighbor distance (s) between the particles in patch. (f) Area-fraction of the Ld membrane phase in the vicinity of particles embedded in the patches.

Please cite this article as: T. Bhatia, et al., Exploring the raft-hypothesis by probing planar bilayer patches of free-standing giant vesicles at nanoscale resolution, with and ... Biochim. Biophys. Acta (2016), http://dx.doi.org/10.1016/j.bbamem.2016.09.001
domains. Fig. 5h shows topography image of a region within the selected region (white box in Fig. 5g) that reveals i) fluctuations of the thinner membrane phase \( (l_0) \) within the macroscopic \( l_d \) domain and ii) particles embedded in the ternary membrane patch are heterogeneously distributed in the form of small clusters. In Fig. 5i, we have plotted the z-height values along the two lines (shown in Fig. 5h) that confirms \( l_0/l_d \) regions in the membrane with a step height difference of \(-1\) nm, as also found in the ternary patches without NKA (Fig. 2d–f). We have shown z-height values (in nm) in the vicinity of a particle (marked by a blue arrow in Fig. 5h) in Fig. 5i with \( z = 0 \) nm indicating the thick phase \( (l_0) \) and \( z = (-1) \) nm indicating the thin phase \( (l_d) \) extracted from the topography image. The different colors are used to indicate different z-height values. A circle is drawn to indicate an equivalent circular area of the particle with radius R. Histogram of z-height for many particles in different patches is shown in Fig. 5k that indicates height distribution between 2 and 6 nm for majority of the particles. The spatial distribution and number density of the particles embedded in the membrane is estimated from Voronoi analysis, as described below.

Fig. 6a shows the Voronoi diagram of a DOPC/chol patch-region shown in Fig. 5d. Number density (N) of the particles is calculated by dividing the entire patch area into Voronoi cells (c.f. section 2.9 in [32]) and counting the number of Voronoi particles/\( \mu m^2 \) giving on the average 100 particles/\( \mu m^2 \) in DOPC/chol patch as shown in Fig. 6b. Fig. 6c shows a plot of the distribution of the normalized Voronoi cells area \( t = A/ \bar{A} \) where \( A \) and \( \bar{A} \) are the area and mean-area of Voronoi cells for the particles respectively. The solid black line is a fit, \( f(t) = c/s^\eta t/\lambda^{\eta} \) with fitting parameters \( \lambda = 3.61 \) and \( \eta = 0.71 \) as found for a random distribution [41]. Fig. 6d shows Voronoi diagram for a DOPC/DPPC/chol patch-region shown in Fig. 5h. Delaunay triangulation presentation is used to calculate the particles distribution and the distance (s) between two particles shown in Fig. 6e displaying a long tail of the distribution. The distribution of particles (bright clusters) is heterogeneous in contrast to that in DOPC and DOPC/cholesterol patches (Figs. 6a, 5a,d). To calculate the membrane phase in the vicinity of the particles, we have selected individual particles in the topography image and have extracted the thickness of the membrane within a circle of area \( 2\pi R^2 \) that encloses the selected particle of equivalent circular radius \( R \), as shown in Fig. 5g. In Fig. 5i, it is evident that particles are located at the \( l_0/l_d \) interfaces which suggest that particles have strong affinity for the interfaces compared to the \( l_d \) and the \( l_0 \) bulk phases.

The density of particles as found by the biochemical activity assays (c.f. Section 3.3) suggest that 40% of NKA are reconstituted in single phase GUVs. This corresponds to \(-121\) NKA/\( \mu m^2 \) in GUVs compared to 100 particles/\( \mu m^2 \) as observed in patches (Fig. 6a). From the crystal structure of NKA, we know that \( \alpha \)-subunit of NKA protruding out of bilayer at the cytoplasmic face correspond to height less than 6 nm and is almost doubled to that protruding out at the extracellular face of the bilayer consisting mainly of the \( \beta \)-subunit of NKA [34,42,43]. Therefore, we suggest that particles of height 1–3 nm correspond to \( \alpha \)-oriented NKA and those of height 4–6 nm correspond to \( \beta \)-oriented NKA. The n/o NKA will show height \(-1–3\) nm as suggested by the crystal structures [42, 43]. Previously, we have detected small domains of the \( l_0 \) membrane phase in the macroscopic \( l_d \) membrane phase and vice versa (Fig. 2) which is consistent even in the presence of NKA, as shown in Fig. 5h,l. We have selected individual particles in the topography image to measure the membrane phase in their vicinity shown in Fig. 5j. From Fig. 6f it is clear that NKA prefer to locate at domain boundaries and is justified by plotting a histogram of the area-fracture of the \( l_0 \) domain in planar bilayer patches for individual 103 particles. Interfacial localization of NKA has been previously suggested in [28,44,45]. The interfacial location of NKA and the organization of NKA into regions with high \( l_0/l_d \) interfacial proliferation are resembling microemulsions well known from oil-water surfactant systems [46], as discussed in the Section 4. Previously, it has been proposed that line active molecules can promote lateral inhomogeneities in membranes [47].

### 4. Conclusions

We have developed a range of new protocols and experimental techniques for preparation and analysis of GUVs, which we have used to address the question about the nature of sub-optical domains in free-standing membranes composed of model “raft” lipid mixtures with and without NKA. The technical advances includes control of the lipid compositional uniformity among GUVs, compositionally controlled GUV formation at physiological buffer conditions by mixing SUVs and fast collapse of GUVs on solid support as planar bilayer patches for further imaging. This combination of techniques has immediate applications to a variety of biophysical problems related to the lateral structure of free-standing lipid/protein membranes. Furthermore, the imaging of planar bilayer patches can be combined with super-resolution imaging techniques to determine lateral size of sub-optical domains and NKA spatial distribution.

Our analysis of GUVs with lipid compositions involving DOPC, DPPC and cholesterol close to the \( l_d/l_0 \) coexistence region revealed transient sub-optical domains up to 100 nm in size in the \( l_d \) and \( l_0 \) phase even at compositions far from the \( l_d/l_0 \) critical point. These thermally induced droplet fluctuations brings the \( l_d/l_0 \) transition back to its origin, as a derivation of the lipid main transition [5, 48]. The pseudo–critical fluctuations of the main transition are similarly associated with sub-optical domains [15]. Numerous studies have shown that the system conditions at which pseudo-critical fluctuations prevails can be strongly extended by the presence of domain boundary active compounds, which includes a plethora of small biologically relevant molecules [49,50]. An immediate application of our techniques is to study lipid micro-domains in the presence of such compounds.

Our studies showed that reconstitution of NKA into single phase DOPC/cholesterol bilayers leads to a completely random distribution of NKA. This picture is changed completely for NKA reconstituted into the \( l_d/l_0 \) coexistence region of the ternary lipid mixture, where NKA primarily appear in clusters. A closer look at the clusters reveals that they have the character of micro-emulsion droplets consisting of sub-optical domains of the \( l_d \) and the \( l_0 \) membrane phases with NKA in the interfacial region. Such interfacial location has been suggested previously based on biochemical experiments [44,45]. The \( l_d/l_0 \) interfacial boundary has a difference in membrane hydrophobic thickness of \(-1\) nm and the free energy cost to expose such edges to the lipid solvent can be minimized if NKA has mixed solubility with respect to the \( l_d \) and the \( l_0 \) phases, described as *hydrophobic matching* [51, 52]. This is agreement with structural finding of a highly asymmetric (polar) hydrophobic transmembrane region with segments which is hydrophobically matching the \( l_0 \) phase, while others match to the \( l_d \) phase [53,28,44]. Also, it agrees with the finding that NKA conformations are highly influenced by cholesterol content [54,55]. NKA thus have an emulsifying effect on the \( l_d/l_0 \) coexistence. The potential role for the function of NKA requires further studies including analysis of snapshots from GUVs with active NKA. It would be advantageous to image sub-optical domains in planar bilayer patches with and without NKA with super resolution techniques like STED (stimulated emission depletion microscopy) or STORM (stochastic optical reconstruction microscopy), in order to measure the lateral size of the features precisely. A systematic study of GUVs with \( l_d/l_0 \) coexistence reconstituted with NKA at well defined concentrations would be desirable to quantify the detailed morphology of features that are hidden below the optical diffraction limit.
In conclusion we have experimentally demonstrated that membrane domains at sub-optical length scales are prevalent close to the leq coexistence region in a ternary raft mixture. This study emphasize two mechanisms for stabilizing small domains: thermal droplet fluctuations and interfacial preference of interfacially active proteins. These mechanisms will prevail even if macroscopic phase separation is absent, the latter can even be the cause of it.

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgements

We thank Bianca Franchi and Hanne Kidmose for technical assistance and DAMBIC (Danish Molecular Bio-Imaging Center) for access to equipment. This work was supported by the Danish Council for Independent Research - Natural Sciences (FNU), grant number 95-305–23443, the Human Frontier Science Program (HFSP), grant number 95–305–73485 (to JHI) and the Danish Council for Independent Research – Medical Sciences, grant number DFF-4183-00011 (to FC).

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[9] K. Jacobson, C. Dietrich, L.A. Bagatolli, Z.N. Volovyk, N.L. Thompson, M. Levi, K. Jacobson, Lipid two mechanisms for stabilizing small domains: thermal droplet fluctuations and interfacial preference of interfacially active proteins. These mechanisms will prevail even if macroscopic phase separation is absent, the latter can even be the cause of it.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2016.09.001.

Supplementary information

There are no con-...