Electroformation of giant unilamellar vesicles from uniform lipid film

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Master Thesis

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ELEKTROFORMACIJA GIGANTSKIH JEDNOSLOJNIH VEZIKULA SA HOMOGENOG LIPIDNOG FILMA

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Sažetak: Plazma membrane (PM) su strukture koje odvajaju unutarstaničnu od izvanstanične tekućine. Sastavljene su od lipidnog dvosloja i membranskih proteina. Svojstva membrane te njezina interakcija sa drugim molekulama i lijekovima istražuju se laboratorijski dobivenim membranskim modelima uz kontrolirane uvjetima. Najčešće korišteni modeli su gigantske jednoslojne vezikule (GUV-ovi). U ovom diplomskom radu, prije elektroformacije GUV-ova korištene su dvije metode za nanošenja filma od gigantskih jednoslojnih vezikula (LUV-ova): Spin-Coating metoda i Coffee Ring metoda. Pripremljeni su GUV-ovi od kolesterola i fosfatidilkolina (Chol/POPC) sa različitim udjelom kolesterola (od 0 do 3). Uspješnost elektroformacije provjeravamo korištenjem fluorescentne mikroskopije i zaključujemo da Coffee Ring metoda nije optimalna za nanošenje filma te da je koncentracija LUV-ova od 3 mg/mL optimalna za Spin-Coating metodu.

Ključne riječi: Biološke membrane, nanošenje filma, kolesterol, Spin-Coating, Coffee Ring Effect

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Master Thesis

ELECTROFORMATION OF GIANT UNILAMELLAR VESICLES FROM UNIFORM LIPID FILM

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University graduate study program Physics, orientation Biophysics

Abstract: The plasma membrane (PM) is a structure that separates intercellular and extracellular fluid. It is composed of lipid bilayers and membrane proteins. Membrane properties and interaction with different molecules are often studied using laboratory-made membrane models under controlled conditions. One of the commonly used membrane models is Giant Unillamilar Vesicles (GUVs). In this thesis, two methods of film deposition of large unillamilar vesicles (LUVs) prior to electroforming of GUVs are tested: Spin-Coating Method and Coffee Ring Effect Method. GUVs containing Chol/POPC (Cholesterol/ Phosphatidylcholine) with different Chol ratios (from 0 to 3) are prepared. We quantify electroformation successfulness using fluorescence microscopy and concluded that the coffee ring effect is not an optimal method for film deposition and that a concentration of 3 mg/ml LUVs is optimal for the spin-coating method.

Key words: Biological membrane, film deposition, cholesterol, Spin-Coating, Coffee Ring Effect

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Content

1. Introduction

It was previously learned that the cell is the fundamental structural and functional unit of an organism. In order to fulfill the needs of the whole organism, the cell receives signals from the environment. Structures that receive and conduct signals within cells by stimuli from the environment are called biological membranes. The biological membranes of all cells (prokaryotic and eukaryotic) represent the barriers that separate the intracellular from the extracellular space and are required for vital chemical and physical processes. The emphasis will be on the biological membrane of eukaryotic cells. The main role of the biological membrane of eukaryotes is to separate the intracellular space from the extracellular space -the plasma membrane (PM) - and to enclose cellular organelles from the cytosol. In this paper, we focus on the plasma membranes (PMs), which, in addition to its aforementioned role as a cell barrier, also play an important role in mediating information between the cell and its environment [1].

Due to the complexity of the native structure, it is difficult to isolate plasma membranes from living cells, so laboratory-made membrane models are used. Mimicking biological membranes allows us to observe and understand the basic properties of the membrane itself and its interaction with other molecules and drugs. The current comprehensive research data on artificial membranes cover a wide range of scientific disciplines including synthetic chemistry, biophysics, pharmacology, biotechnology, and others [2]. In another section of the paper, the structure, function and main composition of the membrane are explained, as well as membrane lipid phases and model membranes prepared in the laboratory.

In this master thesis, different methods of film deposition will be experimentally investigated to achieve successful of Giant Unilamellar Vesicles (GUVs). In previous studies, lipids dissolved in organic solvents were usually used for film deposition on indium-tin oxide coated glass. In this experiment, Large Unilamellar Vesicles (LUVs) are prepared and used for film deposition instead. For the GUV electroformation efficiency and reproducibility the thickness of the lipid film is as important as the homogeneity of the lipid film [3]. Spin coating and Coffee Ring Effect are two methods used for film deposition, and based on the results of the experiment we will mention their advantages and disadvantages.

Moreover, we will check which concentration is the most suitable for the preparation of cholesterol-free GUVs. Then, we will use the previously determined concentration with the highest reproducibility together with some other concentrations to investigate which one gives the best reproducibility of GUVs when the Chol content is increased from 20 mol% - 75 mol%.

2. Structure of plasma membrane

The composition of the plasma membrane (PM) consists of lipids, phospholipids, glycolipids, and proteins. Due to their amphipathic nature - hydrophilic head and hydrophobic tail - lipids form a permeability barrier and form compartments, while membrane proteins perform all other membrane functions. The hydrophilic headgroups interact with water, while the hydrophobic fatty acid tails avoid water and interact with each other. Membranes are composed of two opposing leaflets of lipids and proteins arranged in the bilayer (Fig. 1). The mass ratio of lipids and proteins varies from 1:4 to 4:1 [4]. The membrane bilayer has the important property of selective permeability. Only selected small molecules can pass through the bilayer unhindered, for example, water molecules. However, since the lipid bilayer is impermeable to most molecules and ions that need to enter and leave the cell to maintain cell function, the protein components of the membrane are responsible for transporting selected molecules and ions into and out of the cell using channels and pumps (Fig. 1) [4].

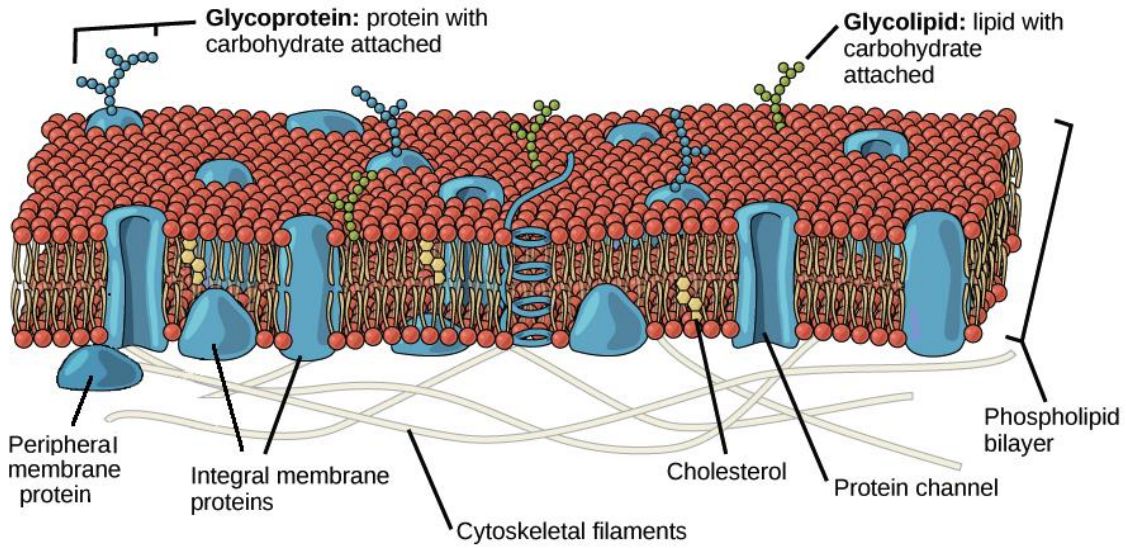

Figure 1. Structure of plasma membrane (PL) [5].

Fatty acids are responsible for the hydrophobicity of lipids. They differ in chain length and degree of saturation and carry a carboxyl group at their ends. The lipid bilayer is always asymmetric, which causes the inner leaflet to differ from the outer leaflet [6]. It is important to remember that membranes are not static but complex dynamic structures characterized by the property of fluidity. This means that the plasma membrane exhibits lateral organization and mobility of lipid and protein components [7]. This movement can be random or direct. This lateral mobility of lipids across the membrane causes membrane-based responses. In lateral mobility, there are two types of diffusion: lateral and transverse (Fig. 2). Lateral diffusion is the rapid movement of membrane components (lipids and proteins) from one side of the membrane to the other. Transverse diffusion, or flip-flop, is the rotation of membrane composition, mainly lipids, from one side of the membrane leaflet to the opposite, and this diffusion is slow. Protein flip-flop has not yet been observed [4,6,7].

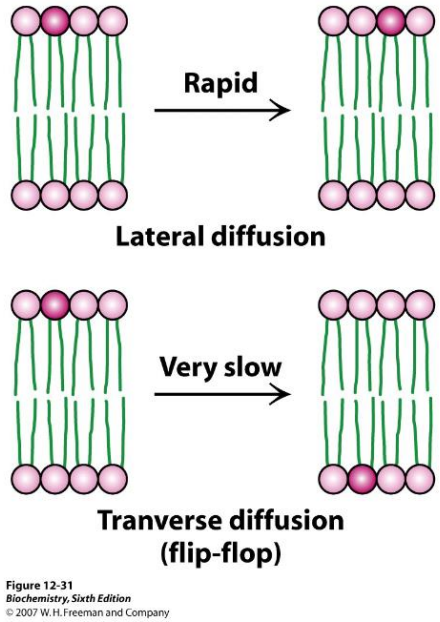

Figure 2. Lateral and Transverse diffusion [4].

Short chains and the unsaturation of hydrocarbon chains increase the fluidity of fatty acids and their derivatives. Membrane fluidity also depends on the cholesterol content in the membrane. The higher the cholesterol content, the less fluid the membrane and vice versa [4,6,7]. More about the cholesterol content in the plasma membrane is explained in the following section of this paper.

3. Major structural lipids in plasma membrane

Lipids are distinguished by their headgroup, the length and degree of saturation of their chain. The three major lipid species of the eukaryotic membrane are: Phospholipids (PL), sphingolipids, glycolipids (lipids containing carbohydrates) and sterols. The diversity of lipid composition plays an important role in membrane organization, which controls cellular functions. The main structure of PLs and sphingolipids consists of four parts: Glycerol or sphingosine, which serves as a template for one or more fatty acids attached to it, phosphate, and alcohol (Fig. 3) [4, 6].

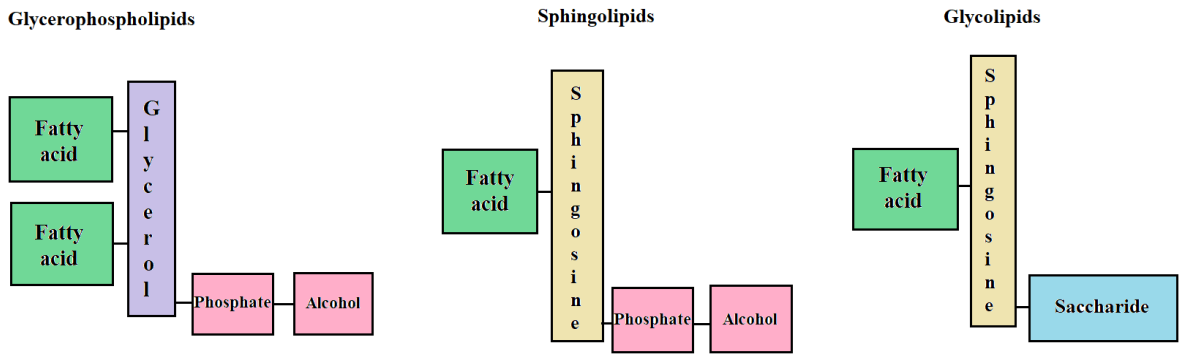

Fig. 3. Schematic structure of phospholipids and glycolipids molecules.

Glycerophospholipids use glycerol as a template for the two most abundant fatty acids. The major glycerophospholipids of the eukaryotic membrane are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), and phosphatidic acid (PA). More than 50% of the membrane lipid composition is PC, hence the spatial arrangement of glycerophospholipids is a planar bilayer. This self-assembly occurred spontaneously because PC has a cylindrical molecular geometry and a cis-unsaturated fatty acyl chain. This type of structure makes PCs fluid at room temperature. Sphingolipids use sphingosine as a template for a fatty acid that has saturated or trans-unsaturated tails. The main sphingolipids in eukaryotic cells are sphingomyelin (SM) and glycosphingolipids, which contain mono-, di- or oligosaccharides [4, 6-8]. Figure 4. shows types of fatty acid configurations.

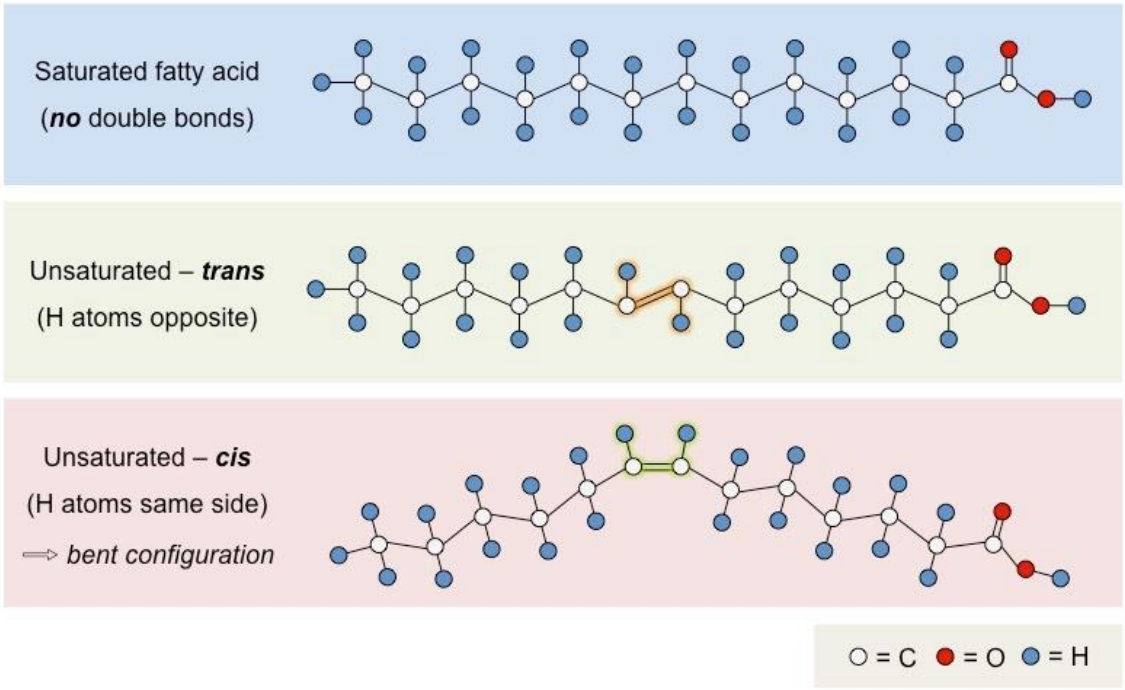

Figure 4. Differences in fatty acid configurations [9].

3.1. Lipid phases in membrane

In eukaryotic cells, lipids can behave in different solid and liquid phases. Advances in scientific techniques have allowed scientists to track and determine the contribution of lipid phases to membrane function. The coexistence of two liquid phases in a single membrane plane has been observed. This behavior of lipid phases gives lipids freedom of movement and thus maintains membrane organization. A lipid phase is formed by the lipid-lipid interaction of opposing bilayer leaflets. Cholesterol content also plays an important role in the formation of coexisting phases and domains in the membrane. The behavior of the different lipid phases depends on the chain length and the degree of saturation of the hydrocarbon chain (Fig. 5.). However, proteins are also present in the membrane. For the different lipid phases, the membrane uses proteins to modify and possibly control the behavior of these phases [4,7].

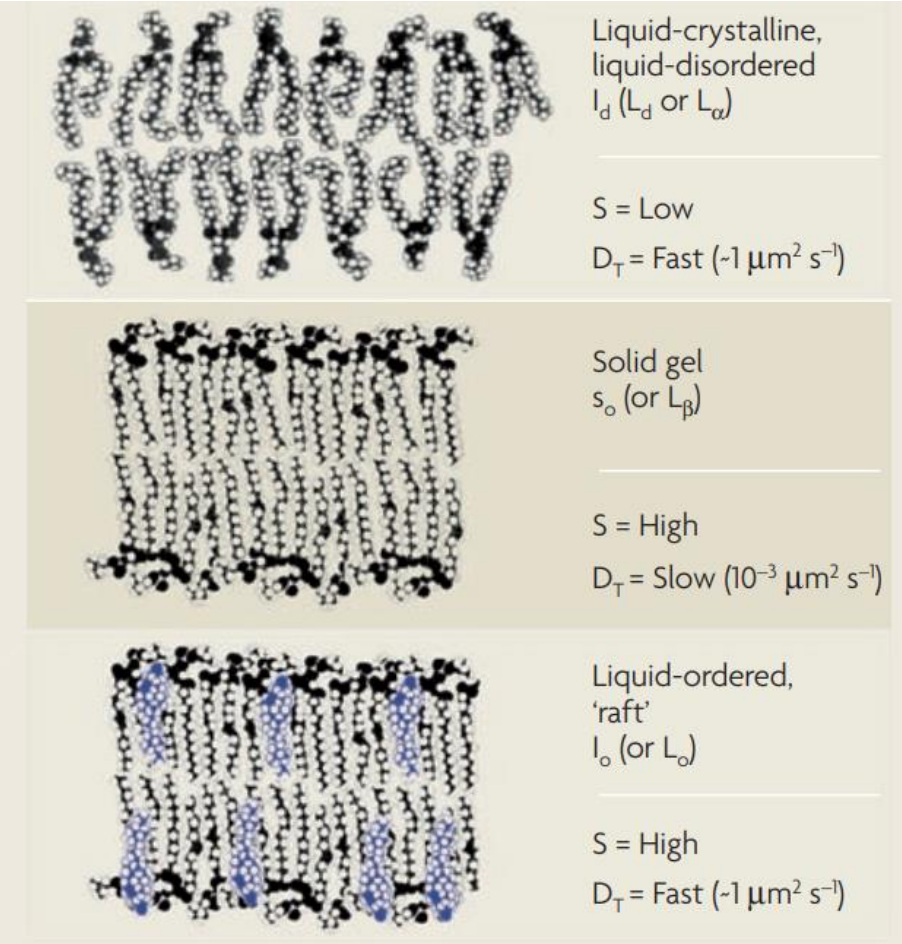

Figure 5. Lipid phases in membrane [4].

Fig 5. shows different lipid phases. The parameter S represents the order of hydrocarbon chain and parameter D_T represents the translation diffusion coefficient. Sterols together with bilayer-forming lipid can form different phases. Example are PC and Chol, together they tend to adopt liquid-ordered (l_0) or liquid-disordered (l_d) phases depending on cholesterol content (Fig. 5.). Structure in the middle represent the solid gel (s_0) phase, this phase happens when lipid bilayer is below transition temperature (T_m) [4, 7].

3.2. Cholesterol

In this work, it is mentioned that Chol plays an important role in membrane fluidity and rigidity, which are responsible for membrane permeability and also for membrane domain formation. Chol is a steroid lipid and contains three well-distinguishable regions: four hydrocarbon rings represent the center of the sterol, with a hydrocarbon tail and hydroxyl group attached to its ends, as shown in Figure 6. Cholesterol penetrates the membrane bilayer by its hydroxyl group interacting with the polar head group of phospholipids (hydrophilic

part) and the sterol rings interacting with the hydrocarbon chain of phospholipids (hydrophobic part) [10].

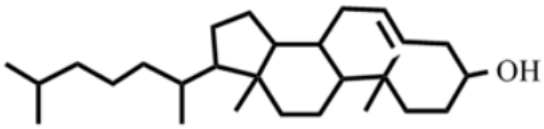

Figure 6. Chemical structure of cholesterol [10].

Along the secretory pathway, cholesterol content increases in the eukaryotic cell membrane. The endoplasmic reticulum has very low cholesterol content, the Golgi apparatus has higher cholesterol content, and the plasma membrane has the highest cholesterol content [10-11].

Table 1. Cholesterol content in biological membranes [8].

	Cholesterol to phospholipid (PL) molar ratio	**Type of membrane**
First group	<10 mol%	Endoplasmic reticulum Golgi apparatus Mitochondria membranes
Second group	10 to 30 mol% (normal)	Plasma membrane
Third group	~ 50 mol% (high, saturation, forming domain - CBD)	Plasma membrane of red blood cell Myelin membrane
Forth group	>50 mol% (saturation of domain, forming cholesterol crystals)	Fiber cell plasma membrane of the eye lens

Table 1. shows the different cholesterol content in the different membrane types. When the molar ratio of Chol/ PL exceeds 1 or (~50 mol%), the membrane becomes saturated with cholesterol and a pure cholesterol domain called CBD (cholesterol bilayer domain) is formed.

CBD is formed until the threshold for Chol solubility is reached (CST), which is at a molar ratio of 2 (66 mol% chol) to PL. Above this threshold, cholesterol crystals form, presumably outside the lipid bilayer [10-11].

3.3. Lipid self-assembly process

In contact with water, amphipathic molecules can assemble into a spherical structure called a micelle (the polar heads face the water environment, the hydrocarbon tails face the interior of the structure where they interact with each other), or a bilayer can be formed from two lipid leaflets by a self-assembly process. For most phospholipids and glycolipids, the lipid bilayer is a more favorable structure than the micelle, because the two bulky hydrocarbon chains do not fit inside the micelle. Especially salts of fatty acids form micelles because they contain a single hydrocarbon chain. The lipid bilayer is a stable structure due to the action of Van der Waals attractive forces between interacting hydrocarbon chains, electrostatic forces, and hydrogen bonds between polar heads and water molecules. The lipid bilayer has the inherent property of expanding and tends to close internally so that the hydrocarbon chains do not interact with the polar solution. Energetically, the most favorable structure is a closed one such as a cell or an organelle [4, 12].

4. Artificial biological membrane models

Liposomes are spherical, self-assembled vesicles formed from synthetic or natural phospholipids and containing a water compartment inside, as shown in Figure 7.

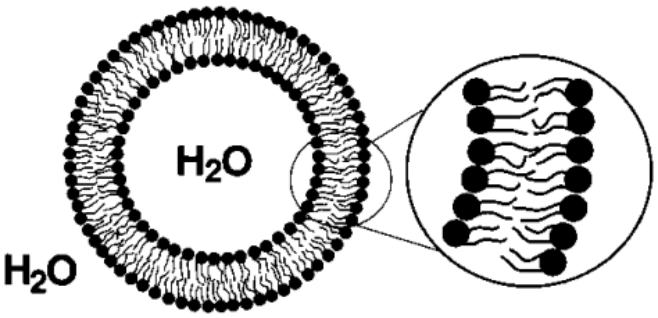

Figure 7. Structure of unilamellar vesicle [13].

The highly complex structure and small transverse size of barely 6 nm provide precise molecular information that is difficult to obtain when studying in vivo samples of cell

membranes. For this reason, artificial membrane models are used to mimic biological membranes under controlled conditions. Unilamellar vesicles are one of the models commonly used to study membrane properties (Fig. 8.) [13]. In the experimental part of this work, LUVs were used for electroformation optimization of GUVs.

Vesicles can vary in diameter and in the number of bilayers, so they can be subdivided into; giant unilamellar vesicles (GUVs) with a diameter of > 1μm, large unilamellar vesicles (LUVs) with a diameter of 200-1000 nm, small unilamellar vesicles (SUVs) with a diameter of < 200nm, mulilamellar vesicle (MLV) which consists of several concentric bilayers, and multi vesicular vesicle (MVV) which consists of several vesicles enclosed in one large vesicle. The structure of all these vesicle types can be seen in Figure 8. [12-13].

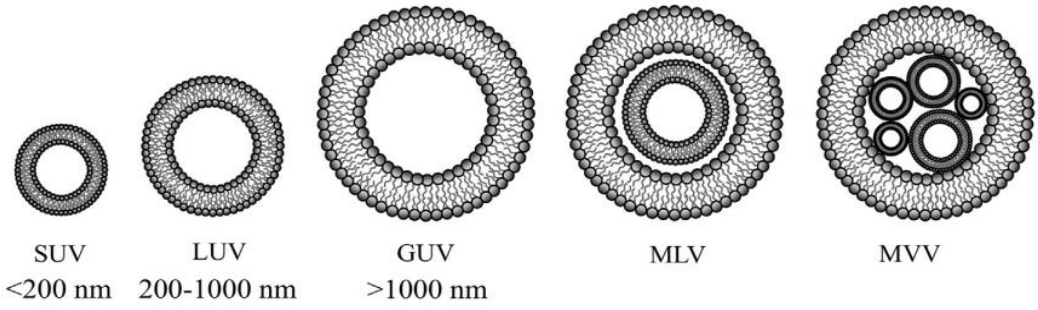

Figure 8. Schematic representation of the main lipid based vesicular systems [14].

Because of the diameter closest to the cell size, GUV is the most commonly used membrane model that allows observation of membrane-related phenomena using light microscopy. On the other hand, LUVs and SUVs are invisible to light microscopy, and there is also a disadvantage for these vesicles, such as the small diameter, which leads to a different packing of lipids compared to biological membranes. For this reason, GUVs are widely used to study membrane properties and their interaction with various biomolecules such as peptides, drugs, and proteins [12-14].

Besides the above, there are other types of membrane models such as lipid monolayers, supported lipid bilayer (SLB), and some advanced planar membrane systems. Lipid monolayers are model made up of a single lipid leaflet at the liquid-air interface. Lipid monolayers are also called Langmuir monolayers (or films). The SLB represents two lipid leaflets, one of which is supported on a solid surface. The SLB one of the most used membrane model for mimicking eukaryotic as a prokaryotic cell due to the wide range of

investigation applications as easy access for surface-sensitive measurement techniques [12, 15-16].

5. Materials and Methods

5.1. Materials

One-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) and Chol were obtained from Avanti Polar Lipids Inc. (Alabaster, AL, USA). The fluorescent dye 1,1'-dioctade-cyl-3,3,3',3'-tetramethylindocarbocyanine Perchlorate ($DiIC_{18}(3)$) was purchased from Invitrogen, Thermo Fisher Scientific (Waltham, MA, USA). Other chemicals of at least reagent grade were obtained from Sigma-Aldrich (St. Louis, MO, USA). Indium-tin oxide-coated glass (ITO, CG-90 INS 115) was purchased from Delta Technologies (Loveland, CO, USA). ITO glass dimensions were 25 mm x 75 mm x 1.1 mm.Mili-Q deionized water, 70% ethanol.

Methods

5.2. Preparation of Large Unilamellar Vesicles (LUVs)

We prepared vesicles from a mixture of POPC and Chol. Lipids dissolved in chloroform were mixed in various ratios. The resulting mixture was stored in a refrigerator at -20° C for further experiments. Prior to the preparation of LUVs, the mixture was taken out from the refrigerator and heated to room temperature. The dry lipid mass was then obtained by evaporating the chloroform in an Eppendorf tube. Lipid mixtures were prepared using different Chol concentrations, with the Chol/POPC mixing ratios ranging from 0 to 3. Since the results were analyzed by fluorescence microscopy, the fluorescent dye DiIC18(3) was also added to the mixture, and the DiIC18(3)/POPC molar ratio was 0.002 [17]. The dried lipids were hydrated with deionized Mili-Q water preheated to 60 °C (to be sure that the temperature was above the phase transition temperature of the POPC/Chol mixture). After shaking and stirring the obtained aqueous solution for ten minutes, the hydrated lipid film detaches from the tube wall and forms MLVs. The LUVs are then prepared by passing this MLVs suspension through a polycarbonate membrane with a pore size of 30 nm. This process is performed using the

Avanti Mini extruder (Fig. 9.) preheated to 60 °C. To avoid the loss of lipid mixture, it was necessary to reduce the dead volume by prewetting the extruder parts. Then, a gas-tight syringe was filled with the lipid mixture and the suspension was extruded in a total of 15 passes to obtain the solution of LUVs [18].

Figure 9. Avanti Mini Extruder [19].

5.3. Deposition of the Liposome Solution

Before deposition of the LUV solution to the ITO -coated glass, the glass was immersed in deionized water for at least 45 minutes and then wiped with 70% ethanol using lint-free wipes. Also, the slides were cleaned with plasma oxygen for 30 seconds to obtain a clean and hydrophilic surface. Two ITO-coated slides are needed for electroformation (detail decription is in section 5.4.). One slide contained the lipid film and the other was cleaned by the same procedure, but only served to seal the chamber with the aqueous solution [20].

To achieve the best efficiency in the preparation of GUVs, the thickness of the lipid film needs to be about 30-60 nm. In previous studies, researchers investigated the most suitable and efficient method to deposit the lipid film uniformly. This is important for the production of GUVs, because such nonuniformities lead to a lack of reproducibility. The conventional droplet deposition method cannot produce such uniform films [20-22]. In the following section, two possible improvements are presented: the spin coating method and the coffee ring effect.

5.3.1. Spin-coating method

Reproducibly controllable lipid film thickness was achieved using the spin-coating method. The lipid solution is dropped onto the flat electrode surface, which is subsequently rotated very fast (ω ~ 600 rpm) in order to achieve a homogenous film (Fig. 10.) [23].

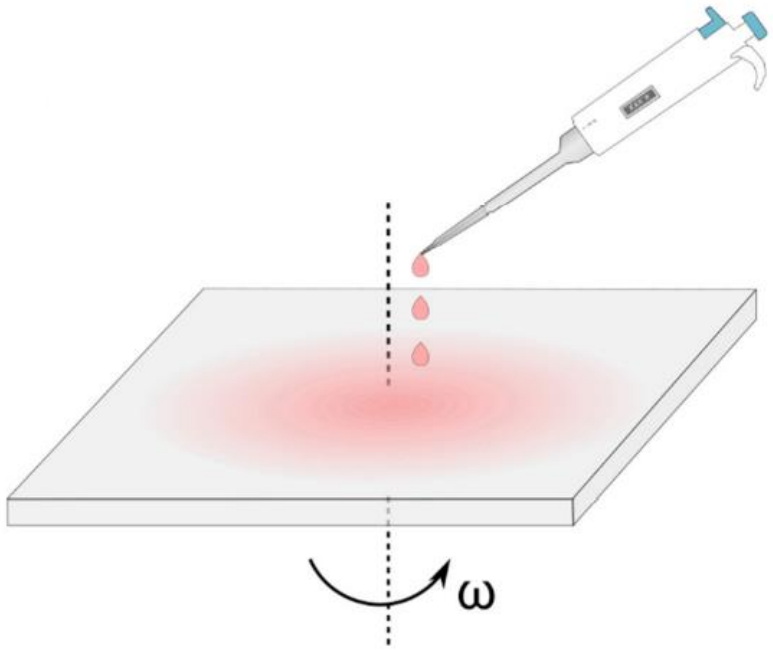

Figure 10. Spin-coating of lipid solution by fast rotation of the electrode immediately after the deposition [22].

In this work, 350µl of LUVs solution was applied to ITO-coated glass slides. 350 µl was used as this volume is sufficient to cover the entire ITO-coated slide. The Sawatec SM -150 spin coater (Fig. 11.) was used to spin the glass at 600 rpm for 4 minutes (the final speed was reached in 1s) and a thin uniform lipid film was generated [3].

Figure 11. Sawatec SM-150 spin-coater.

5.3.2. Coffee ring effect method

Coffee Ring effect describes a phenomenon in which a drying droplet deposits most of its material on the periphery, forming a ring-like stain. The droplets are deposited progressively larger on top of one another. Since a larger droplet has more volume, it will create a larger diameter ring and, in the process, will smear and flatten the ring from the previous droplet. This process will leave an area of uniform lipid thickness inside (Fig. 12.). Authors of this method showed that diameter of vesicle populations hasn't significantly changed compared to the single droplet deposition, but the multi-droplet preparations displayed a much lower

percentage of nonunilamellar vesicles. Another advantage of this method is using low mass of lipid which reduces costs of the experiments.

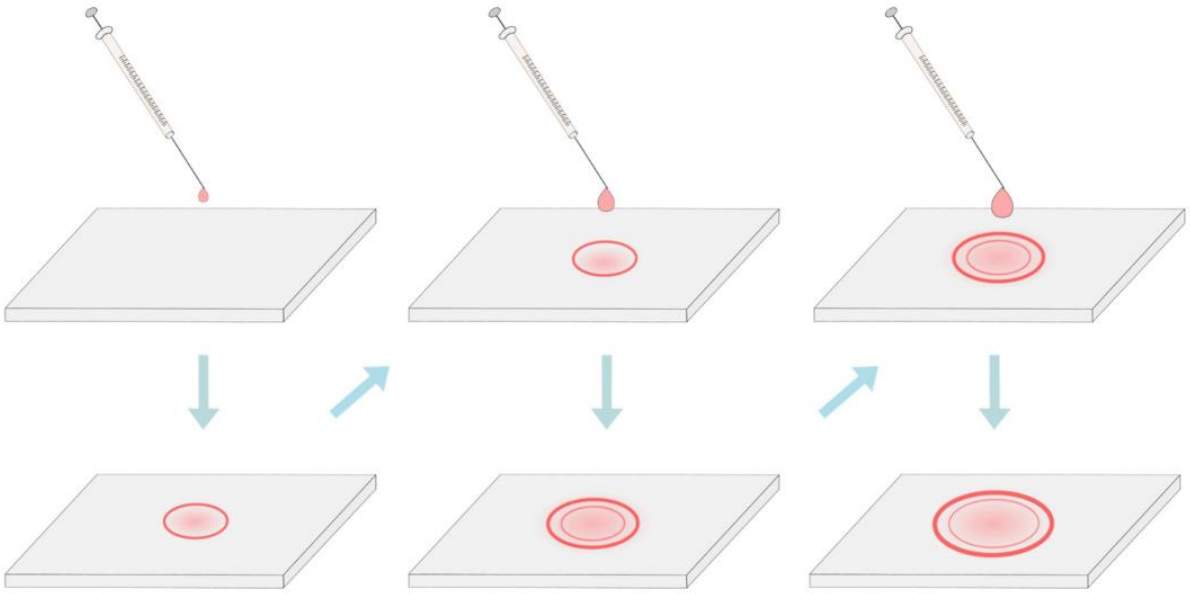

FIgure 12. Deposition of lipids utilizing the coffee ring effect. After drying, ring-like strain is formed because most of the material is carried away toward the periphery. This is known as the coffee-ring effect. By depositing progressively larger droplets, the ring from the previous droplets gets smeared and flattened, thus leaving behind an area of uniform lipid film thickness [22].

In this work, LUV suspension was filled into a Hamilton syringe mounted on the syringe holder (Fig. 13.). Different liposome concentrations were used (0.05 mg/ml, 0.5 mg/ml, and 4 mg/ml). A drop of 0.1 µL was poured onto a pre-cleaned ITO-coated glass slide and evaporated. The ring-shaped strains of lipid material remained on the substrate due to the coffee ring effect. The procedure is repeated with 0.2 µL with casting drops exactly over the previously dried lipid strains using the same liposome suspension. The same was repeated with 0.3, 0.4, 0.6, 0.9, 1.2 and 1.6 µL drop. After the last drop of LUV suspension was dried, two more drops of 2.1 and 2.6 µm deionized Mili-Q water were added in the same way. Each circular multilayer film (prepared with different concentrations) contains 265 ng, 2.65 µg, and 21.2 µg of lipid dry mass [18].

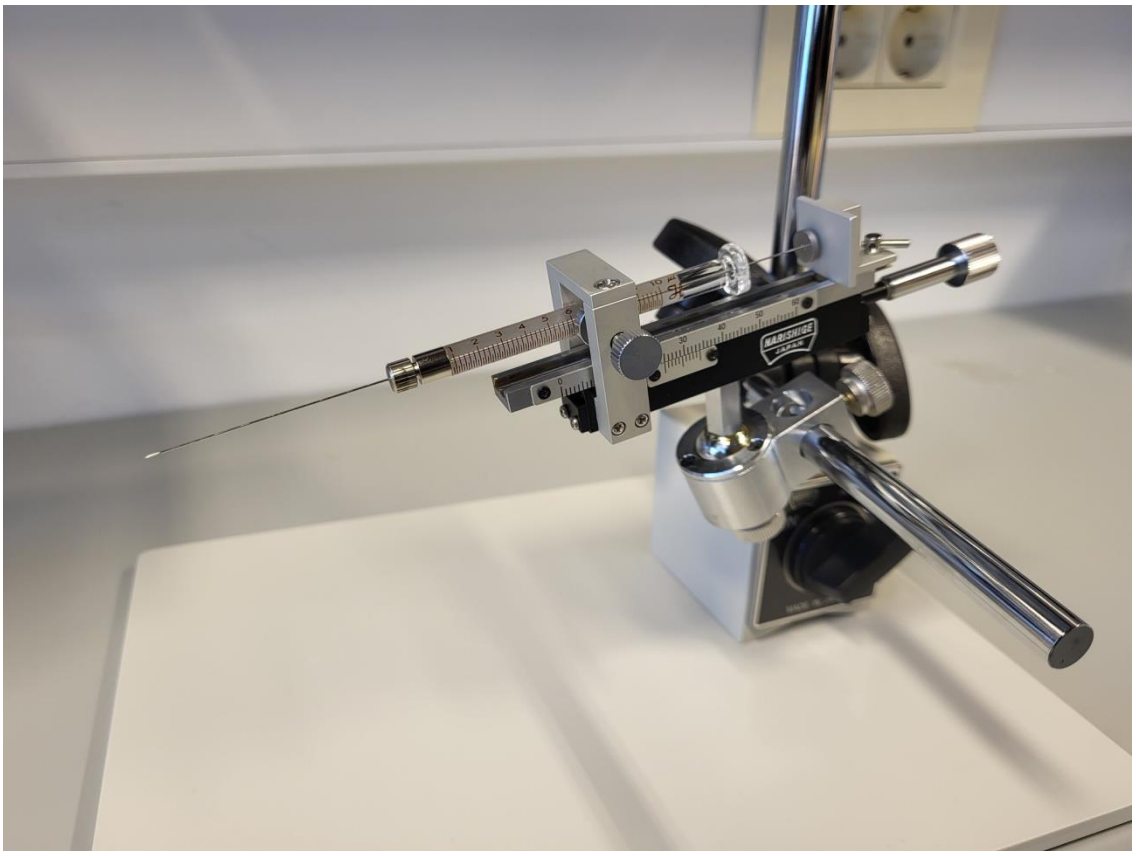

Figure 13. Apparatus for the coffee ring effect.

5.4. The electrofomation method

After preparing a lipid film using the two methods described above, the film must be rehydrated to produce GUVs. We used the electroformation method for this step. The setup of the electroformation chamber is shown in Figure 14. ITO-coated glass slides were cut with a diamond pen cutter (25 mm x 75 mm) to obtain the electrodes. The electrodes were separated by a 1.6 mm thick Teflon spacer. A piece of copper tape was attached to the ends of the two ITO-coated glass slides to make better contact with the alternating current function generator. The spacer was attached with vacuum grease to the ITO-coated slide containing the dried LUVs suspension. Another ITO-coated slide was then attached to the spacer with the conductive side facing inward. Mili-Q deionized water was injected as an internal chamber solution and then a plug was placed on the opening and sealed with vacuum grease. The chamber is designed to prevent contamination of the internal solution by contact of the solution with the vacuum grease. After assembly, the chamber was connected to a voltage of 2 V and a frequency of 10 Hz and placed in an incubator at 60 °C (to ensure that the

temperature was above the phase transition temperature of the lipid mixture). The voltage source was turned off after 2 hours and the sample was then left in the incubator for another hour. The alternating current causes electroosmotic movement of the fluid which destabilizes the lipid film, promotes hydration of the film, and thus facilitates the production of GUVs [3, 24-25].

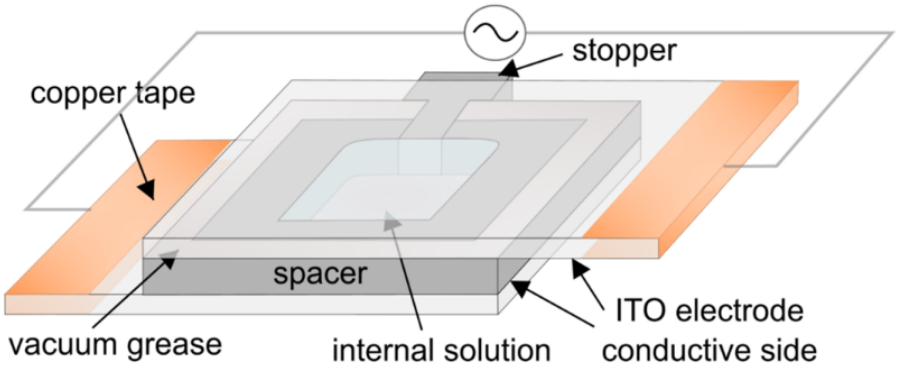

Figure 14. Schematic representation of electroformation chamber used in this experiment [3].

5.5. Fluorescence Imaging

The principle of fluorescence microscopy is that light energy (a photon) strikes the sample, which then absorbs that energy. Since some of the energy is always lost, the sample emits light energy of a different wavelength (a second photon) a few nanoseconds later. The energy of the incident light energy is always greater than the emitted light energy. When the energy decreases, the wavelength increases. Therefore, the light emitted from a sample usually has a longer wavelength than the absorbed (excited) light. Light with a short wavelength (in the blue region) has a higher energy than light with a long wavelength (in the red region) [26].

In order to search the entire volume of the chamber, we scanned 13 points on the chamber surface as indicated in Figure 15. The diameter of 50 vesicles was measured per image, if possible. Up to 50 vesicles from the image were selected randomly to avoid biased measurements [17]. Images were obtained using a fluorescence microscope (Olympus BX51). Vesicle diameters were measured using the line tool in Fiji software [27].

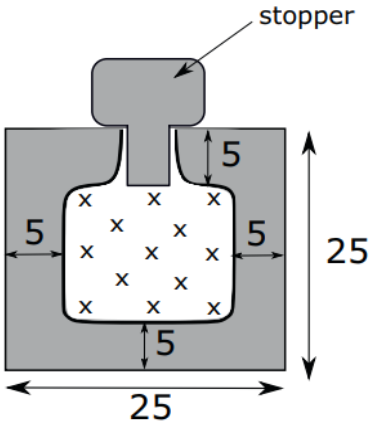

Figure 15. Label "x" at the surface chamber presents the position at which microscope images were taken [17].

5.6. Data Analysis

Data analysis was performed using the R programming language [28]. Student's t-test was used for comparisons. Normality was assessed using qq plots and histograms.

6. Results and Discussion

6.1. Effect of different film deposition

As a fluorescent probe was added to the LUVs suspension, the yield efficiency and reproducibility (as the amount of defect) of the vesicles could be studied using fluorescent microscopy. We repeated the Coffee Ring Effect method 3 times with different liposome concentrations (0.05 mg/mL, 0.5 mg/mL, and 4 mg/mL). The 0.05 mg/mL concentration should have produced the best results according to the original article [18]. However, we could not reproduce those results and actually saw practically no vesicles in the sample. We attributed this lack of vesicles to an insufficient concentration of lipids, and tried to repeat the procedure with a concentration of 4 mg/mL. This lipid concentration proved to be too high (Fig. 16). The third attempt was with a concentration of 0.5 mg/mL. According to Figure 16., we see that vesicles indeed formed, but the yield was much lower than in the original paper [18]. One possible explanation for our lack of reproducibility is that unlike the paper [18] in which a motorized stage of an inverted microscope was used, we applied the drops manually using a microsyringe holder. Since the drops used to create the film had a volume of 0.1 - 2.6 µL, it was difficult to deposit a drop exactly at the center of the previously dried one. The

method also has one additional downside – the preparation time is very long due to having to wait for the drops to dry for a long time.

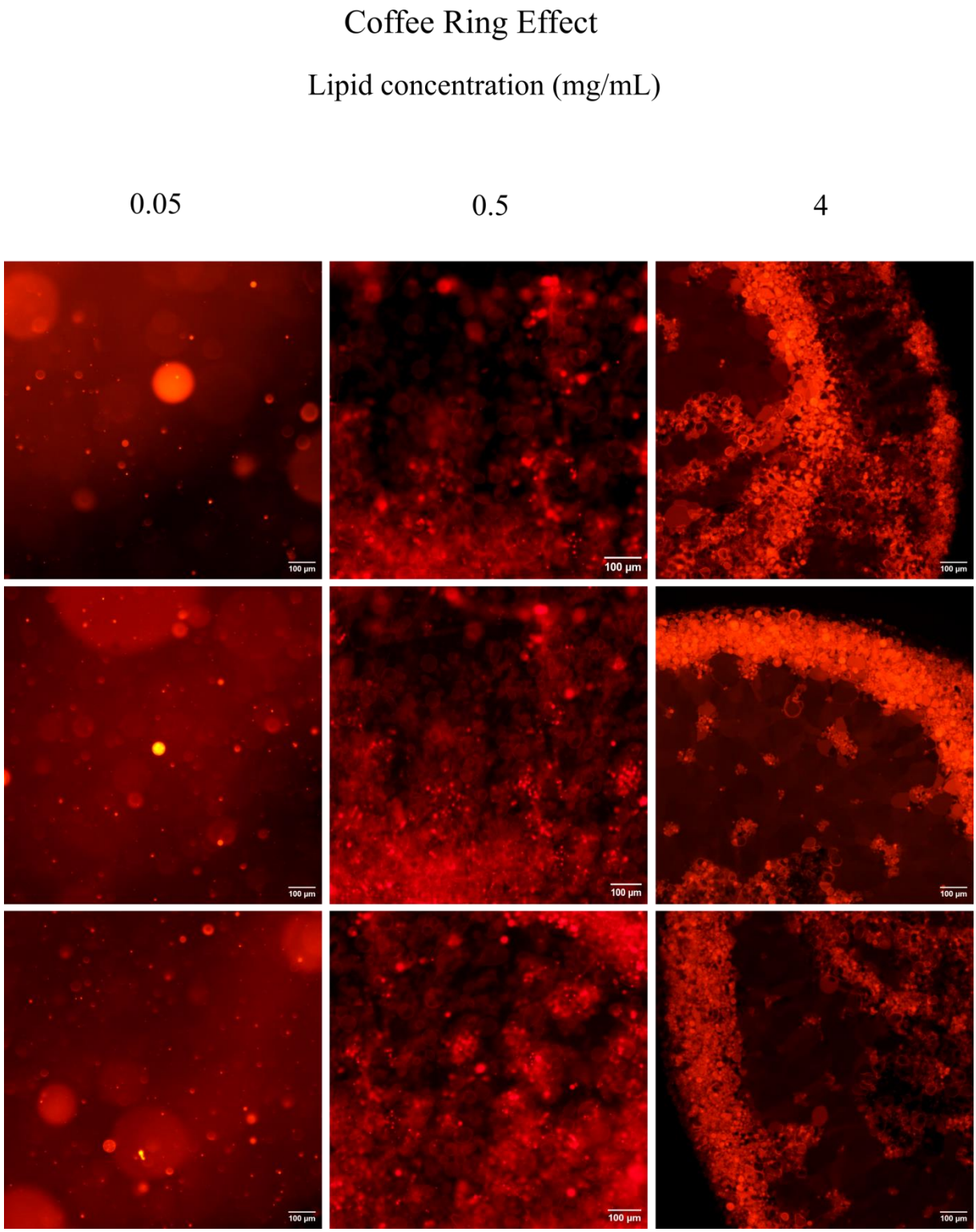

Figure 16. Fluorescence microscopy images represent the Coffee Ring method. Each column of the figure shows a different liposome concentration. Each concentration was performed using a frequency-voltage combination of 10 Hz and 2 V. Scale bar on the right bottom of each image denote 100 µm.

6.2. Effect of Cholesterol concentration

6.2.1. POPC:Chol = 1:0

Following the recommendations from a recent article [3], we decided to use total lipid concentrations of 3 mg/mL, 4 mg/mL and 5 mg/mL in this study. We compared the homogeneity of the GUV population as well as their size and the amount of defects.

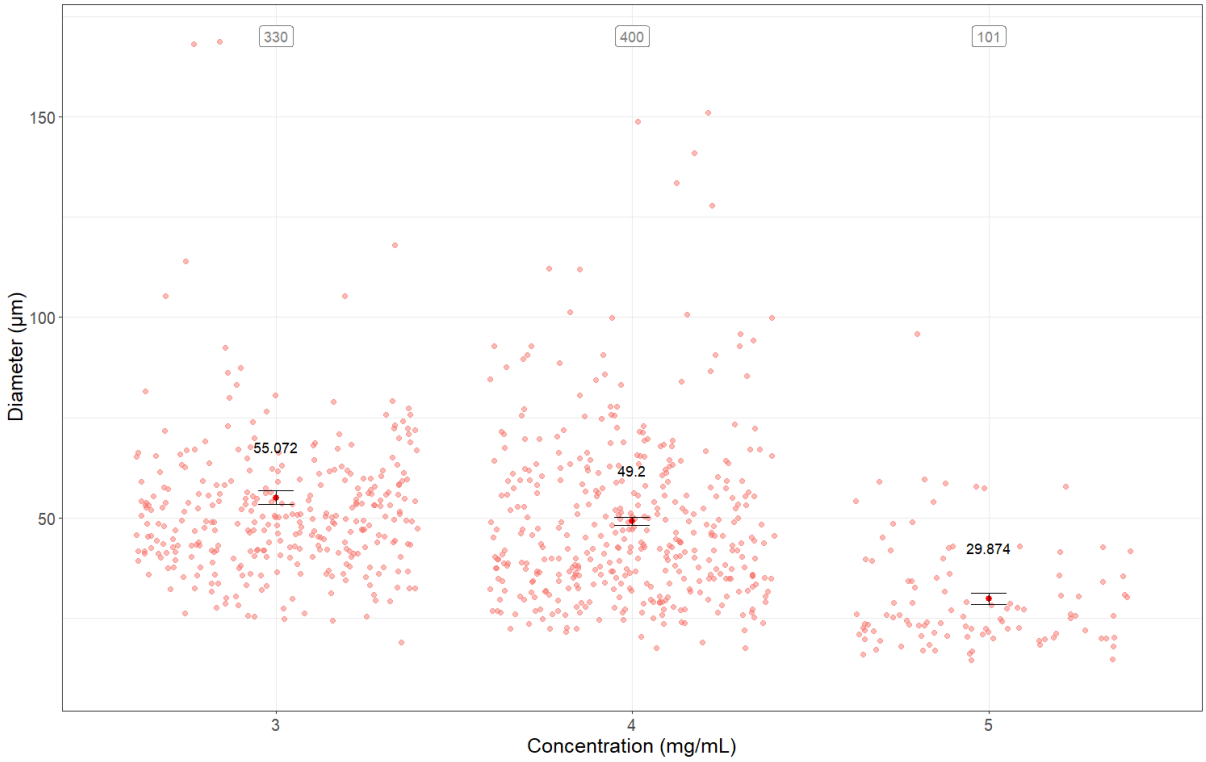

Figure 17. GUVs size depending on the liposome concentration. Each concentration was performed using a frequency-voltage combination of 10 Hz and 2 V. Top of the panel display average number of tracked vesicles per sample. The mean value of each sample is shown as a red point.

Knowing that adding Chol makes it harder for GUVs to form, we started off with GUVs containing only POPC. According to the obtained results the most successful formation of GUVs was achieved with a lipid concentration of 3 mg/mL (Fig. 17.). If we compare the results for concentrations of 3 and 4 mg/mL, we observe a very small difference in the mean value of the vesicle diameters. Also, according to the number of measurements, concentrations of 3 and 4 mg/mL have approximately the same number of vesicles, while the concentration of 5 mg/mL deviates in terms of the number of vesicles from the previous two. These result ssuggest that 5 mg/mL is a too high lipid concentration. The fluorescence

microscopy images also confirm the higher quality in two samples with lower lipid concentrations (Fig. 18).

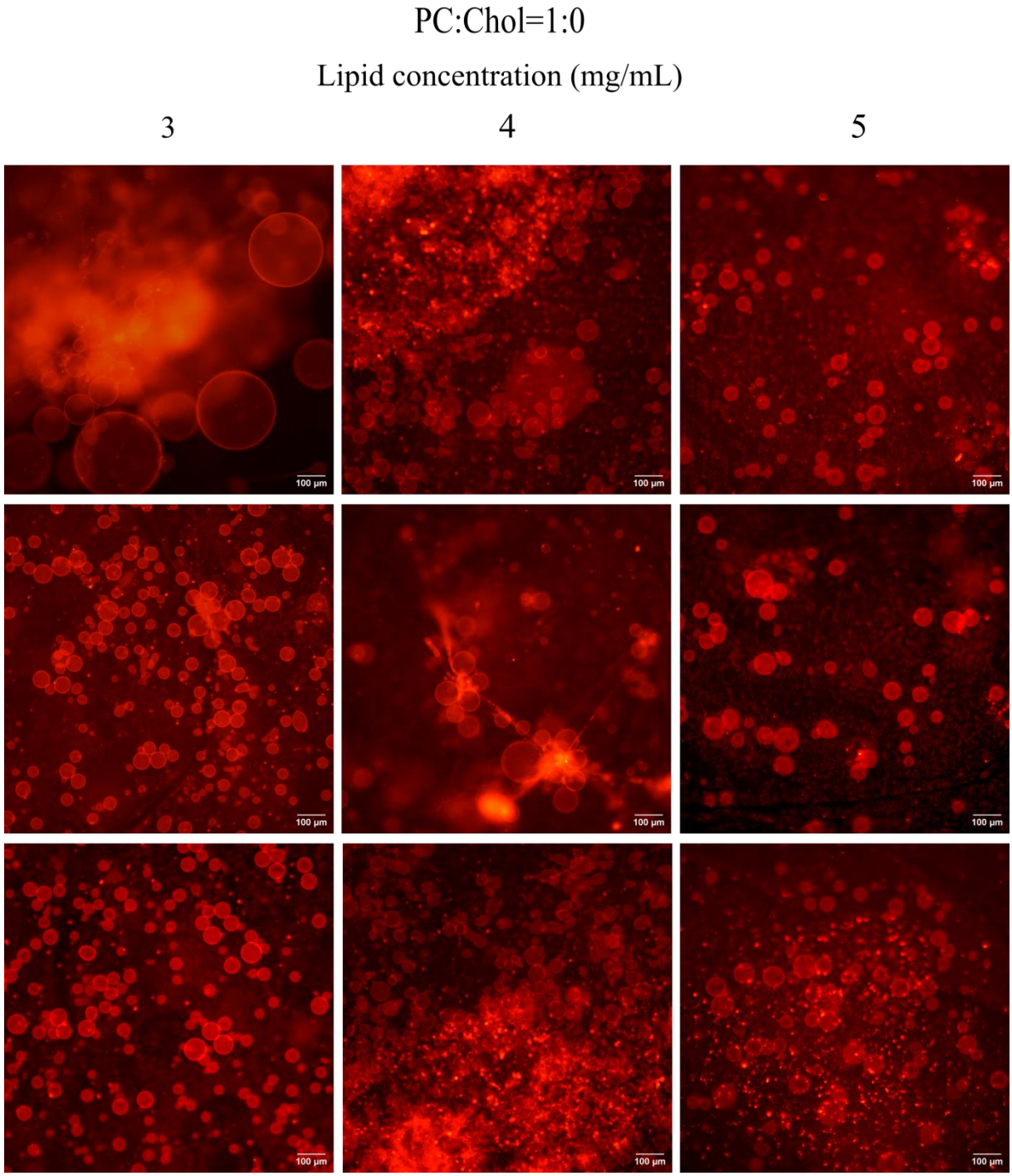

Figure 18. Fluorescence microscopy images represent GUVs size depending on the liposome concentration. Each column of the figure shows a different liposome concentration. Scale bar on the right bottom of each image denotes 100 µm.

If we compare figures 16. with figure 18., we can see a much higher reproducibility in figure 18., which represent the result using a spin-coater.

Indeed, liposome concentrations of 3 mg/mL and 4 mg/mL will continue to be used in further measurements with different cholesterol fractions. In addition to these two, we will also test a concentration of 2 mg/mL.

6.2.2. POPC:Chol = 1:1

Figure 19. GUVs size depending on the liposome concentration and POPC/Chol mixing ratio (50% Chol). Each concentration was performed using a frequency-voltage combination of 10 Hz and 2 V. Top of the panel display average number of tracked vesicles per sample. Different colors represent different samples.The mean value of each sample is shown as a red point.

After analyzing samples with pure POPC GUVs, we proceeded with experiments on an equimolar mixture of POPC and Chol.

According to Figure 19., just like with pure POPC GUVs, a liposome concentration of 3 mg/mL gave the best results. This concentration, in addition to having the most tracked vesicles per sample, also shows the highest homogeneity.

According to Figure 20., we can see the best reproducibility of the sample with a liposome concentration of 3 mg/mL. A concentration of 4 mg/mL shows a slightly worse reproducibility, while samples using a concentration of 2 mg/mL have a lower yield and produces a lot more defects.

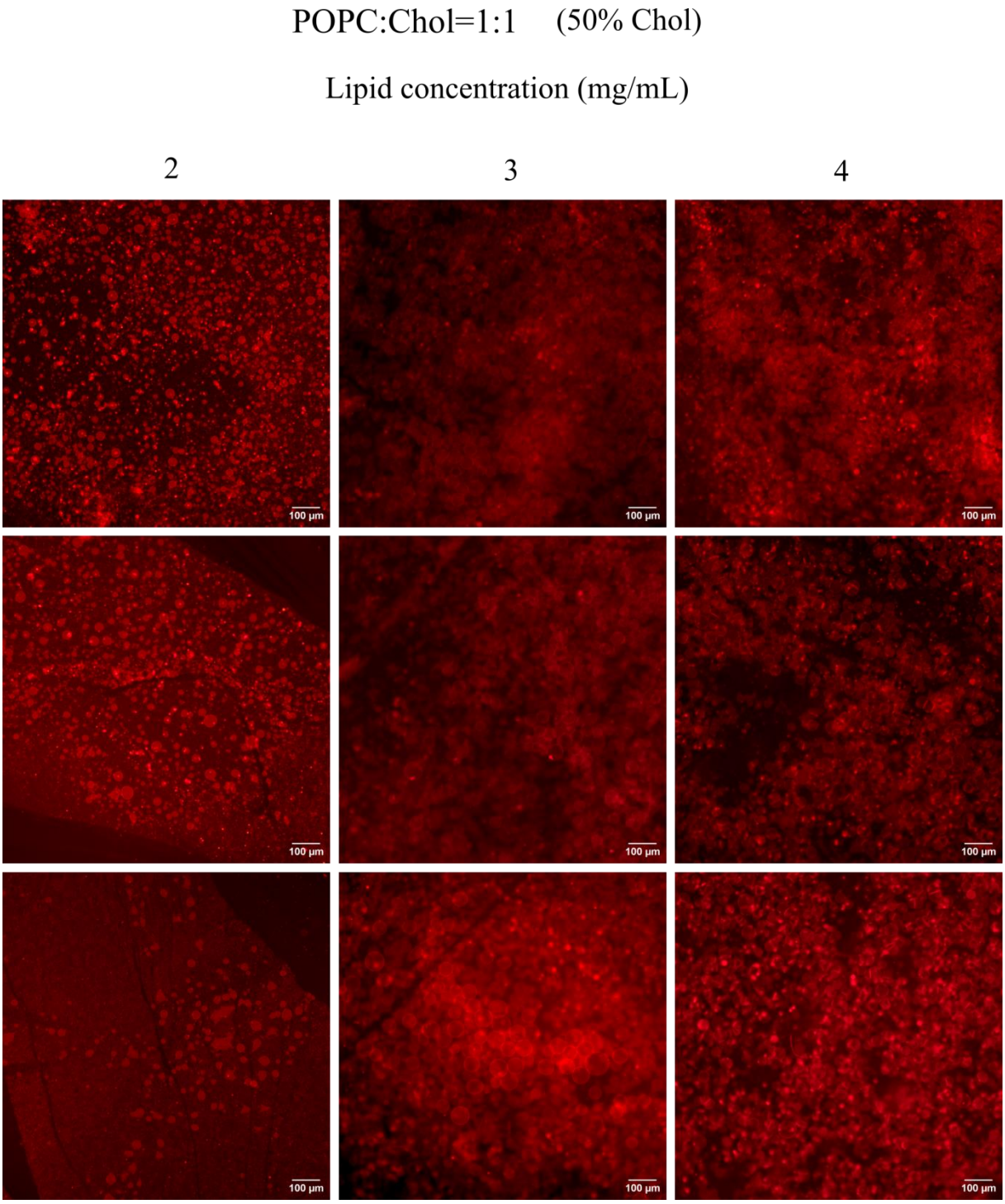

Figure 20. Fluorescence microscopy images represent GUVs size depending on the liposome concentration and POPC/Chol mixing ratio. Each column of the figure shows a different liposome concentration. Scale bar on the right bottom of each image denotes 100 µm.

6.2.3. POPC:Chol = 1:0.25

Lookingat Figure 21., we can again see that the concentration of 3 mg/mL gave the best results. The fact that this concentration gave the best results for all Chol concentrations is not

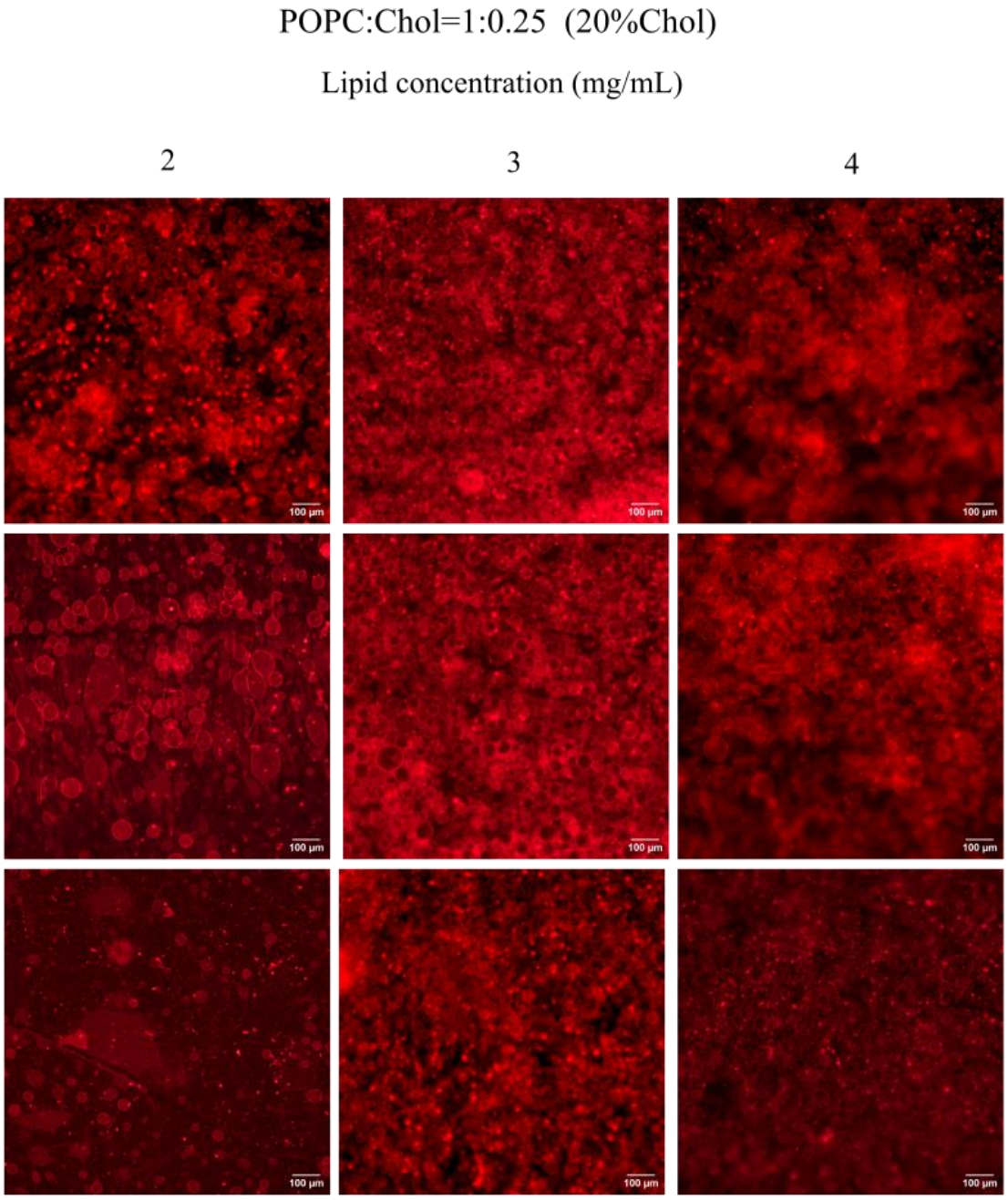

Figure 21. Fluorescence microscopy images represent GUVs size depending on the liposome concentration and POPC/Chol mixing ratio. Each column of the figure shows a different liposome concentration. Scale bar on the right bottom of each image denotes 100 µm.

surprising, since a recent study found that such concentrations lead to a lipid film thickness of ~30 nm which seems to be optimal for GUV electroformation [3].

Figure 22. shows a comparison for two different Chol concentrations using a same lipid concentration of 3 mg/mL. In line with findings in recent studies [3, 17], an increase in Chol content leads to a decrease in electroformation successfulness ($p = 2.2 \cdot 10^{-16}$, unpaired Student's t-test).

Figure 22. Comparison of electroformation successfulness for different Chol concentrations. Different colors represent different samples. X denotes the mean of all values. The errorbars denote the mean ± sd values.

These results can be attributed to higher rigidity and lower membrane permeability of GUVs containing more Chol [10].

6.2.4. POPC:Chol=1:3

The attempt to obtain GUVs with a cholesterol content of 75% was not successful (for all concentrations used) as can be seen from Figure 23.

One of the reasons for the results obtained in this way is the drying of the lipid mixture with high cholesterol content. Drying is necessary to replace the organic solvent with an aqueous buffer. During the drying of the lipid mixture, cholesterol crystals are formed. These crystals do not participate in the membrane formation. Consequently, using such a high Chol concentration prevented the formation of MLVs needed for the later production of LUVs. One of the solutions for this problem is the rapid solvent exchange (RSE) method. RSE is novel strategy that is used for lipid mixture for direct transfer between the organic solvent and aqueous buffer [29]. With the RSE method dry phase would be avoided. When going through dry phase, Cholesterol crystals are formed that later do not participate in GUVs formation, so Chol mixing and molar ratio would be different. Chol molar ratio will always be lower than Chol mixing ratio if sample is passing through dry phase) [29].

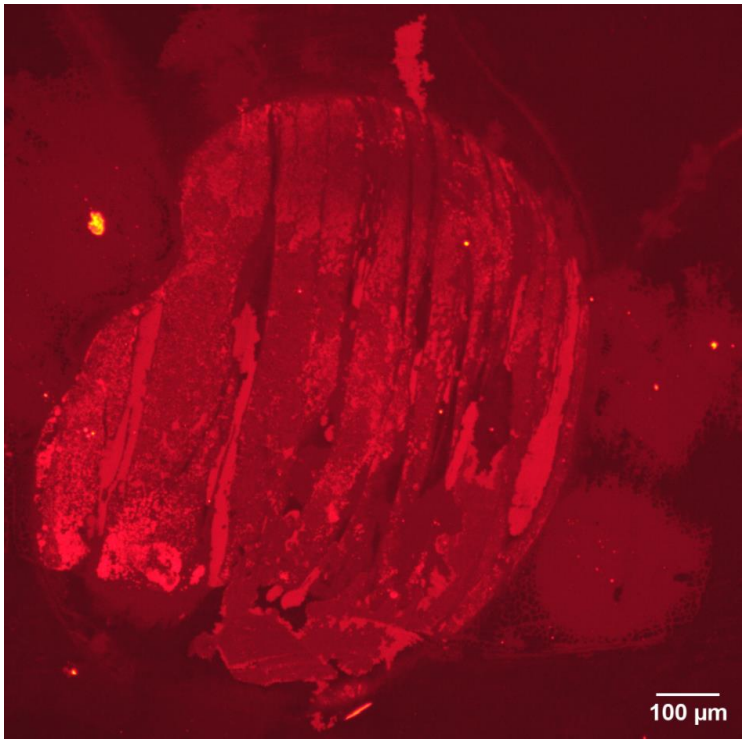

Figure 23. Fluorescence microscopy images represent GUVs size depending on the liposome concentration and POPC/Chol mixing ratio.

7. Conclusions

Creating artificial vesicles with sizes similar to the sizes of eukaryotic cells is of interest to researchers who want to explore membrane properties in a controlled environment. However, in order to get reliable results, a reproducible technique for their production is needed. Electroformation is the most commonly used method nowadays, but the traditional drop-deposition technique for film deposition leads to uneven film thicknesses. This causes low reproducibility and sample homogeneity.

Two alternative approaches for uniform film deposition have recently been suggested by researchers – the spin-coating [23] and coffee-ring method [18].

Contrary to the original study [18], we were not able to create reproducibly uniform films using the coffee ring method. This could be because we were not using a motorized stage during the deposition of rings. Furthermore, even if we were able to reproduce the original results, there is still the issue of long preparation time due to having to wait for the drops to dry. The only advantage of the Coffee Ring method compared to the spin-coating method is that much less lipid is needed when performing film deposition. Consequently, we conclude that the spin-coating method is a better alternative for production of uniform lipid films.

Proceeding with the spin-coating method, we tested the effect of various lipid concentrations and Chol concentrations on the final result. We concluded that performing electroformation using a liposome concentration of 3 mg/mL produces the best results for all tested conditions. In line with recent studies, the yield and average diameter of GUVs decreased when the concentration of Chol was increased. Moreover, using a Chol/POPC ratio of 3, we were not able to produce any GUVs. This is probably a consequence of artifactual Chol crystal formation during the lipid film drying stage. The problem could potentially be solved by using the RSE method during the protocol. This approach was applied to lower Chol concentrations by Baykal-Caglar et al. [20], but we think it could be extended to higher concentrations as well.

8. References

[1] G. M. Cooper, R. E. Hausman; ***Stanica – Molekularnipristup***, trećeizdanje, Medicinskanaklada, Zagreb, 2004.

[2] A. M. Seddon, D. Casey, R. V. Law, A. Gee, R. H. Templera, O. Ces (2009.); ***Drug interactions with lipid membranes***. *Chemical Society Reviews,* Volume 38, 2497–2812

[3] Z. Boban, I. Mardešić, W. K. Subczynski, D. Jozić, M. Raguz (2022.); ***Optimization of Giant Unilamellar Vesicle Electroformation for Phosphatidylcholine/Sphingomyelin/Cholesterol Ternary Mixtures***. Membrane, 12, 525

[4] J. M. Berg, J. L. Tymoczko, L. Stryer; ***Biokemija***, Školska knjiga, Zagreb, 2013.

[5] Openstax. Biology; Components and Structure.

https://cnx.org/contents/GFy_h8cu@9.85:QOGUelqL@9/Components-and-Structure

[6] G. van Meer, D. R. Voelker, G. W. Feigenson (2008.); ***Membrane lipids: where they are and how they behave***. *Nature Reviews Molecular Cell Biology*, Volume 9

[7] K. Jacobson, P. Liu, B. C. Lagerholm (2019.); ***The Lateral Organization and Mobility of Plasma Membrane Components***. *Cell*, Volume 177, Issue 4, 806-819

[8] T. M. Konyakhina, J.Wu , J. D. Mastroianni, F. A. Heberle, G. W. Feigenson (2013.); ***Phase diagram of a 4-component lipid mixture: DSPC/DOPC/POPC/chol***. *Elsevier*, Volume 1828, Issue 9, 2204-2214

[9] BioNinja. Type of fatty acids. https://ib.bioninja.com.au/standard-level/topic-2-molecular-biology/23-carbohydrates-and-lipids/types-of-fatty-acids.html

[10] W. K. Subczynski, M. Pasenkiewicz-Gierula, J. Widomska, L. Mainali, M. Raguz (2017.); ***High Cholesterol/Low Cholesterol: Effects in Biological Membranes Review***. *Springer: Cell BiochemBiophys,* 75:369–385

[11] S. L. Veatch, S. L. Keller (2002.); ***Organization in Lipid Membranes Containing Cholesterol***. *Phys. Rev. Lett,* 89

[12] I. Czolkos, A. Jesorka, O. Orwar (2011.); ***Molecular phospholipid films on solid supports***. *Soft Matter*, 7, 4562–4576

[13] F. M. Menger, M. I. Angelova (1998.); ***Giant Vesicles: Imitating the Cytological Processes of Cell Membranes***. *Accounts Of Chemical Research*, 31, 789-797

[14] G. Milcovich, S. Lettieri, F. E. Antunes, B. Medronho , A. C. Fonseca, J. F. J. Coelho, P. Marizza, F. Perrone, R. Farra, B. Dapas, G. Grassi, Mario Grassi, S. Giordani (2017.); ***Recent advances in smart biotechnology: Hydrogels and nanocarriers for tailored bioactive molecules depot***. *Elsevier*, 249, 163-180

[15] L. A. Cliftona, R. A. Campbell, F. Sebastiani, J. Campos-Terán, J. F. Gonzalez-Martinez, S. Björklund, J. Sotre, M. Cárdenas (2020.); ***Design and use of model membranes to study biomolecular interactions using complementary surface-sensitive techniques***. *Elsevier*, Volume 277

[16] J. A. Jackman, Nam-Joon Cho (2020.); ***Supported Lipid Bilayer Formation: Beyond Vesicle Fusion***. *Langmuir*, 36, 1387−1400

[17] Z. Boban, A. Puljas , D. Kovač, W. K. Subczynski, M. Raguz (2020.); ***Effect of Electrical Parameters and Cholesterol Concentration on Giant Unilamellar Vesicles Electroformation***. *Springer: Cell BiochemBiophys*, 78:157–164

[18] E. Oropeza-Guzman, M. Ríos-Ramírez, J. Carlos Ruiz-Sua´rez (2019.); ***Leveraging the Coffee Ring Effect for a Defect-Free Electroformation of Giant Unilamellar Vesicles***. *Langmuir*, 35, 16528−16535

[19] Avanti Polar Lipids. Equipement. https://avantilipids.com/divisions/equipment-products

[20] E. Baykal-Caglar, E. Hassan-Zadeh, B. Saremi, J. Huang (2012.); ***Preparation of giant unilamellar vesicles from damp lipid film for better lipid compositional uniformity***. *Elsevier*, 1818, 2598-2604

[21] Reeves, J. P., &Dowben, R. M. (1969); ***Formation and properties of thin-walled phospholipid vesicles***. *Journal of Cellular Physiology*, 73, 49–6

[22] Z. Boban, I. Mardešić, W. K. Subczynski, M, Raguz (2021.); ***Giant Unilamellar Vesicle Electroformation: What to Use, What to Avoid, and How to Quantify the Results***. *Membranes*, 11, 860

[23] Estes, D.J.; Mayer, M. ***Electroformation of giant liposomes from spin-coated films of lipids***. Colloids Surf. B Biointerfaces 2005, 42, 115–123

[24] Herold, C., Chwastek, G., Schwille P., Petrov, E.P (2018.); ***Efficient electroformation of supergiant unilamellar vesicles containing cationic lipids on ITO-coated electrodes***. Langmuir 2012, 28, 5518–5521

[25] T. Pott, H. Bouvrais, P. Meleard (2008.); ***Giant unilamellar vesicle formation under physiologically relevant conditions***. *Elsevier*, 154, 115–119

[26] M. J. Sanderson, I. Smith, I. Parker, M. D. Bootman (2014.); ***Fluorescence Microscopy***. Cold Spring HarbProtoc, 10

[27] Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., &Schmid, B., et al. (2012). ***Fiji: an open-source platform for biological-image analysis***. Nature Methods., 9, 676

[28] R Development Core Team (2008). R: A language and environment for statistical computing. http://www.r-project.org

[29] J. T. Buboltz, G. W. Feigenson (1999.); ***A novel strategy for the preparation of liposomes: rapid solvent exchange***. Biochimica et BiophysicaActa 1417, 232-245