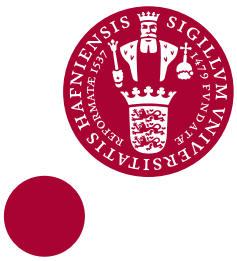

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Preparing giant unilamellar vesicles (GUVs) of complex lipid mixtures on demand: Mixing small unilamellar vesicles of compositionally heterogeneous mixtures

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ABSTRACT

Giant unilamellar vesicles (GUVs) are simple model membrane systems of cell-size, which are instrumental to study the function of more complex biological membranes involving heterogeneities in lipid composition, shape, mechanical properties, and chemical properties. We have devised a method that makes it possible to prepare a uniform sample of ternary GUVs of a prescribed composition and heterogeneity by mixing different populations of small unilamellar vesicles (SUVs). The validity of the protocol has been demonstrated by applying it to ternary lipid mixture of DOPC, DPPC, and cholesterol by mixing small unilamellar vesicles (SUVs) of two different populations and with different lipid compositions. The compositional homogeneity among GUVs resulting from SUV mixing is quantified by measuring the area fraction of the liquid ordered–liquid disordered phases in giant vesicles and is found to be comparable to that in GUVs of the prescribed composition produced from hydration of dried lipids mixed in organic solvent. Our method opens up the possibility to quickly increase and manipulate the complexity of GUV membranes in a controlled manner at physiological buffer and temperature conditions. The new protocol will permit quantitative biophysical studies of a whole new class of well-defined model membrane systems of a complexity that resembles biological membranes with rafts.

1. Introduction

The lipid bilayer is the basic building block of the cell membranes [1], which serves to structure and compartmentalize living matter in the form of cells and sub-cellular entities [2]. The complexity of cellular membranes in composition and dynamic organization has motivated the development of a variety of simpler model systems that serve as strong test beds for understanding more complex biological membranes [3]. Vesicles are free-standing bilayer models whose size, geometry, and composition can be tailored with high precision. These include bilayers in the form of vesicles of sizes ranging from 50 nm (small unilamellar vesicles, SUVs) to 100 μm (giant unilammellar vesicles, GUVs). The vesicle membrane involves a self-assembly of lipid molecules in water, owing its stability to mainly weak physical forces of electrostatic and colloidal nature [4]. The size of GUVs (1–100 μm) and their curvature enable us to visualize these individually, using an optical microscope, and GUVs have found application in numerous biophysical contexts in which membrane composition, tension, and geometry is controlled and manipulated using microscopy techniques such as, membrane flickering analysis [5,6], micropipette aspiration [7,8], or fluorescence imaging of lateral membrane organization [9]. The lipid composition of vesicles can be varied from a single lipid component to mixtures of lipids (synthetic or natural lipid extracts) containing proteins [10,11,12,13,14,15,16], or fragments from natural cell membrane [17,18].

The classical technique for the formation of giant liposomes is first to dissolve lipids (or mixtures of lipids) in an organic solvent, and a film of lipid solution is then spread on a support (such as glass [19]). The organic solvent is evaporated and the lipids are hydrated at a temperature above the main chain melting transition temperature (T_m) of the lipid components in the mixture in order to prepare giant liposomes [20,21,22,23]. Vaz et al. [24] and Keller et al. [25] have successfully used this "gentle hydration technique" for the reconstitution of integral membrane protein glycophorin and K^+-channel respectively, into large vesicles by rehydrating SUVs (instead of dried organic solution of lipids) containing the purified membrane proteins (called proteoliposomes). The advantage of SUV-based hydration method is that the risk of denaturing the membrane proteins from organic solvents is minimized. However, the large vesicles prepared by hydration methods are found

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to be mostly multilamellar, pausilamellar and very few unilamellar. In addition, the lipid compositional variation among those vesicles remains unexplored.

It was first shown by Angelova et al. that a high yield of giant unilamellar vesicles is expedited if rehydration of dried lipids plaque is assisted by an AC electric field [26,27,16,28]. The process is known as electroformation. It was reported by Husen et al. [27] that the electroformation based GUV preparation method involving dissolution of lipids in an organic solvent produce a system of GUVs with small variability in composition. Electrofusion of a calculable composition of single or two-component GUVs prepared by electroformation to prepare single GUV of three lipid components has been also reported [29], which shows improved compositional homogeneity. GUVs are also prepared by a lipid or surfactant stabilized water–oil (w/o) emulsion [30] or water–oil–water (w/o/w) double emulsion [31].

Recently the single population proteoliposomes-based electroformation of GUVs has been used where SUVs were prepared containing the purified membrane proteins with endogeneous lipids [18] or come from a defined reconstitution protocol involving a premixed lipid composition [32,10,11,12,13,14]. This is one of the standard methods used for the successful reconstitution of membrane proteins and channels in GUV membranes, but works for a limited range of lipids. Also, the lipid composition and heterogeneity among vesicles in above mentioned studies are not compared with the electroformed vesicles made using the conventional deposition method (mixing organic solutions of lipids).

GUVs prepared by all of the above mentioned methods (by the single population SUV-based mixing or by mixing organic solutions of lipids) have found applications in a variety of biophysical problems, but they have some limitations [22]. This shows up in studies of more complex membranes, e.g. for lipid mixtures containing high-melting lipids, where the high temperature needed for electroformation increases the risk of denaturation of peptides and proteins, peri-oxidation of unsaturated lipids, electrolysis, or other chemical degradation during electroformation [33]. The rate limiting factor in making different GUVs is the apparatus and in order to prepare GUVs with new composition, it is required to prepare new samples (batches of SUVs or mixing organic solutions of the lipids). Therefore, the need arises for a fast and reliable new technique for formation of GUVs with well-defined composition under general buffer conditions in a physiologically relevant temperature range, which can also be used to reconstitute membrane proteins and channels in GUV membranes.

In the present article, we present such a new technique in the case of a three-component lipid mixture consisting of DOPC (low-melting lipid), DPPC (high-melting lipid) and cholesterol. We show that if SUVs of two different populations with the overall desired composition are mixed, dried and subjected to electroformation at physiological temperature (below the T_m of the lipid components in the mixture), the resulting GUVs are compositionally homogeneous. This allows for a controlled tuning of the lipid environment in GUVs without any need to prepare new SUV samples. This particular mixture was chosen since it is the one that most model membrane studies use for investigating putative raft formation [27]. The special feature of this ternary mixture is that it represents a fluid–fluid phase separation region (liquid-ordered/liquid-disordered, l_o/l_d). The compositional variation in GUVs is evaluated based on a 3D-analysis of confocal image stacks of GUVs displaying lateral phase coexistence. The area fraction of the l_o/l_d domains is found to be consistent with recent thermodynamic measurements of tie lines in the same membrane system. We discuss under which system conditions the method is applicable and then outline its perspectives and limitations.

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 Naphtopyrene (NaP) were purchased from Molecular Probes and Sigma, respectively. Chloroform was of HPLC grade purchased from Rathburn (Micro-lab, Aarhus, Denmark). Stock solutions of lipids in chloroform are prepared separately: 8.65 mM DOPC (containing membrane dye NaP 0.4 mol%), 8.65 mM DPPC:chol (53.8%:46.2% containing membrane dye RhPE 0.4 mol%), 3.4 mg/ml (5.43 mM) of DOPC:chol (60%:40% containing membrane dye NaP 0.4 mol%) and 3.4 mg/ml of DPPC (containing membrane dye RhPE 0.4 mol%). Glucose and sucrose were from Sigma. Ultra-pure MilliQ water (18.3 MOhm cm) was used in all steps involving water. The osmolarity of solutions was checked using an osmometer (Osmomat 030, Gonotec GmbH, Berlin, Germany).

2.1. Preparation of small unilamellar vesicles (SUVs)

Lipids mixtures in chloroform of 8.65 mM DOPC (containing membrane dye NaP 0.4 mol%) and 8.65 mM DPPC:chol (53.8%:46.2% containing membrane dye RhPE 0.4 mol%), 3.4 mg/ml (5.43 mM) of DOPC:chol (60%:40% containing membrane dye NaP 0.4 mol%) and 3.4 mg/ml of DPPC (containing membrane dye RhPE 0.4 mol%) are prepared. Around 500 μl of the 8.65 mM DOPC, 8.65 mM DPPC:chol (53.8%:46.2%), 1 ml of 3.4 mg/ml of DOPC:chol (60%:40%) and 1 ml of 3.4 mg/ml of DPPC is put into a flask and chloroform is removed from sample by using a rotary evaporator at 50 °C for about an hour. The sample-flask is kept in vacuum for about an hour to remove any residual chloroform at room temperature (23 °C). 500 μl of milli-Q water is added to the flask to hydrate the lipids and is mixed using the rotary evaporator without vacuum-tight conduit at R. T. for about an hour. The flask containing DPPC SUVs is kept at a temperature of around 45 °C during hydration and mixing. The hydrated lipid sample is transferred in an eppendorf and 500 μl of milli-Q water is re-added to the sample flask and mixed, resulting in an overall 1 ml volume (measured with a micropipette) of the 4.325 mM of DOPC, 4.325 mM DPPC:chol (53.8%:46.2%), 3.4 mg/ml of DOPC:chol (60%:40%) and 3.4 mg/ml of DPPC. A tip-ultrasonicator (Misonixs 3000, Qsonica, Newtown, CT operating at frequency 20 kHz) is used to prepare SUVs of 1 ml each of the lipid solutions in water inside a glass vial kept in an ice-bath (to prevent heat-induced chemical degradation of lipids) in the following sequence: one step of sonication for 10 s and break for 5 s at 2 W power, for total sonication and break time of 20 min and 10 min respectively. In this way, we have prepared SUVs in water of concentration (1) 4.325 mM of DOPC containing membrane dye NaP, 0.4 mol%, (2) 3.4 mg/ml of DOPC:chol (60%:40%) containing membrane dye NaP, 0.4 mol%, (3) 3.4 mg/ml of DPPC containing membrane dye RhPE, 0.4 mol%, and (4) 4.325 mM of DPPC-chol (53.8%:46.2%) containing membrane dye RhPE, 0.4 mol%. For prolonged use, we have stored the SUVs at (−20 °C). SUVs are sonicated (following the same protocol as mentioned above) and extruded using a Nuclepore polycarbonate membrane filters with pore size of 100 nm at 37 °C, prior to use.

2.2. Confocal microscopy

For confocal microscopy, a Zeiss LSM 510 Meta confocal laser scanning fluorescence microscope (Carl Zeiss GmbH, Jena, Germany) is used. GUVs were transferred to an eight-well microscopy chamber (Nunc Lab-Tek, Thermo Scientific, Waltham MA, USA) and imaged with a 40×, C-Apochromat, water-immersion objective with NA = 1.2. Two-channel image stacks were acquired in multi-track mode, using Argon lasers of wavelengths 458 nm and 543 nm for NaP and RhPE excitation, respectively. The lasers were directed to 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 raw confocal fluorescence image stacks were used for analysis without deconvolution.

3. Results

3.1. Preparation of giant unilamellar vesicles (GUVs)

We have prepared two different GUVs samples composed of, I: DOPC (52%):chol (22%):DPPC (26%) and II: DOPC (30%):chol (20%):DPPC (50%). We have mixed for sample I: 20 μl of 4.325 mM DOPC SUVs with 18.57 μl of 4.325 mM DPPC:Chol (53.8%:46.2%) SUVs, and for sample II: 23 μl 3.4 mg/ml DOPC:chol (60%:40%) SUVs with 27 μl of the 3.4 mg/ml DPPC SUVs in an eppendorf. Ternary mixture phase diagram and lipid composition of the samples within the coexistence region is shown in Fig. 1a. The mixed SUV suspension of samples is diluted with pure water to a concentration of 1.1 mg/ml. A home-made electroformation chamber consisting of two parallel Pt-wires, connected to an external AC power supply is used for electroswelling of GUVs. Many well separated 2 μl drops of SUV suspension are deposited onto Pt-electrodes. The Pt-wire chambers coated with samples are kept inside a tightly closed container (a desiccator catalog no. 24 782 61 from Schot-Duran, with minimal pressure drop inside enough to hold the container tight during incubation), in dark for 10–12 h at ~4 °C for dehydration of SUV suspension deposits. GUVs are prepared by rehydrating the dried SUV deposition with 200 mM sucrose solution, using an AC field at ~37 °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). For observations at R.T., the GUVs are cooled down to 23 °C after the electroformation is completed at a rate of 0.02 °C/min.

50 μl of GUVs of samples I and II are transferred to an observation chamber filled with 500 μl of 200 mM glucose solution for confocal fluorescence observations at room temperature. Fig. 1b-c shows GUVs of samples I and II. GUVs of sample I are around 5–100 μm diameter in size and mostly unilamellar and only few multilamellar, displaying two-phase coexistence. To investigate whether GUVs prepared by fusion of two populations of SUVs with heterogeneous lipid components display homogenized mixing of lipids during the electroformation processes, we have investigated the lipid compositional heterogeneity among GUVs by measuring the area fraction of the l_o/l_d domains using the two dyes NaP (green) and RhPE (red) which prefer the l_o and l_d phase, respectively: the method is described in [27,35,36]. We have observed that sample II does not lead to the formation of GUVs displaying lateral domains structures in a reproducible manner unless the temperature is raised above the main chain melting transition temperature of DPPC. For both samples, the yield, unilamellarity, and domain formation in GUVs are found to be reproducible, if SUVs are in the fluid state. This implies that the mixing method of SUV populations work for the fluid-phase, e.g. l_o/l_d phases, but cannot be used to prepare GUVs if one of the lipid components in the mixture is in the gel-phase during electroformation, as in sample II (see Discussion and conclusions).

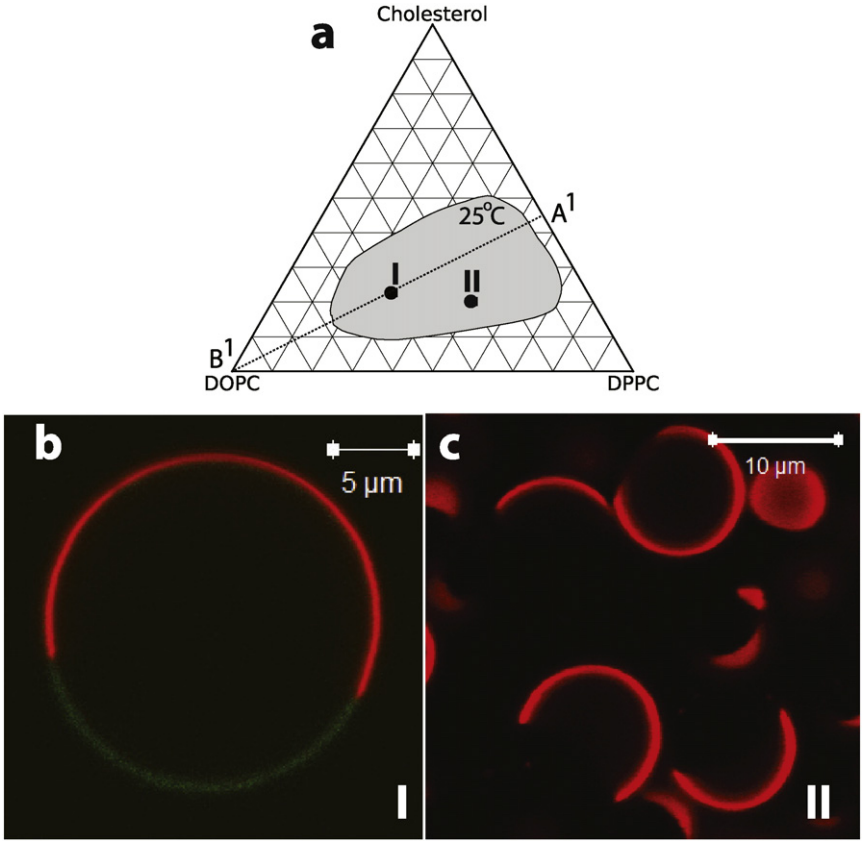

Fig. 1. (a) Phase-diagram of the ternary lipid mixture displaying the liquid–liquid coexistence region at 25 °C. The black dots represent samples I and II. A^1B^1 is a line on which all the lipid compositions by mixing A^1 = DPPC:chol (53.8%:46.2%) and B^1 = DOPC in different molar ratios will lie. (b–c) Confocal fluorescence images of GUVs of samples I and II. The red and green color represents the RhPE-dye and NaP dye that prefers the l_d and l_o membrane phase respectively.
a, taken from [34].

3.2. Quantification of domain areas in GUVs

Fig. 2a shows a three-dimensional image (reconstructed from the confocal 3D stacks) of a GUV of sample I, having round, micron sized, l_o/l_d domain structures on the surface of the vesicle. The area of l_d and l_o domains in GUVs having lateral phase coexistence is quantified by a 3D-analysis of confocal image stacks of each GUVs, a method developed by Husen et al. [27]. Fig. 2b shows a color map of the l_o area fraction measured in the GUVs prepared using the electroformation starting from a mixture of lipids dissolved in chloroform where the different colors represent l_o phase area fraction varying between 0 and 1, as described in [27].. The location of sample I is shown in the Fig. 2b.

For a GUV, first we correct for its translational motion and align the confocal stacks to calculate radius and center of mass. Then the whole vesicle surface is divided into a triangulated mesh with a uniform resolution. The fluorescence intensity of the dyes is measured on the surface of GUV within each mesh point (acting like one pixel) . In this way, we have measured the fluorescence intensities of the RhPE (red) and NaP (green) dyes which originate from the l_d and l_o phases respectively. A 2D-histogram representing counts of the two membrane dyes on the surface of a GUV is shown in Fig. 2c. Two well-separated regions in the histogram are used for the precise segmentation (shown by a black line in the histogram that separates the two membrane phases) of the l_o and l_d domain areas on the surface of a vesicle. Fig. 2d shows the variation in the l_o area fractions for a batch of vesicles prepared of sample I. The values of the l_o area fractions are centered around a mean value of $38.7 \pm 1\%$ (SEM, $N = 14$). The error has contributions from both the compositional variation among vesicles and the variation from the vesicle area detection from the acquired confocal stacks. Furthermore, since the coexistence only constitutes a small part of the total phase diagram (as shown in Fig. 2b), the variations of the area fractions are systematically amplified compared to the corresponding compositional variations. The given standard error on the area fraction thus constitutes an upper bound on the standard deviation of the compositions. Fig. 2d, suggests that the lipids in the membranes are well mixed and that the vesicle sample is uniform. The sensitivity and quality of method is clear as the lo phase area fraction closely matches with the value previously found for the same composition prepared by lipid dissolved in organic solvents, and can be compared using the color map in the Fig. 2b.

4. Discussion and conclusions

A well-investigated ternary model membrane system consisting of DOPC, DPPC, and cholesterol was chosen with a composition such that it belongs to a point in the l_o/l_d coexistence region, as suggested by previous studies. The l_o-domain area fractions for a batch of GUVs prepared of sample I is shown in the Fig. 2d suggest that the lipids are mixed well and a compositionally uniform vesicle sample has been prepared. The sensitivity of the area fraction to the thermodynamic state of the membrane serves as general control that the state is not measurably shifted during mixing. At room temperature, SUVs of DOPC, DOPC:chol (60%:40%) and DPPC:chol (53.8%:46.2%) are in the liquid-phase [37,38,39] and SUVs of DPPC are in the gel-phase. We find that lipids are mixed thoroughly if the SUV suspensions of two different populations are in the liquid-phase (as in the sample I). If one of the lipid components of SUVs is in the gel-phase (as in the sample II at 37° C) then it is required to perform electroformation above the main chain melting transition temperature of all the lipid components, otherwise the

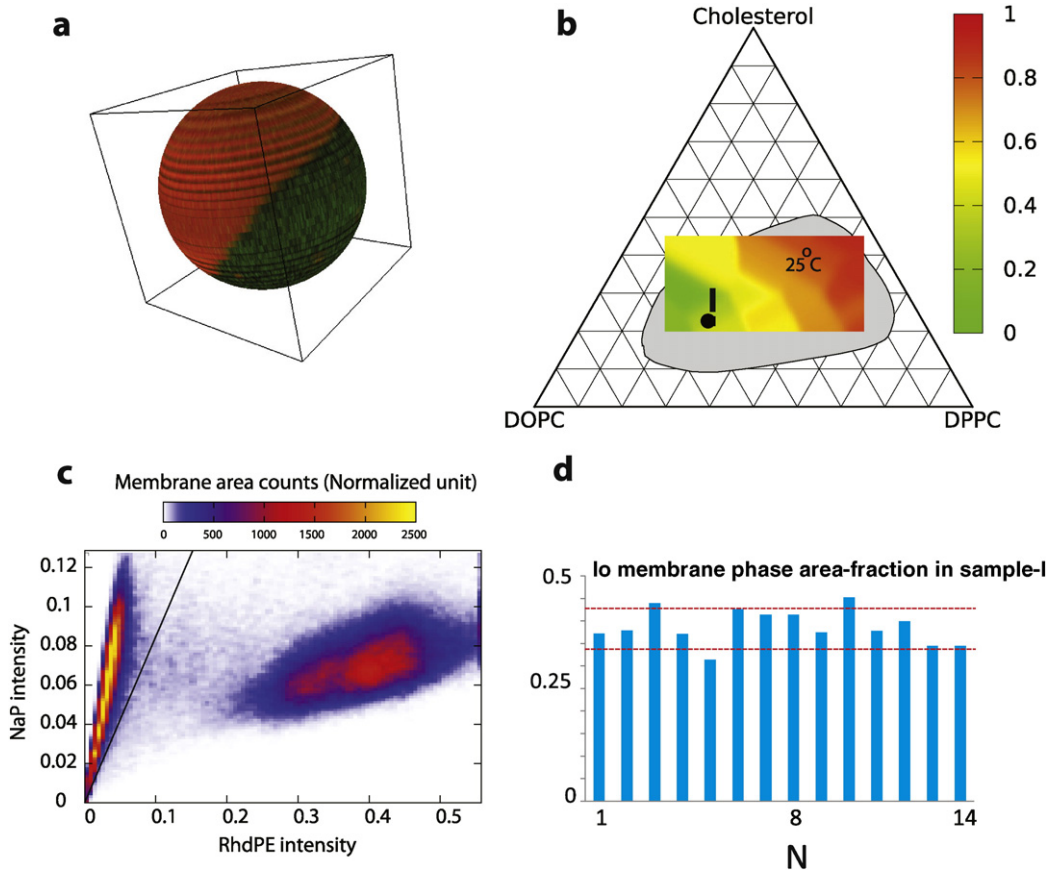

Fig. 2. (a) 3D-reconstructed image of a GUV of sample I using the confocal scans displaying co-existing l_o/l_d phases. Green color represents the l_o phase shows the lipid composition of the (NaP dye) and red color represents the l_d phase (RhPE dye). (b) The color map denotes the l_o phase area fraction as reported in [27] for the l_d/l_o coexistence region. The black dot represents sample I. (c) A 2D-histogram displaying the fluorescence intensities of NaP and RhPE on the vesicle surface (of sample I). (d) The area fraction of the l_o membrane phase is plotted for the 14 randomly chosen GUVs analyzed. The standard deviation ($\pm 3.9\%$) is indicated by the width between the two red-dashed lines.

SUVs do not mix properly (producing irreproducible results). If SUVs are kept at −20 °C for long-term storage then prior to mixing, the protocol requires sonication and extrusion methods to produce SUVs from large and multi lamellar vesicles (LUVs, MLVs) [40]. This result indicates that a necessary condition for vesicle mixing to be successful is that the vesicles are SUVs and the lipid components are miscible in a fluid state at the system conditions where electroformation takes place.

The protocol described here, may be of interest for studying membrane proteins in mixed membrane systems, where the proteins are provided in SUVs with a fixed lipid composition. Previously reported methods of protein reconstitution from a single population of proteoliposomes into large vesicles involve freeze–thaw [41], gentle hydration [24,25] and electroswelling [32,10,11,12,13,14]. It is clear from all these previous studies that the electroswelling produce high yield of giant unilamellar vesicles, as compared to other conventional methods. Using our method, membrane proteins reconstituted in proteoliposomes of one fixed lipid composition can be transferred into GUVs with a different lipid composition, including both low and high melting lipids simultaneously, at physiological relevant conditions. Hence it is not necessary to go through the elaborate work of producing new batches of SUVs with different lipid compositions in order to prepare a desired new three-component lipid composition. With our method, it is possible to access all the lipid compositions in the l_o/l_d coexistence region of the membrane lying along the dotted line A^1B^1 (Fig. 1a) by mixing a single SUV population of DPPC:chol (53.8%:46.2%) with DOPC SUVs of varying concentration and could be further generalized to work with three populations of SUVs to reach an overall composition (x_1,x_2,x_3) if the molar ratios of the populations are chosen appropriately (see Supplementary material). The vast literature on the phase behavior of binary lipid systems may here prove to be a valuable resource [37]. The protocol described in this paper is to our knowledge a novel and so far the only method that permits quantification of the compositional homogeneity in GUVs of complex lipid membranes.

We show that electroformation based on two populations of SUVs with the overall desired composition produce a system of GUVs with small variability in composition. SUVs of different populations with fixed lipid compositions can be mixed in the fluid-phase to reach an overall composition that can be tuned in a controlled manner for the l_o/l_d coexistence region, as requested. In this way, the complexity of the model membranes can be increased on demand.

Transparency document

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2015.09.020.

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