Seeing different lateral organization in membrane bilayer made of various ternary mixtures of phospholipids, sphingomyelin and cholesterol by fluorescent microscopy

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Odjel za fiziku

Gorana Glavurtić

Seeing different lateral organization in membrane bilayer made of various ternary mixture of phospholipids, sphingomyelin and cholesterol by fluorescent microscopy

Diplomski rad

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TEMELJNA DOKUMENTACIJSKA KARTICA

Sveučilište u Splitu
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Odjel za fiziku Diplomski rad

Proučavanje lateralne organizacije membranskog dvosloja načinjenih od različitih trokomponentnih smjesa fosfolipida, sfingomjelina i kolesterola uz primjenu fluorescentne mikroskopije

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Sažetak:
Vezikule predstavljaju lipidne membranske dvosloje koje svojim dimenzijama i biofizikalnom svojstvima modeliraju staničnu membranu. Osnovna gradivna jedinica vezikule je lipid, koji se sastoji od hidrofilne "glave" i hidrofobnog "repa" te omogućuje stvaranje lipidnog dvosloja u vodenoj otopini. Lipidni dvosloj je heterogen i sastoji se od različitih vrsta lipida. Najviše zastupljeni su fosfolipidi, sfingolipidi i steroli. Eksperiment je proveden za različite trokomponentne smjese fosfatidilkolina, sfingomijelina i kolesterola. Odabrali smo četiri različite vrste fosfatidilkolina (sa dvije stearinske, palmitinske i miristrinske masne kiseline kao i sa kombinacijom oleinske i palmitinske kiseline). Odabrani fosfatidilkolini su imali različitu duljinu i stupanj zasićenosti masnih kiselina. Različite lipidne domene se formiraju u vezikuli ovisno o njenom molekularnom sastavu. Molarni udjeli lipidnih komponenti u vezikuli su odabrani na osnovu prethodno objavljenih faznih dijagrama za trokomponentnu smjesu sfingomijelina, fosfatidilkolina sa kombinacijom oleinske i palmitinske masne kiseline i kolesterola. Promjenom lipidnog sastava (koncentracije kolesterola i ostalih lipida u vezikuli) formirat će se različite domene, uključujući uređene (L_o) i neuređene (L_d) tekuće domene. Dobiveni rezultati za vezikule načinjene od sfingomijelina, fosfatidilkolina sa kombinacijom oleinske i palmitinske masne kiseline i kolesterola su potvrdili rezultate objavljene u literaturi. Ovisno o dužini i stupnju zasićenosti masnih kiselina fosfatidilkolina eksperimentalno su detektirane različite lipidne domene za jednaki molarni udio kolesterola i sfingomijelina. Broj formiranih domena u vezikulama koje sadrže fosfatidilkoline sa zasićenim masnim kiselinama (sa dvije stearinske, palmitinske i miristrinske masne kiseline) ovisi o temperaturi faznog prijelaza, T_m.

(25 stranica, 11 slika, 35 literaturnih navoda, jezik izvornika: engleski)

Ključne riječi: membranski dvosloj, GUVs, POPC, DSPC, DPPC, DMPC, sfingomijelin, kolesterol, "splav" domene, metoda s dvije fluorescentne probe, Bdp-Chol, DiI-C_{18}(3)

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Master's thesis

Seeing different lateral organization in membrane bilayer of various ternary mixture of phospholipids, sphingomyelin and cholesterol by fluorescent microscope

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Summary:
Vesicle is lipid membrane bilayer which simulates the cell membrane in terms of its dimension and biophysical properties. The basic building blocks of vesicle is lipid, which consists of hydrophilic "head" and hydrophobic "tail" and forms lipid bilayer in aqueous environment. Lipid bilayer is heterogeneous and it consists of many different kinds of lipid. The most abundant are phospholipid, sphingolipids and sterols. Experiment was performed for different three-component mixture of phosphatidylcholine, sphingomyelin (SM) and cholesterol (Chol). Four different kinds of phosphatidylcholine were chosen (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2- dimyristoyl-*sn*-glycero-3-phophocholine (DMPC)). Chosen phosphatidylcholines have different length and degree of saturation of their acyl chains. Different membrane domains are formed depends on its lipid composition. All molar ratios of vesicle lipid components were chosen based on previously reported phase diagram for ternary mixture of SM, POPC and Chol. Different membrane domains, including liquid-ordered (L_o) and liquid-disordered (L_d), will be form depends on concentration of cholesterol and other lipids in vesicle. Results obtained for vesicles made of SM, POPC and Chol confirmed literature data. Depends on length and degree of saturation of phospholipid fatty acid different lipid domains were experimentally detected for the same cholesterol and SM molar content. The number of formed domains in vesicles that contain DSPC, DPPC and DMPC depends on phase transition temperature, T_m.

(25 pages, 11 figures, 35 references, original in: english)

Key words: membrane bilayer, GUVs, POPC, DSPC, DPPC, DMPC, sphingomyelin, cholesterol, "raft" domain, two-dye method, Bdp-Chol, DiI-C_{18}(3)

Supervisors: Doc. dr. sc. Marija Raguž
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1. Introduction

The basic structure of most cellular membrane is bilayer composed of amphiphilic components, called lipids. Lipids are molecules that have a hydrophobic “tail” and hydrophilic “head” based on their mentioned features they form a lipid bilayer in aqueous environment as the most favourable conformational structure. Hydrophobic “tails” are facing each other and hydrophilic “heads” are in contact with water. This structure of lipid bilayer will be formed spontaneously at normal physiological conditions. Cellular membrane, beside their basic building blocks (lipids) contains proteins. Proteins can be immersed into the membrane bilayer, in one or both layers, anchored into one layer or forming pores and channels in the membrane through which is performed transport of nutrients and small molecules (Fig. 1) [1], [2]. The cell membrane contains many different types of lipids but phospholipids are the major components. The most represented phospholipids in eukaryotic cell membrane are various phosphatidylcholines (PC) and phosphatidylethanolamines (PE) [3]. The cell membrane is largely composed of sphingolipids, in particular with a sphingomyelin as dominant sphingolipid. Another important component of membrane is cholesterol, which affects membrane fluidity by ordering lipid hydrocarbon chains and membrane packing, especially when sphingomyelin is present [4]. For decades lipids in eukaryotic cell membrane were considered as randomly organized building blocks, this model of biological membrane known as “fluid mosaic model” has been accepted from Singer and Nicholson [5]. Nowdays, the lipid bilayer is the basic building block of eukaryotic cell membranes, which serves as structure of living matter in the form of cells [6]. The complexity of cellular membranes in composition and dynamic organization has motivated the development of variety simpler model systems that serve as example for understanding more complex membranes. The most commonly used model systems for cellular membrane are vesicles. Vesicles (lat. *vesicula* = little bladder, blister) are freestanding bilayers models whose size, geometry and composition can be tailored with high precision. The vesicles membrane involves a self-assembly of lipid molecules in water, owing its stability to mainly weak physical forces of electrostatic and colloid nature [3], [7]–[10]. Vesicles are spherical bilayers of various sizes from small unilammellar vesicles (SUVs) 20 nm-100 nm through larger unilammellar vesicles (LUVs) 100 nm-400 nm to giant unilamellar vesicles (GUVs) 1µm-100µm [11].

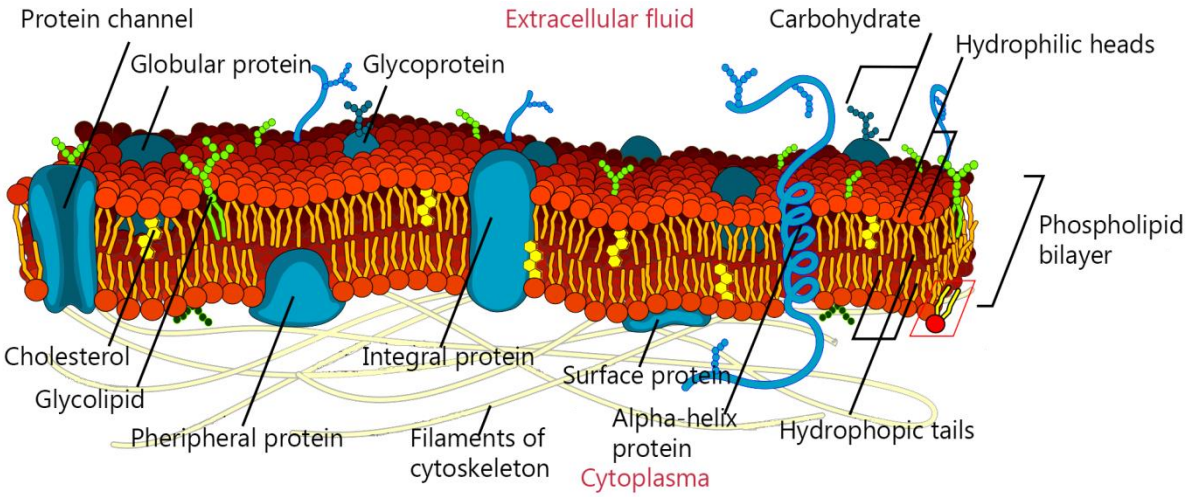


Figure 1. Illustration of the cell membrane [12].

1.1. GUVs, 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. Using microscopy related techniques such as phase contrast and fluorescence microscope, we are able to visualise giant unilamellar vesicles (GUVs) individually because of their suitable size (5-100 μm) and their curvature [3], [8]. GUVs have found application in many biophysical aspects in which membrane composition, tension and geometry is controlled and manipulated using microscopy techniques, such as micropipette aspiration, fluorescence imaging of lateral membrane structures, mechanical and biophysical properties of lipid-lipid interaction, also lipid-DNA, lipid-peptide and lipid-protein interaction [7], [9]. Experiments using GUVs as model membrane system are performed at level of single vesicles so heterogeneity in shape and size and the presence of multilamellar vesicles are excluded. GUVs can be form of single lipid species or mixture with no more than three to four component [7]. In recently reported studies, it is also possible to form GUVs from natural lipid extracts and native membranes [10], [13].

1.2. Lipids, the basic components of GUVs

Lipids are amphiphilic molecules, they have one polar end (“head”), soluble in water and the other that is nonpolar or soluble in fat (“tail”), so they form double layer with polar ends pointing outwards and the nonpolar ends pointing inwards in aqueous solution. Formed lipid bilayer keeps the watery interior of the cell separate from the watery exterior. The four major classes of membrane lipids, based on the properties of “head” group, are phospholipids, glycolipids, sterols and archaebacterial ether lipids, the first three are membrane lipids of eukaryotic cell (Fig. 2).

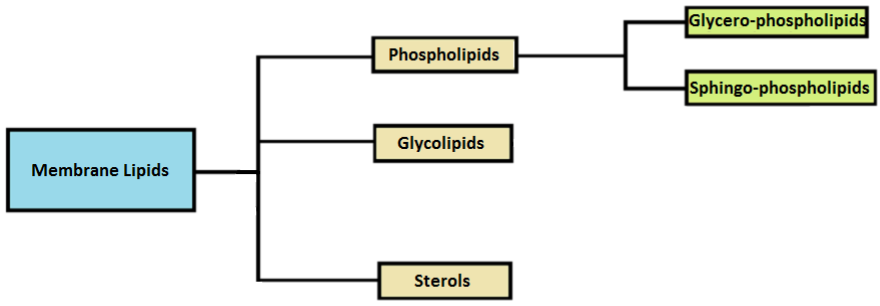


Figure 2. Classification of membrane lipids.

A phospholipid molecule is constructed from four components: fatty acid, a platform to which the fatty acids are attached, a phosphate and an alcohol attached to the phosphate. The two hydrophobic acyl chains are representing the “tails” and the main hydrophilic part consisted of glycerol or sphingosine molecule and phosphate group, that can be modified, represent a “head” of the phospholipid molecule.

Phospholipid platform can be built of glycerol, a small 3-carbon alcohol, or sphingosine, a more complex alcohol. If the backbone (or platform) of phospholipid is built from glycerol then they make a group of glycerol-phospholipids and if the backbone is built from sphingosine than they make a group of sphingo-phospholipids. The glycerol-3-phosphate is backbone of all glycerol-phospholipids to which two fatty acid chains and alcohol are attached. The glycerol-phospholipids are distinguished from various attached alcohol moieties and the common alcohol moieties are amino acid serine, ethanolamine, choline, glycerol and the inositol. The most commonly used phospholipids in forming GUVs are

phosphatidylcholines (POPC, DPPC, DSPC, DMPC, DOPC), phosphatidylethanolamines (DMPE, DPPE, DOPE), phosphatidylserines (DOPS, DMPS) and phosphatidylglycerols (DMPG, DPPG) [14]. Structure of POPC, DSPC, DPPC and DMPC molecules are shown in Fig. 3.

The second group of phospholipids found in membrane are sphingo-phospholipids; they have a sphingosine for backbone, an amino alcohol that contains a long unsaturated hydrocarbon chain. In the sphingomyelin, the most common sphingolipid who make up to 10-20% of plasma membrane lipids, the amino group of sphingosine backbone is linked to a fatty acid by an amide bond and the primary hydroxyl group of sphingosine is esterified to phosphoryl choline [14]. Sphingomyelin (SM) plays several important features for structural role in biological membrane, such as low degree of unsaturation, an asymmetric molecular structure and their extensive hydrogen-bonding properties (Fig. 3) [15]. Sphingomyelin likes to interact with cholesterol and other sterols and they established a co-localization or “raft” domains, the SM/sterol-rich domains, in cell plasma membrane. Large amount of evidence regarding SM favourably interaction with cholesterol indicates that the functional role of SM is be a regulator of cholesterol distribution within cellular membrane and cholesterol homeostasis in cell [15].

Sterols are unique class of membrane lipids with specific structure compared to other lipids. Their structure is composed of carbon rings and the common feature of sterols is hydrophobicity. Sterols have a regulatory role in the cell; one of many examples is that the many human sex hormones are synthesized with cholesterol as the precursor beside their construction roles in membrane [16]. Cholesterol (Chol) is composed of four linked hydrocarbon rings (a sterol part) and at one end of sterol it is linked a hydrocarbon tail that has a two double bond between C5 and C6, two methyl groups at C10 and C13 and a polar hydroxyl group at C3 (Fig. 3). The effective length of a cholesterol molecule in the membrane has been estimated to correspond to a 17-carbon long, all-trans acyl chain [14], [16]. The cholesterol is an important structural component of the cellular membrane where it regulates lipid bilayer dynamic and structure by modulating the packing of phospholipids molecules [17]. The cholesterol molecule is oriented in the membrane in a way that the long axis lies parallel to the phospholipid acyl chain, increasing the order in the upper acyl chain region of the membrane while decreasing packing constraints at the terminal methyl groups

[17]. The components of membrane such as diverse phospholipids and cholesterols are affecting lateral structures of membrane and their heterogeneity.

In aspect of lipid-raft hypothesis, cholesterol and sphingomyelin have a main role because of their tendencies of mutual packing. Cholesterol prefers SM over any other PC with equal acyl chain and one of the main reasons is hydrogen bound generated by the amide group of SM molecule and the 3-hydroxyl group of cholesterol. This Chol-SM preference manifests the liquid-ordered phase, so called “raft” domains, enriched in cholesterol and sphingomyelin [16], [18], [19].

1.3. Fluorescent probes

Fluorescent probes are used in the experiment to provide a visualization of vesicles. The application of probes allows us direct visualization of different lipid domains in vesicles. It is possible to distinguish liquid-ordered (L_o) over liquid-disordered phase (L_d phase) considering preferential incorporation properties of selected probes. Fluorescent probes that we used in our experiment are 23-(dipyrrometheneboron difluoride)-24-norcholesterol (Bdp-Chol) and 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate (DiI-C_{18}(3)) and they have a selective participation for certain domains [20]. Bdp-Chol is green fluorescent dye, it is analog of cholesterol which have a fluorescent fragment attached to the hydrocarbon tail (Fig. 3). Based on previous studies it is known that Bdp-Chol prefers a liquid-order phase (L_o phase) in lipid mixture DOPC:BSM:Chol = 40:40:20 [mol %] [20], [21]. Therefore Bdp-Chol is a great candidate for our experiment where we want to distinguish two different domains from each other, L_o and L_d phases. DiI-C_n are a class of cationic lipophilic fluorescent probes that have been used extensively in the study of membrane biophysical properties because of their large molar absorptivity and reasonable photostability [22]. It is orange-red fluorescent dye, a lipid analog with 18-carbons long saturated acyl chains and has a fluorescent fragment attached to the “head” (Fig. 3). It is also known that this probe prefers L_d over L_o phase in lipid mixture SM:DOPC:Chol = 27:50:23 [mol %] [20], [23]. Both used probes are characterized by exceptional photostability [20]–[23].

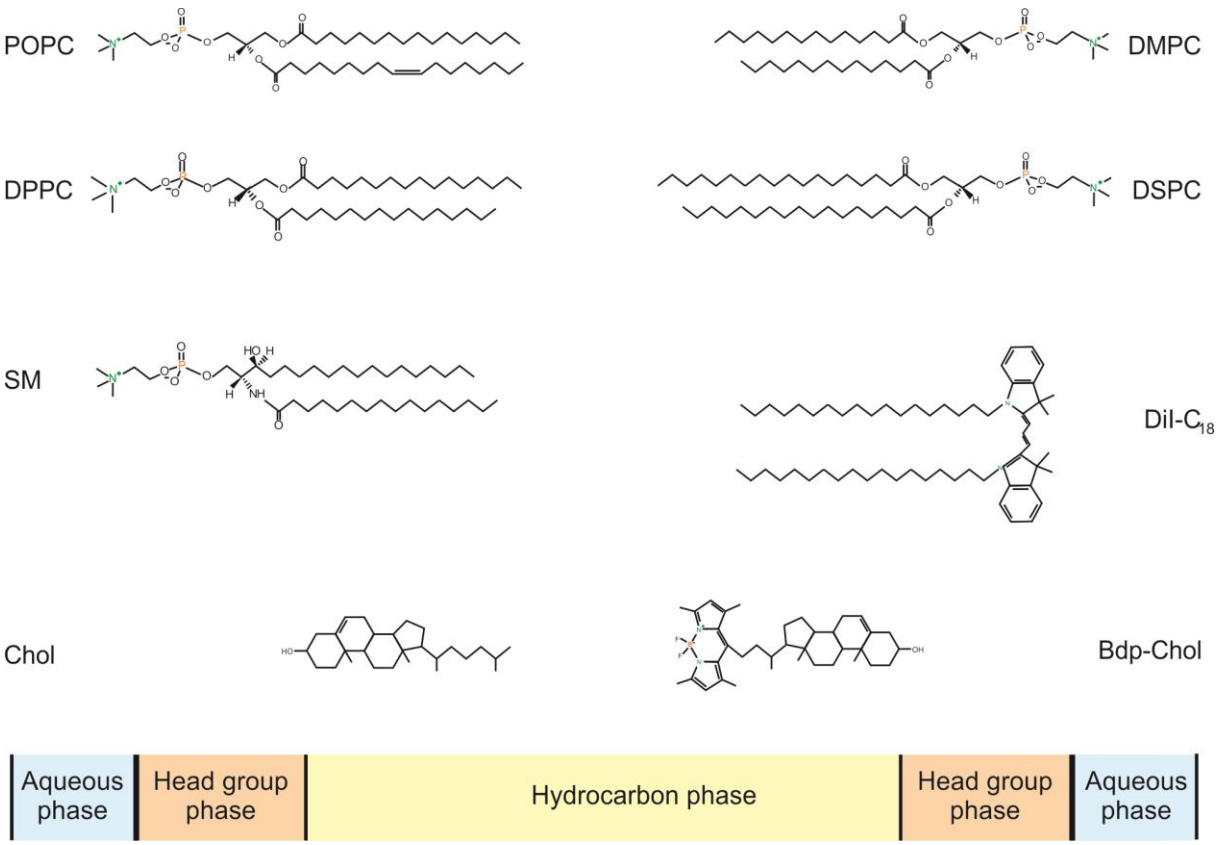


Figure 3. Chemical structure of fluorescent probes Bdp-Chol and DiI-C_{18}(3) together with the structure of phospholipids (POPC, DPPC, DMPC, DSPC), cholesterol (Chol) and sphingomyelin (SM). Approximate locations of these molecules across the lipid bilayer membrane are illustrated.

2. Aim of thesis

We made four different ternary mixtures of lipids for vesicles that contain SM: X: Chol; X stand for one of PC lipids (POPC, DSPC, DPPC and DMPC). Each preparation was repeated at least a couple times to make results more credible. The main aim of this experiment is to visually distinguish different membrane phases using fluorescent microscope. Appropriate fluorescent probes were chosen for that cause, (Bdp-Chol and DiI-C_{18}(3)). Changing the PC lipids, which are different in length and degree of saturation of their acyl chains, we wanted to found out if that affects incorporation of fluorescent probes in a specific domain. The main goal was to detect “raft” domains (L_o phases), which are formed of tightly packed sphingomyelins and cholesterols.

3. Material and Methods

3.1. Material

Chemicals and materials that we used in the experiments:

- Phospholipids: POPC (16:0, 18:1) -1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
  DSPC (18:0) - 1,2-distearoyl-*sn*-glycero-3-phosphocholine
  DPPC (16:0) - 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine
  DMPC (14:0) - 1,2- dimyristoyl-*sn*-glycero-3-phophocholine
- Cholesterol (ovine wool, > 98%), (cholest-5-en-3β-ol)
- Sphingomyelin (chicken egg, > 99%), (16:0) SM, Hexadecanoyl Sphingomyelin
- Fluorescent probes:
  1. DiI-C_{18}(3) - 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate
  2. Bdp-Chol (Bodipy Cholesterol) - 23-(dipyrromethene boron difluoride)-24-norcholesterol
- Chloroform
- Purified water or MilliQ-water
- Indium tin oxide (ITO)-coated glass microscope slides
- Vacuum grease
- Teflon spacers (dim. ~5x25x0,39 mm^3)
- Parafilm
- Binder clips
- Copper tape or any other conduction type
- 1 mL syringe with 22+gauge needle (Pasteur pipettes) for extracting vesicles from growth chamber
- Multimeter for measuring resistance and AC voltage
- Single generator with sign wave output (minimal power)
- Incubator capable of maintaining 60-65°C
- Very thin plastic needle for smearing stock solution on the ITO-coated glass microscope slides
- Plastic molds for making growth chamber (or capacitor) for vesicles
- Fluorescence microscope

3.2. Methods

3.2.1. Preparation of giant unilamellar vesicles (GUVs)

The method that was used to prepare the giant unilamellar vesicles (GUVs) is electroformation modified by S. L. Veatch [24].
Electroformation is procedure that implies preparation vesicles from stock solution of phospholipids, cholesterol and sphingomyelin, labelled with fluorescent probes, under influence of AC electric field. The AC electric field create a gentle mechanical agitation what causes the lifting of lipid film from the support, layer by layer and helps vesicles to form and fuse [25]. The formation of vesicles depends on the strength of the AC field and also the thickness of the dried lipid film, the thinner the lipid film the better preparation of unillamelar vesicles [25], [26]. The direct evidence of the interaction between the AC field and vesicles is that the vesicles vibrate at the same frequency as the applied field. A second effect of AC field is that the obtained vesicles do not exhibit thermal fluctuations of their contour [25]. Labelling with fluorescent probes enable us to visualize different phase behaviour of lipids and also different domain by their composition. This method of labelling provides us to determine “raft” domains, sphingomyelin/cholesterol rich domains.

Preparation of GUVs:

1. Stock solution of ternary mixture of SM:X:Chol (X = POPC, DSPC, DPPC, DMPC) and both probes DiI-C_{18}(3) and Bdp-Chol were made in chloroform. All phospholipids (POPC, DSPC, DPPC, DMPC, SM) and cholesterol were dissolved in chloroform at concentration of 25 mg/ml. Fluorescent probes were dissolved in chloroform at concentration of 0,1 mg/ml. We prepared GUVs for three different cholesterol molar ratios (20, 35 and 50%). For vesicles containing 20 mol % of Chol we made ternary lipid mixtures of SM:X:Chol = 35:45:20 [mol %]. For vesicle containing 35 mol % of Chol the following lipid compositions for vesicles preparation were SM:X:Chol = 30:35:35 [mol %] where X stands for POPC, DSPC, DPPC or DMPC. The special preparation was made for vesicles containing POPC with 50 mol % of Chol. Lipid mixture was SM:POPC:Chol = 22:28:50 [mol %]. Molar ratio of total lipids and either fluorescent probe, DiI-C_{18}(3) and Bdp-Chol, was 0,2 [mol %] in each sample.

2. One ITO-coated microscope slide (dim. 75x25 mm^2) is used for making one growth chamber for vesicle preparation. Microscope slide was scored with a diamond-tipped scribe in order to make two equal pieces for the growth chamber. Cutted slides should be wiped out of dust and also can be cleaned gently with ethanol, but more aggressive cleaners could damage the conducting layer. To determine conducting side of the slides we measured the resistance across the surface by multimeter, the conducting side of the slides will register a small resistance (< 1 kΩ).

3. Put an ITO-coated microscope slide in a plastic mold, which has a groove for slide, with conducting side faced up. Deposit the stock solution (lipid/chloroform solution) on the conducting face and spread the lipids into thin and uniform film, this can been achieved using a very thin plastic needle. The deposit was spread to the half of microscope slides and also the edges of slide (~5 mm) are clean. Lipids were placed under partial vacuum for at least 30 min to remove any excess solvent (chloroform).

4. The edges of slides on the conducting face coat with thin layers of vacuum grease the width of the Teflon spacer (~5 mm). Two Teflon spacers were put on layers of vacuum grease. On the edges of another slide also put a thin layer of vacuum grease and then assemble the growth chamber that two conducting faces are facing inward and are separated by the two Teflon spacers (Fig. 4.A). Vacuum grease and Teflon spacers were used to reduce the evaporation of stock solution. Maintain an overhang of more than 5 mm at the short edge so that slides can be easily attached to current. Use the binder clips to clamp the sandwich together.

5. Fill growth chamber with purified water using pipette from one side, make sure to avoid air bubbles trapped in chamber (Fig. 4.B). Growth chamber will be filled using capillary action and gravity. Also do not over fill with water this will wash lipids from the chamber. When the growth chamber is fully filled with purified water seal off both open sides with vacuum grease and wrap with a thin piece of parafilm to give a stable structural support and to prevent evaporation. Clean excess vacuum grease from chamber ends with ethanol to provide good electrical connections. On both ends of chamber fix the copper tape that is long 2,5 cm, this provides electrical connection.

6. Attach alligator-clip, from the signal generator, directly to both ends of growth chamber to make an electrical connection. The whole growth chamber with cable and alligator-clip put into shallow plastic bowl which has slots on both side for cable, this bowl provides better electrical isolation for chamber in incubator (Fig. 4.C). Place growth chamber in incubator on temp. 60-65°C and make final electrical connections to the signal generator. Set the signal generator to output a sinusoidal wave of 1 V and 10 Hz and verify at the capacitor leads with the multimeter. Grow vesicles in incubator under se AC electric field for ~2 h.

   6. a. After AC electric field vesicles stay in incubator for ~1 h without current and frequency.

   6. b. Put the growth chamber in dark to cool down for at least 20 min.

7. When vesicles growth is complete, detach growth chamber from voltage and remove binder clips. To open growth chamber put away parafilm from one side and then gently split one microscope slide from other slide so the Pasteur pipettes can be put into chamber to draw the vesicle mixture. Put the content of the Pasteur pipettes into a secondary storage container. Vesicles are diluted in warm growth solution heated to the growth temperature (sudden change in temperature may break delicate vesicles). Better results were obtained after at least 12h of vesicles rest in secondary storage container, after that we looked vesicles under fluorescent microscope.

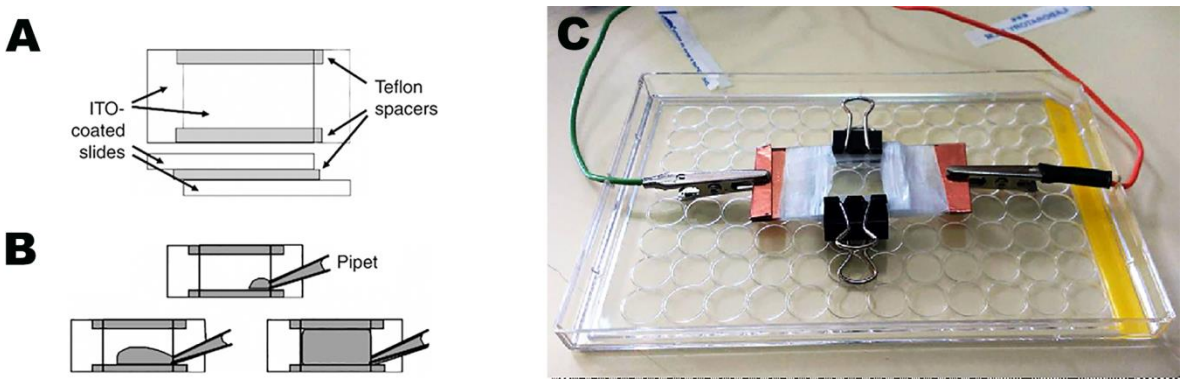


Figure 4. (A) Schematic of growth camber made of two ITO-coated glass slides and two Teflon spacers [24]. (B) Growth camber must be filled with water from one open end using capillary action [24]. (C) Image of composed growth camber with cable and alligator-clip ready to put into incubator.

3.2.2. Fluorescence microscope

A fluorescence microscope is an optical instrument that uses process of fluorescence to generate an image of specimen. Completely or partially fluorescent dye samples are used in fluorescent microscopy. The basic operating principle is that the sample is illuminated with light of a specific wavelength which is absorbed by the fluorophore, causing them to emit light of longer wavelengths (lower energy levels). Typical components of fluorescent microscope are a light source (xenon arc lamp or mercury-vapour lamp, LEDs or laser), the excitation filters, the dichroic mirror, the emission filter and detector (CCD camera or ocular). The filters and dichroic mirror are chosen to match the spectral excitation and emission characteristics of the fluorophore used to label the specimen [27]. We used an epifluorescent microscope that differs from fluorescent microscope in light path, in epifluorescent microscope the excitation of the fluorophore and detection of the fluorescence are done through the same light path (through the same objective). The basic principle of light path in epifluorescent microscope is shown in Fig 5. The light source passes through the excitation filters, that narrows the wavelength of incoming light to only those used to excite the sample, then the light comes to the dichroic mirror, which reflects the excitation light to the sample and simultaneously transmits only the emitted light from the sample to the detector (CCD camera or ocular). The excitation light passes through objective lens to the sample. The emission light (fluorescence of sample) is emitted from sample through the same objective that is used for the excitation. An emission filter between the objective and the detector can filter out the remaining excitation light from fluorescent light because only reflected excitatory light reaches the objective and the most of the excitation light is transmitted through the sample [27], [28].

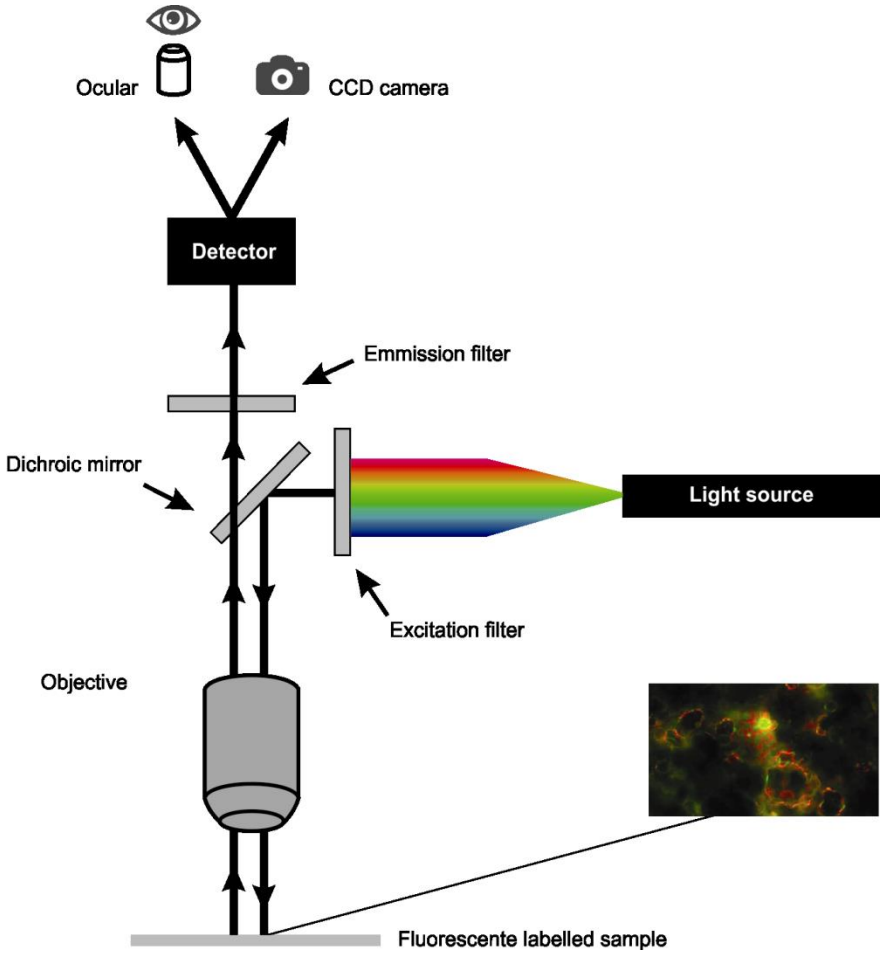


Figure 5. Schematic diagram of light path in an epifluorescent microscope.

The physical background of fluorescent microscopy is fluorescence activity of susceptible molecules (fluorescent probe), which emit light from electronically excited states which are created by physical, mechanical or chemical mechanism. Fluorescent probe absorbs electromagnetic radiation of one wavelength and then emits a wave of longer wavelength (lower energy). Basic processes of fluorescence are classically presented by a Jablonski energy diagram [29], (Fig. 6). The fluorescence process is governed by three important events: 1) absorption of photon by electron-excitation of fluorescent probe, 2) internal conversion-excited electron loses its energy and falls to the lower energy level, 3) fluorescence-return of the electron to the ground state and emits a photon of lower energy then absorbs one. All above mentioned processes differ in timescale. The electronical state of any molecule is determinate by distribution of negative charge and the overall molecular geometry. As most molecules, the electron of probe are normally at the lowest

energy state or ground state (S_0 in diagram). When a photon with appropriate energy interacts with a molecule (probe) the photon may be absorbed, causing an electron to jump to one of the levels of an excited state (S_1 or S_2 in diagram). By 'appropriate energy' we mean an amount corresponding to the energy difference between the ground and excited states. This transition process is very fast, on the order of 10^{-15} seconds. An excited-state electron rapidly loses its energy to vibration (heat) and then falls to the lowest level of the first (S_1) excited state. This process is called internal conversion and it happens on the order of 10^{-12} seconds. From there the electron may fall to one of the sub-levels of the ground (S_0) state, emitting a photon with energy equivalent to the energy difference of the transition. This happens on a time scale of nanoseconds (10^{-9} – 10^{-8} seconds) after the initial photon was absorbed. Since the emitted photon has less energy than the absorbed photon it is at a longer wavelength. This explains the process of fluorescence that converts light of one wavelength (colour) to another and leads to the display of highly saturated colours in fluorescent labelled samples.

Likewise, emission of a photon through fluorescence or phosphorescence is also measured in terms of quanta. The energy in a quantum (Planck's Law) is expressed by the equation:

$$E = h\nu = hc/\lambda$$

where E is the energy, h is Planck's constant, ν and λ are the frequency and wavelength of the incoming photon, and c is the speed of light. Planck's Law dictates that the radiation energy of an absorbed photon is directly proportional to the frequency and inversely proportional to the wavelength, meaning that shorter incident wavelengths possess a greater quantum of energy. The absorption of a photon of energy by a fluorophore, which occurs due to an interaction of the oscillating electric field vector of the light wave with electrons in the molecule, is an all or none phenomenon and can only occur with incident light of specific wavelengths known as absorption bands. In general, fluorescence investigations are conducted with radiation having wavelengths ranging from the ultraviolet to the visible regions of the electromagnetic spectrum (250 to 700 nanometers) [27]–[29].

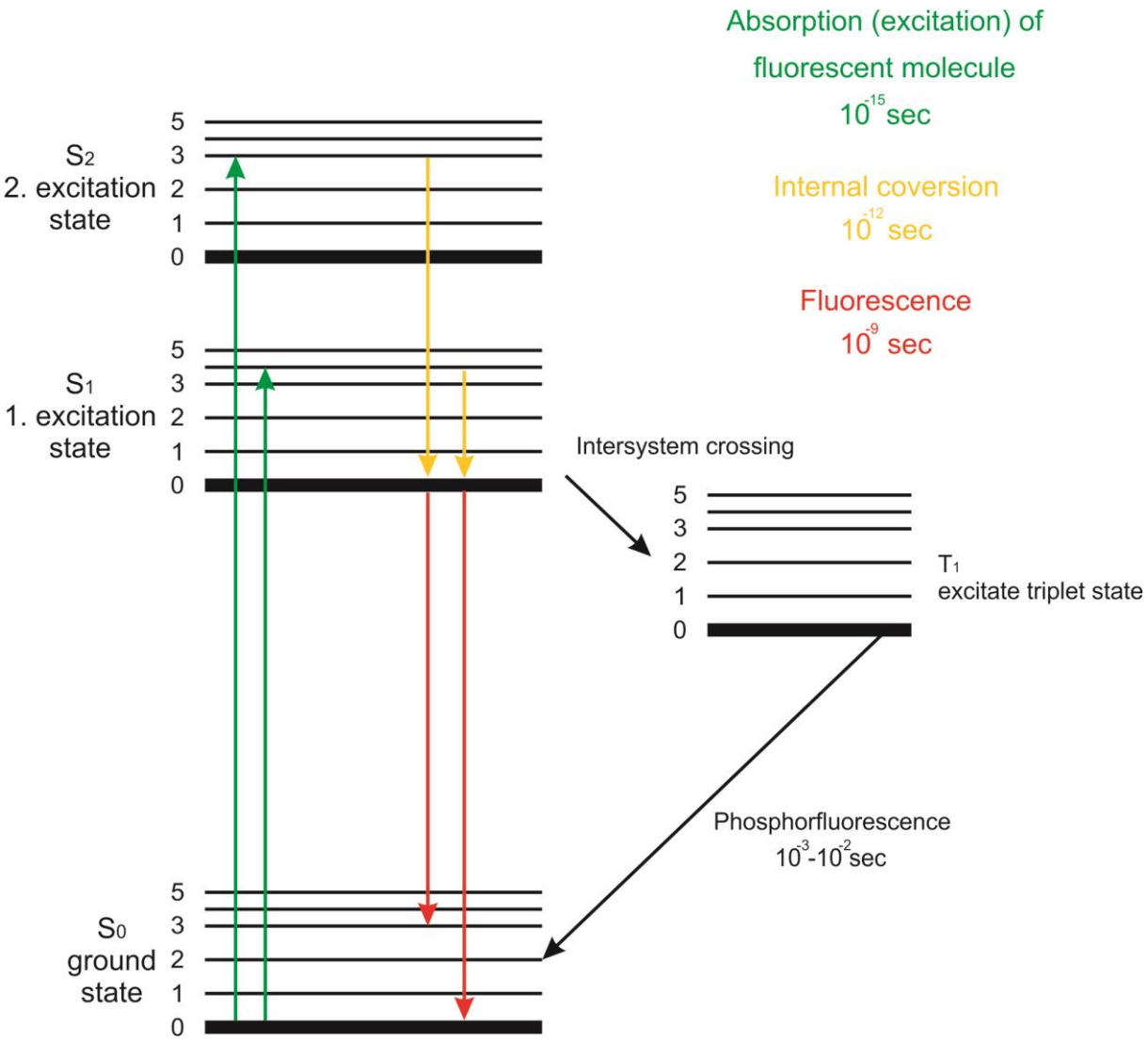


Figure 6. Jablonski energy diagram illustrates the ground state (S_0), the first (S_1) and second (S_2) excited states as a stack of horizontal lines. The bold horizontal lines represent electronic energy states, while thinner lines denote vibrational/rotational sublevels of states. The three main processes are represented: green arrows- absorption (excitation) of fluorescent molecule, yellow arrows- internal conversion and red arrows- fluorescence. They all differ in timescale.

All images were obtained using the upright epifluorescent microscope Zeiss Axio-Imager M1 and processed with Axio Vision Rel 4.7. software. The fluorescence spectra for used probes are: E_x/E_m = 495nm/507nm for Bdp-Chol and E_x/E_m = 550nm/565nm for DiI-C_{18}(3). Used filters were Rhodamine-HE and FITC (Alexa 568). We used Plan-Apochromat x63/1.4 oil objective (Zeiss).

4. Results and Discussion

All molar ratios of vesicles components were chosen based on previously reported phase diagram for ternary mixture of SM, POPC and Chol [30]–[32] (Fig. 7). We selected three specific points in phase diagram. For chosen molar ratio different domains (L_o and L_d) coexist inside of the membrane bilayer. The same molar ratios were retained for other three preparations with DSPC, DPPC and DMPC. We want to emphasize at this point that there is no phase diagrams for lipid mixtures of SM, X and Chol (X = DSPC, DPPC, DMPC) in literature. It is known that cholesterol can promote separation between a low T_m lipid (T_m- phase transition temperature) and a high T_m lipid at an intermediate temperature when the fraction of one lipid is too low to phase separate in the absence of Chol, this result were obtained with detergent independent method [30], [33]. According to phase diagram of three component bilayer mixture that contains cholesterol together with low-melting lipid and high-melting lipid there are areas of coexisting liquid-ordered and liquid-disordered phase [32], [33], shown on Fig. 7. In our experiment POPC is low-melting lipid while others are high-melting lipids (DSPC, DPPC, DMPC and SM). As has been previously reported, Bdp-Chol incorporates in L_o phase in GUVs made of BSM:DOPC:Chol = 40:40:20 [mol %] (BSM- brain sphingomyelin), the L_o partitioning is over 80%. It was determined from intensity line profiles of scanning confocal fluorescence images obtained by scanning confocal microscope [21]. Bdp-Chol has a fluorescent motif attached to hydrocarbon tail, which does not disturb interaction of cholesterol with sphingomyelin and forming L_o phase [21]. DiI-C_{18}(3) prefers L_d over L_o phase in lipid mixture when lipids have the same length of unsaturated acyl chain as probe [20], [23]. In case of DSPC (PC lipids with 18 carbons long saturated chains) is not observed the same result, DiI-C_{18}(3) is incorporated into L_o phase due its approximate hydrophobic matching of dye and lipid chain length [23], [34]. DiI-C_{18}(3) fits better in environment made of chemical structure analogues, such as POPC and DSPC.

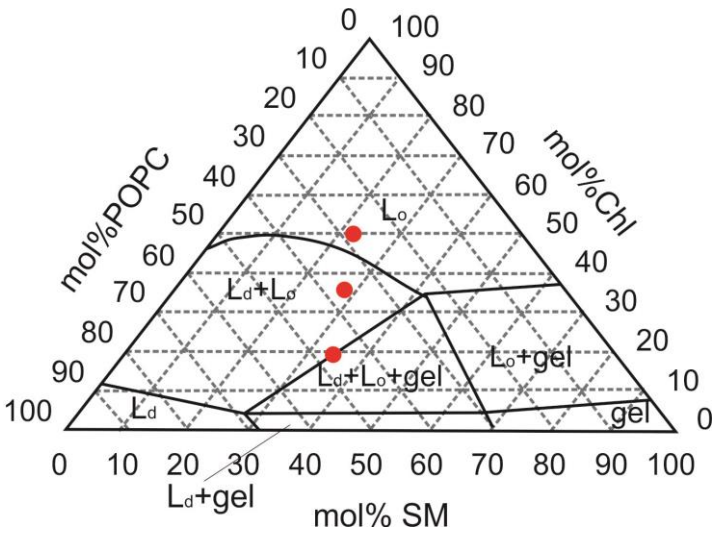


Figure 7. Phase diagram for ternary mixture of SM, POPC and Chol [30]–[32].

4.1. GUVs formed of SM:POPC:Chol mixture

SM:POPC:Chol = 35:45:20 [mol %]

Results presented in Fig. 8.a, b, c show coexistence of two phases, L_o and L_d. We distinguish two coexisting liquid phases based on incorporation of fluorescent probes, Bdp-Chol and DiI-C_{18}(3). Dominant phase for this ratio is L_d. DiI-C_{18}(3) incorporates into L_d phase (Fig. 8.a) and Bdp-Chol probe incorporates into L_o phase (Fig. 8.b) as was previously reported in [20], [21]. These visible small domains of L_o phase, so called "raft" domain, are enriched with tightly packed SM and Chol.

SM:POPC:Chol = 30:35:35 [mol %]

Results presented in Fig. 8.d, e, f show two coexisting liquid phases, L_o and L_d, at this molar ratio. Using the same fluorescent probes allow us to distinguish L_d (Fig. 8.d) from L_o phase (Fig. 8.e). Small domains of L_d phase are visible on entire surface of the vesicle (Fig. 8.d). Bdp-Chol is more homogeneously distributed on the membrane surface what indicates size increase of L_o phase (Fig. 8.e). Based on Fig. 8.d and e, we can conclude that used fluorescent probes incorporate into opposite vesicle domains (Fig. 8.f).

SM:POPC:Chol = 22:28:50 [mol %]

The distribution was homogeneous for both probes, Bdp-Chol and DiI-C_{18}(3) (Fig. 8.g, h, i). Based on the obtained results, we can conclude that only L_o domain forms what is expected based on phase diagram presented in Fig. 7.

In membranes made of lipid mixtures, SM/POPC/Chol, the L_d phase is enriched with low T_m lipids (T_m(POPC)=-2 °C) in contrast of L_o phase, which is enriched with high T_m lipids (T_m(SM)≈37°C) and Chol, as was previously reported in [30]–[32]. POPC (16:0, 18:1) has one saturated and one unsaturated acyl chain, therefore it prefers partitioning in L_d phase due its molecular structure. In experiment with POPC, Bdp-Chol probe incorporates into L_o phase; therefore it can be considered as so-called indicator of "raft" domains.

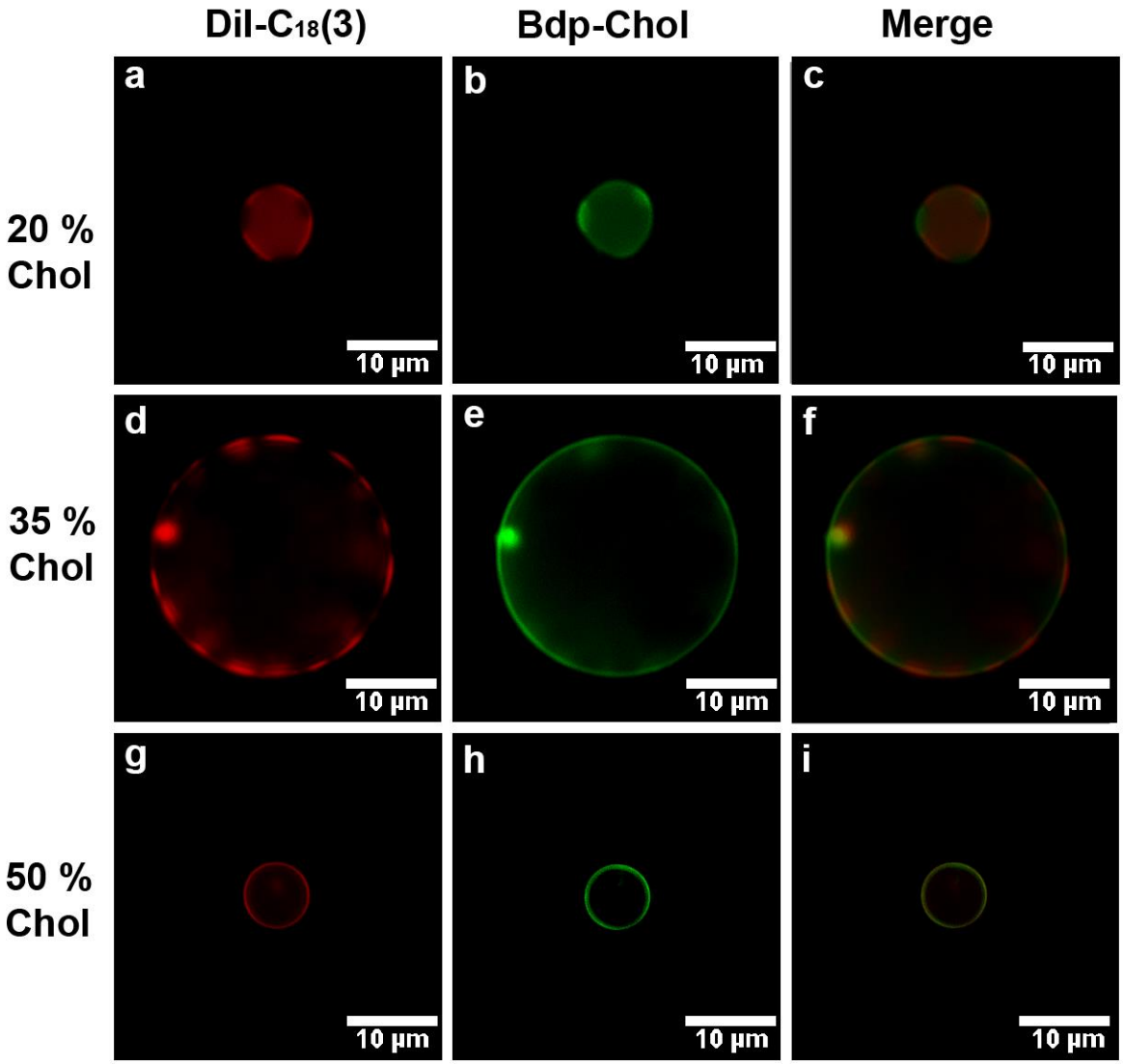


Figure 8. Fluorescent images of GUVs composed of SM/POPC/Chol lipid mixtures labelled with DiI-C_{18}(3) (red) and Bdp-Chol (green). Displaying L_o/L_d phase coexistence, the fluorescent probes show different preferential partitioning, DiI-C_{18}(3) prefers L_d and Bdp-Chol prefers L_o phase. Results for SM:POPC:Chol = 35:45:20[mol %] are presented in (a, b, c), for SM:POPC:Chol = 30:35:35[mol %] in (d, e, f) and for SM:POPC:Chol = 22:28:50 [mol%] in (g, h, i).

4.2. GUVs formed of SM:DSPC:Chol mixture

SM:DSPC:Chol = 35:45:20 [mol %]

Results show the coexistence of two separate phases, both probes are incorporated into the same phase (Fig. 9.a, b). We assume that the labelled area represents L_o phase and the dark area, without probe, probably represents L_d or even gel phase. There is no recorded coexistence of two phases for lipid mixture containing two saturated, high-melting T_m, lipids (DSPC and SM) and cholesterol so far. The assumption of existing L_o phase ("raft" domain) has been made based on previous studies [33], [35], [36], which indicate that Chol prefers packing with saturated PC lipids and high T_m. DSPC fulfils both properties (T_m(DSPC)=55°C) [33]. The red dye is present only in L_o phase while the green one is present in both phases. According to the literature cholesterol is excluded from gel phase [33]. Based on this we can conclude that other domain is L_d. The approximate hydrophobic matching of dye and lipid chain of DSPC (both have two 18 carbons long saturated chains) is obvious. Remarkable difference between T_m temperature of SM and DSPC (T_m(SM)≈37°C, T_m(DSPC)= 55°C) could be the reason for coexistence of two phases in this lipid mixture. More extensive experiment should be performed in order to confirm this conclusion.

SM:DSPC:Chol = 30:35:35 [mol %]

Uniform distribution of DiI-C_{18}(3) and Bdp-Chol is visible in Fig. 9.d and e. We can conclude that only one phase (L_o) was formed and that increase in cholesterol content contributes to formation of one uniform phase.

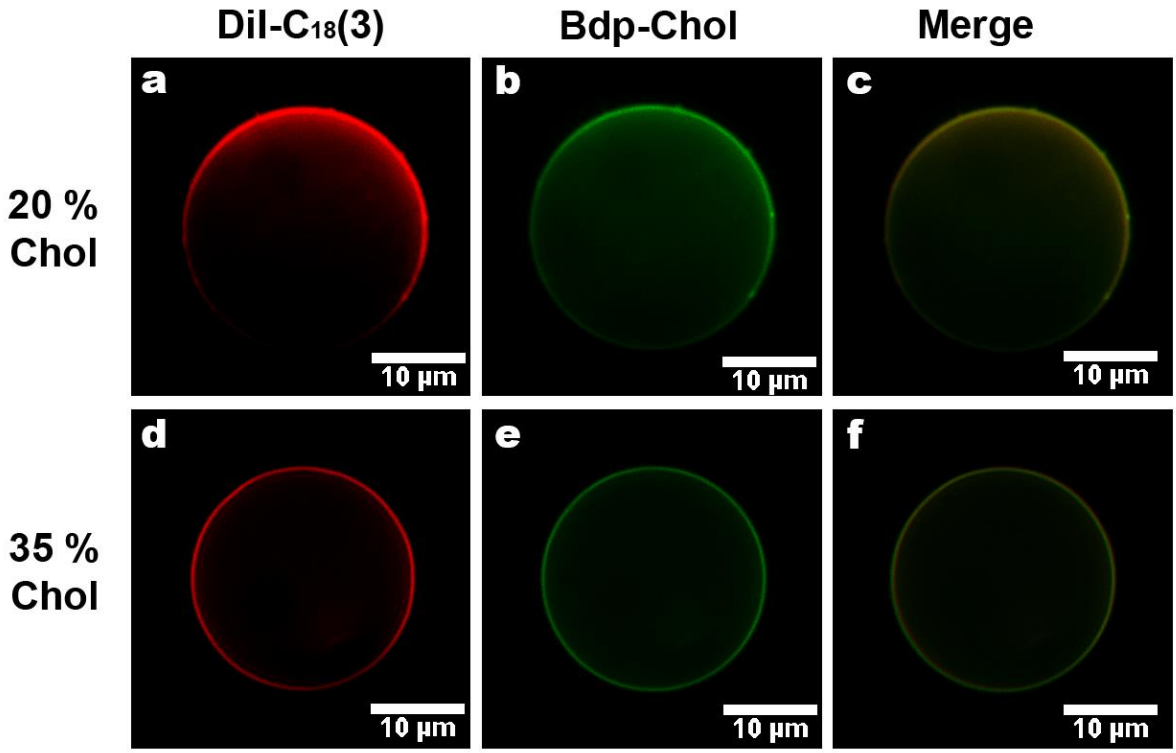


Figure 9. Fluorescent images of GUVs composed of SM/DSPC/Chol lipid mixtures labelled with DiI-C_{18}(3) (red) and Bdp-Chol (green). Displaying L_o/L_d phase coexistence, the fluorescent probes show the same preferential partitioning, DiI-C_{18}(3) and Bdp-Chol both prefer L_o phase. Results for SM:DSPC:Chol = 35:45:20[mol %] are presented in (a, b, c) and for SM:DSPC:Chol = 30:35:35[mol %] in (d, e, f).

4.3. GUVs formed of SM:DPPC:Chol mixture

SM:DPPC:Chol = 35:45:20 [mol %] and SM:DPPC:Chol = 30:35:35 [mol %]

The uniform distribution of DiI-C_{18}(3) and Bdp-Chol was observed for both Chol concentrations what is visible on Fig. 10. Based on this we assume that one uniform L_o phase forms in membrane with this lipid composition.

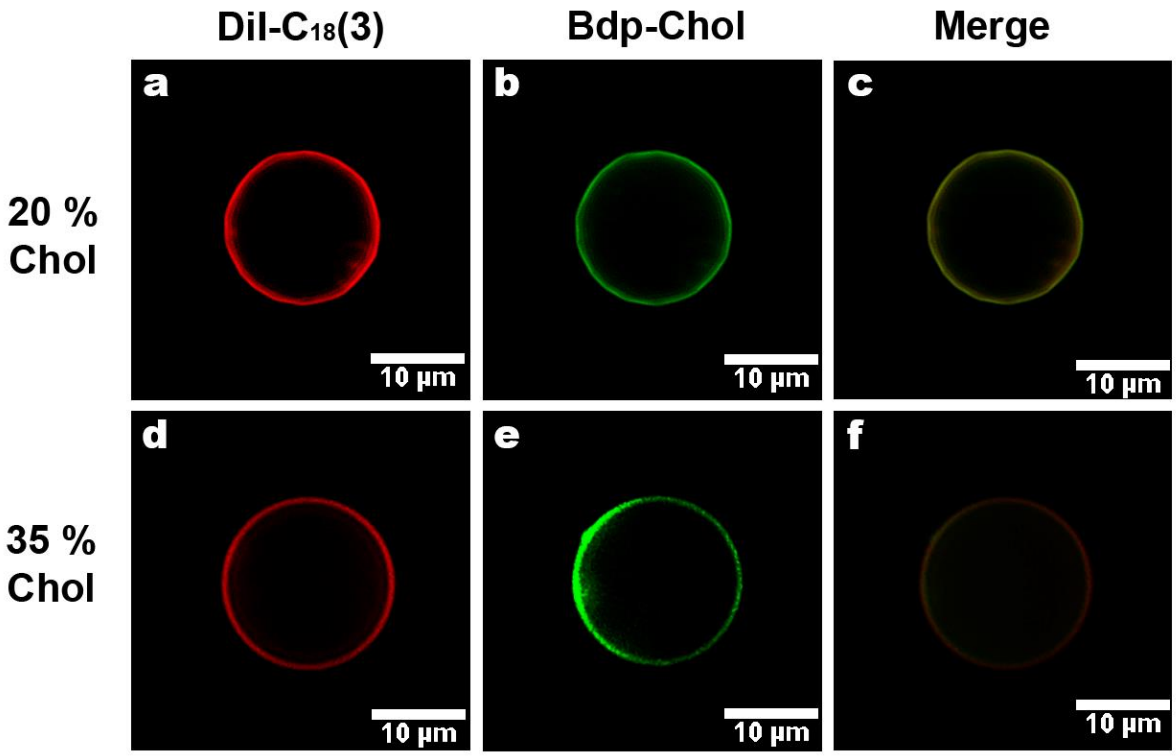


Figure 10. Fluorescent images of GUVs composed of SM/DPPC/Chol lipid mixtures labelled with DiI-C_{18}(3) (red) and Bdp-Chol (green). One uniform phase, probably L_o, is observed. Results for SM:DPPC:Chol = 35:45:20[mol %] are presented in (a, b, c) and for SM:DPPC:Chol = 30:35:35[mol %] in (d, e, f).

4.4. GUVs formed by SM:DMPC:Chol mixture

SM:DMPC:Chol = 35:45:20 [mol %] and SM:DMPC:Chol = 30:35:35 [mol %]

The uniform distribution of DiI-C_{18}(3) and Bdp-Chol was observed for both Chol concentrations what is visible on Fig. 11. We also assume that one uniform L_o phase forms in membrane with this lipid composition.

We made the same conclusion for membranes with SM:X:Chol (X=DPPC, DMPC) composition for both Chol concentrations. In order to prove this assumption more extensive experiment, which would include more PC lipids with different length of acyl chains and wider range of Chol molar concentration, should be done.

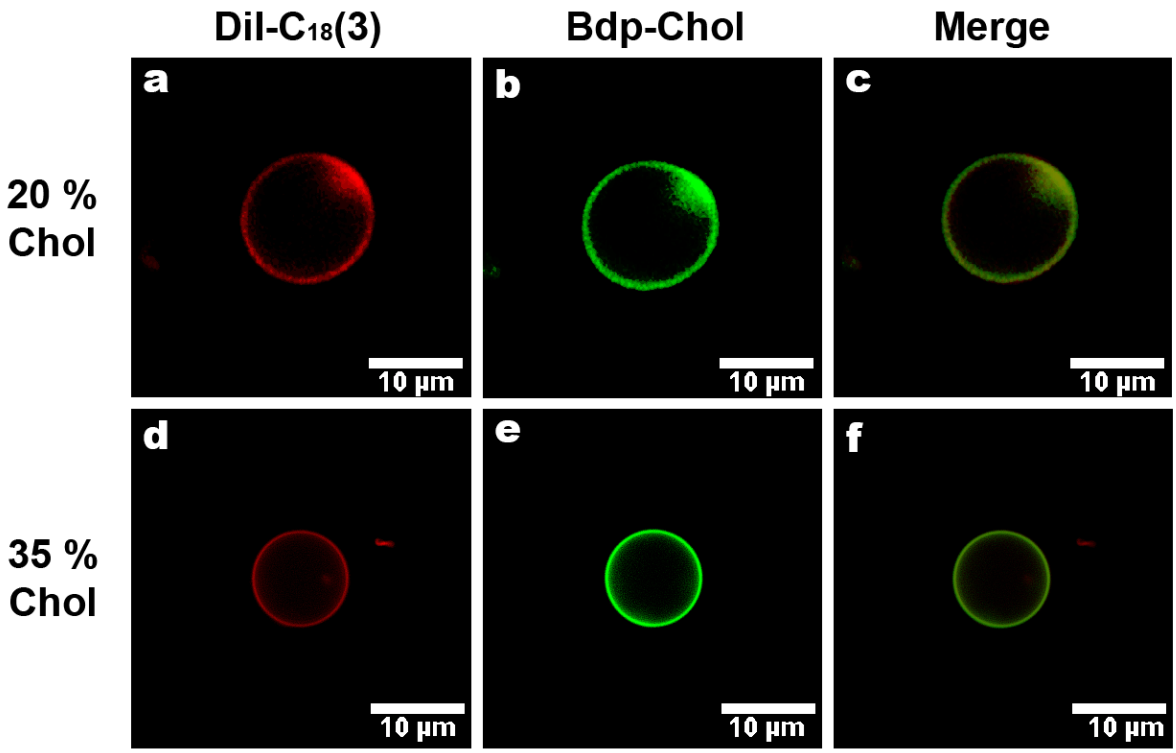


Figure 11. Fluorescent images of GUVs composed of SM/DMPC/Chol lipid mixtures labelled with DiI-C_{18}(3) (red) and Bdp-Chol (green). One uniform phase, probably L_o, is observed. Results for SM:DMPC:Chol = 35:45:20[mol %] are presented in (a, b, c) and for SM:DMPC:Chol = 30:35:35[mol %] in (d, e, f).

5. Conclusion

The aim of this experiment was to investigate domain coexistence in ternary lipid mixture with SM, Chol and various PC lipids. Selected PC lipids differ in degree of saturation and length of acyl chains. According to previously reported studies and phase diagrams [30]–[32], for ternary mixture GUVs formed of saturated, unsaturated lipids and sterols (cholesterol) it is known that there are areas of coexisting liquid domains. Our results obtained for membranes made of SM, POPC and Chol confirm already published literature data. The coexistence of two liquid phases, L_o and L_d, was observed for SM:POPC:Chol = 35:45:20 [mol %] and SM:POPC:Chol = 30:35:35 [mol %] samples and existence only L_o phase for SM:POPC:Chol = 22:28:50 [mol %]. Based on this it can be concluded that the electroformation method of vesicles preparation and subsequent recording images by fluorescent microscopy method was suitable choice to perform for this experiment.

In order to detect how degree of saturation and length of lipid chains affect our data we investigated GUVs with saturated PC lipids, which differ in the length of acyl chains (DSPC, DPPC and DMPC). These lipids differ in phase transition temperature; T_m. Results show that lipid mixture with shorter PC lipids, DPPC (16:0) and DMPC (14:0), form only one uniform phase (probably L_o). Interesting results have been reported for GUVs made of SM, DSPC and Chol. In preparation with 20% of Chol the coexistence of two phases was reported (L_o and L_d) while in preparation with 35 % of Chol only one phase (probably L_o) was detected. These results are not in agreement with data presented in phase diagram for ternary mixture of SM, POPC and Chol. Different membrane domains form in vesicles made of lipid mixtures with saturated and unsaturated PC phospholipids. Longer length of acyl chains and higher T_m of DSPC compared to DPPC and DMPC apparently affect phase formation in membranes made of SM/X/Chol mixtures (X = DSPC, DPPC, DMPC) when Chol content is 20%. In order to prove this assumption more extensive experiment, which would include more PC lipids with different length of acyl chains and wider range of Chol molar concentration (between 10 to 35 %), should be done. These results could provide new information of phase transition temperature for these lipids that could be foundation to make phase diagrams.

6. References

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