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Posted Date: 22 February 2024

doi: 10.20944/preprints202402.1317.v1

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Article

Electroformation of Giant Unilamellar Vesicles from Damp Lipid Films with a Focus on Vesicles with High Cholesterol Content

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Abstract: Giant unilamellar vesicles (GUVs) are membrane model used to study membrane properties. Electroformation is one of the methods used to produce GUVs. During electroformation protocol dry lipid film is formed. Drying of the lipid film induce Cholesterol (Chol) demixing artifact, in which Chol forms anhydrous crystals, where these crystals does not participate in formation of vesicles. This leads to a lower Chol concentration in the vesicle bilayers compared to the Chol concentration in the initial lipid solution. To address this problem, we propose novel electroformation protocol that includes rapid solvent exchange, plasma cleaning and spin-coating methods to produce GUVs. We tested the protocol focusing on vesicles with high Chol content using different spin-coating durations and vesicle types deposition. Additionally, we compared the novel protocol using completely dry lipid film. The optimal spin-coating duration for vesicles made out of phosphatidylcholine/Chol mixture was 30 s. Multilamellar vesicles (MLVs), large unilamellar vesicles (LUVs) obtained by extrusion of MLVs through 100 nm membrane pores and LUVs obtained by extrusion of previously obtained LUVs through 50 nm membrane pores were deposited on electrode for 1.5/1 Chol/phosphatidylcholine lipid mixture and the results were compared. Electroformation using all three deposited vesicle types gave a high GUVs yield, but deposition of LUVs obtained by extrusion of MLVs through 100 nm membrane pores gave the most reproducible results. Using deposition of these LUVs, we produced GUVs with high yield for 6 different Chol concentrations (from 0 % to 71.4%). Using protocol that includes dry lipid film GUVs yield was lower and Chol demixing artifact was induced, proving that the lipid film should never undergo drying when Chol content is high.

Keywords: GUV; electroformation; cholesterol; damp lipid film; lipid film deposition; cholesterol demixing artifact

1. Introduction

Vesicles are membrane models consisting of one or more lipid bilayers filled with aqueous solution. They are commonly used to investigate membrane properties under controlled conditions [1,2]. Depending on their structure, lipid vesicles can be unilamellar, multilamellar and oligolamellar. Unilamellar vesicles have only a single lipid bilayer, multilamellar vesicles (MLVs) contain multiple lipid bilayers arranged in concentric circles and oligolamellar vesicles contain smaller vesicles within the outer bilayer. With respect to their size, unilamellar vesicles are usually divided into three groups: small (SUVs) (< 100 nm), large (LUVs) (100 nm – 1 µm) and giant (GUVs) (> 1 µm). SUVs and LUVs are the most commonly produced by extrusion and often used for drug delivery studies or in protocols for formation of GUVs [3] or supported lipid bilayers [4]. Amongst these three groups, researchers studying membrane properties and organization are most interested in GUVs due their size similar to eukaryotic cells.

The first attempt to form GUVs was performed by Reeves and Dowben [2]. In their approach, the lipid mixture is deposited on to the electrode and dried to form a dry lipid film that is rehydrated and the aqueous solution is driven between the lipid stacks due to osmotic pressure (gentle hydration). Because of the amphiphilic nature of lipids, it is unfavorable for the hydrophobic acyl chains of lipids to be exposed to the aqueous solution, so the lipid bilayers enclose into vesicles. Even though this method is straightforward and simple, it results in a low GUVs yield with many defects [5].

One of the most commonly used methods for the production of GUVs today is electroformation, which was developed by Angelova and Dimitrov in 1986 [6]. This method improved the previous approach by applying an external electric field to the lipid film in addition to hydration. In their protocol, lipids are dissolved in an organic solvent and deposited on an electrode. The organic solvent is then evaporated using a stream of inert gas and vacuum. The electrode with the lipid film is used to construct an electroformation chamber, which is filled with a desired aqueous solution and connected to an external alternating electric field. The electric field and osmotic pressure promote the detachment of the lipids from the electrode, leading to the formation of GUVs [7]. Compared to samples obtained using the gentle hydration method, the vesicles formed by electroformation have a higher yield, a lower number of defects and a higher proportion of unilamellar vesicles [5].

The electroformation method has evolved significantly since its inception, with various modifications to the protocols tested [8–10] in order to improve the method's reproducibility and enable compatibility with a wider range of lipid mixtures. Some groups attempt to improve the method by replacing the lipids dissolved in an organic solvent with an aqueous solution of SUVs or LUVs [11]. It was concluded that the use of an unilamellar vesicles aqueous solution improved the efficiency of GUVs formation compared to deposition of lipids dissolved in an organic solvent. It was also shown that the formation of GUVs with buffers as internal solutions is easier when using buffer loaded SUVs or LUVs compared to the previous approach where the buffer would be applied to a dry lipid film [11]. Another advantage of this modification is the possibility of improved proteoliposomes formation, due to reduced protein denaturation when the organic solvent is removed from the protocol [11–13].

Another issue with the traditional protocol was the use of the drop-deposition method for lipid film deposition [6,7,9]. The problem with this approach is formation of lipid films of nonuniform thickness resulting in high vesicle heterogeneity in the sample and low experimental reproducibility. Various alternative approaches have been tested to address this issue [14–16]. The one we found to be optimal in terms of ease of use and quality of the final result is the spin-coating technique in which uniform lipid films are obtained by depositing the lipid solution onto a flat surface and spinning it at a high angular velocity [15,17–20].

The initial electroformation protocol also turned out to be incompatible with lipid mixtures containing a high cholesterol (Chol) concentration. This is due to precipitation of Chol into anhydrous Chol crystals during the lipid film drying phase [21]. When the film is rehydrated, these anhydrous Chol crystals do not participate in the bilayer formation, resulting in a lower Chol concentration in the vesicle bilayers compared to the Chol concentration in the initial lipid solution. Chol demixing can be avoided by using the rapid solvent exchange (RSE) method [22,23]. In this method, lipids dissolved in an organic solvent are first mixed with an aqueous solution. By suddenly decreasing the pressure, the organic solvent is evaporated, leaving behind an aqueous solution of MLVs. In order to avoid Chol demixing artifact during GUVs preparation, Baykal-Caglar et al. tested electroformation from a damp lipid film formed by depositing MLVs, produced by RSE method, onto the electrode [3]. The final results were positive, indicating a higher Chol content in formed GUVs, compared to GUVs obtained using the original protocol. However, the described protocol is very long due to the long lipid film drying (22 - 25 h), and the MLVs were deposited using the drop-deposition method, inevitably resulting in a non-uniform film.

In addition to the deposition techniques and the properties of the lipid mixtures, the cleanliness of the electrodes has also been shown to have an influence on the electroformation results. Pretreatment of the electrodes with plasma improved the efficiency of the formation of GUVs

containing buffers with physiological charge levels. This is probably due to the fact that the plasma makes the electrode surface more hydrophilic, facilitating the hydration of the lipid film and the subsequent formation of the lipid bilayers [24].

In our previous study, we adapted the traditional electroformation protocol to incorporate all of the aforementioned improvements, allowing us to bypass the dry lipid film phase and produce a uniform damp lipid film (Figure 1) [25]. Inspired by the vesicle fusion method for the preparation of supported lipid bilayers [26–28], the adapted protocol uses spin-coating to deposit an aqueous solution of LUVs onto a plasma treated electrode. The hydrophilized surface induces LUV rupturing, and the formation of lipid bilayers on the electrode surface. The bilayers later detach and form GUVs under the influence of osmotic pressure and an alternating electric field. With respect to the approach of Baykal-Caglar et al., these modifications significantly shorten the duration of the protocol while also increasing the experiment reproducibility. In order to make the protocol compatible with high Chol concentrations, the deposited LUVs were obtained by extruding a solution of MLVs produced by RSE method, and the lipid film was not dry after spin-coating.

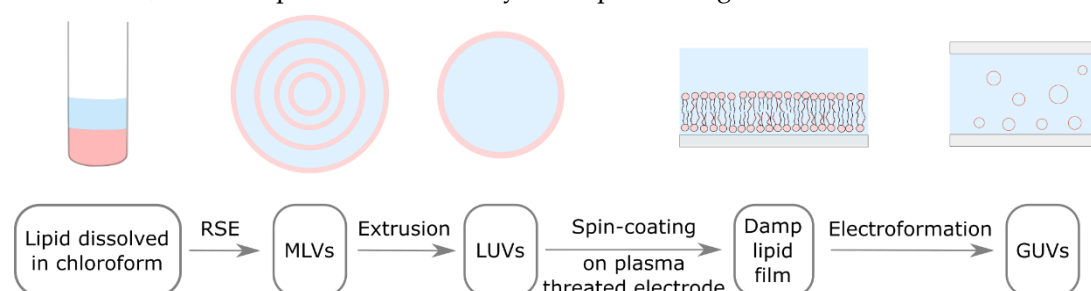


Figure 1. Adapted electroformation protocol. Lipids dissolved in organic solvent (pink) are mixed with aqueous solution (light blue). MLVs are formed using RSE method. MLV solution is extruded through filter with membrane pores in order to obtain LUVs. LUV solution is spin-coated on plasma treated electrode, where these vesicle rupture and form damp lipid film. Electrode with damp lipid film is used to build electroformation chamber, where lipid bilayers detach and form GUVs under the influence of osmotic pressure and an alternating electric field.

The efficiency of the protocol was tested using 1/1 Chol/1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) and 1/1/1 Chol/POPC/sphingomyelin lipid mixtures. Compared to the protocol involving a drying step, the new protocol resulted in a similar or better GUV's yield, while having the potential to significantly reduce the Chol demixing artifact [25]. In order to further increase the reproducibility and yield of the obtained samples, this study will focus on further optimization of the new protocol.

Special focus is placed on GUVs with a high Chol content for which the Chol demixing issue is more pronounced. Within the lipid bilayer Chol interacts with phospholipids and forms different domains depending on its membrane concentration. For vesicles with a Chol content lower than ~15 mol%, the lipid bilayer is in a liquid disordered phase. At 30 – 50 mol% it is in a liquid ordered phase. Cholesterol bilayer domains (CBDs) containing only Chol molecules with no phospholipids start forming when the Chol content is greater than 50 mol%. After reaching the Chol solubility limit at ~66 mol%, excess Chol precipitates in the form of Chol crystals [29].

Modeling membranes with such a high Chol concentration is important for groups such as ours that study the properties of the eye lens plasma membranes [21,30,31] or for groups performing atherosclerosis research [32]. The protocol may also be beneficial for the formation of proteoliposomes by reducing the denaturation of proteins that occurs during the preparation of GUVs from lipids dissolved in an organic solvent or during film drying.

2.1. Materials

1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) and Chol were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA), while the fluorescent dye 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine Perchlorate (DiI_{C18}(3)) was acquired from Invitrogen, Thermo Fisher

Scientific (Waltham, MA, USA). Lipids were stored at -20°C when not in use. ITO-coated glass (ICG-90 INS 115, resistance 70–100 Ω) measuring $25 \times 75 \times 1.1$ mm was procured from Delta Technologies (Loveland, LO, USA). Fresh ITO-coated glass was employed for each preparation to ensure efficient formation of GUVs [33]. Mili-Q deionized water (Merck, Rahway, NJ, USA), preheated to 60°C , served as the internal chamber solution.

2.2. Preparation of Multilamellar Vesicles Using the Rapid Solvent Exchange Method

Initially, multilamellar vesicles (MLVs) were generated using a home built RSE device to circumvent the cholesterol demixing issue. A lipid mixture dissolved in chloroform was prepared by blending 25 mg/ml of POPC, 20 mg/ml of cholesterol (Chol), and 1 mg/ml of DiIC₁₈(3) in appropriate proportions. The Chol/POPC mixing ratios were maintained between 0 and 2.5 (Chol mixing concentration of 0% to 71.4%), while the DiIC₁₈(3)/POPC mixing ratio was fixed at 1/500. The total lipid mass amounted to 2.1 mg. Subsequently, 400 μL of Mili-Q water was introduced into the solution, and the mixture was vortexed (Vortex IR, Star Lab, Blakelands, UK) at an angular velocity of 2200 rpm. Throughout the vortexing process, the pressure was regulated to approximately 0.05 bar using a vacuum pump (HiScroll 6, Pfeiffer Vacuum, Asslar, Germany). The sample was maintained at the specified pressure for 90 seconds to ensure complete removal of residual organic solvent.

2.3. Preparation of Large Unilamellar Vesicles

The MLVs solution was extruded utilizing an Avanti Mini Extruder (Avanti Polar Lipids, Inc, Alabaster, AL.), passing through either a 50 or a 100 nm diameter pores of polycarbonate filters (Nuclepore Track-Etch Membrane, Whatman, UK) 15 times to achieve a uniform LUV suspension. To prevent loss of the lipid suspension, filters and membranes were pre-wetted with Mili-Q water. Additional Mili-Q water was added to the LUV solution to adjust a lipid concentration of 3.5 mg/ml.

2.4. Preparation of the Damp Lipid Film

Prior to conducting the experiments, the ITO-coated glass was submerged in Mili-Q deionized water. Subsequently, the glass was wiped using lint-free cloths saturated with 70% ethanol. Following this, the glass underwent treatment with oxygen plasma for a duration of 1 minute utilizing a plasma cleaner (PDC-002- HPCE with the PLASMAFLO PDC-FMG -2 attachment, Harrick Plasma, Ithaca, NY, USA) connected to a vacuum pump (HiScroll 6, Pfeiffer Vacuum, Assler, Germany).

Unless specified otherwise, a volume of 550 μL of LUV suspension was applied onto the hydrophilic plasma-treated ITO-coated glass electrode and promptly spin-coated using a spin-coater (SM -150, Sawatec, Sax, Switzerland). The electrode was spun at 600 rpm, achieving the final speed within 1 second. The duration of spin-coating, unless specified otherwise, was maintained at 30 seconds to guarantee the formation of a damp, uniform lipid film. To prevent unintended evaporation, the coated ITO-coated glass was transferred into a Petri dish and promptly utilized to assemble an electroformation chamber.

2.5. Electroformation Protocol

The electroformation chamber was constructed by sandwiching two 25×37.5 mm ITO-coated glasses with a 1.6 mm thick Teflon spacer in between. Electrodes were made by slicing a 25×75 mm ITO-coated glass using a diamond pen cutter. To form the electroformation chamber, the lipid-coated glass was sealed to the Teflon spacer with vacuum grease. Following the addition of 280 μL of Mili-Q water, the stopper was sealed using vacuum grease, ensuring no contact between grease and water to prevent any detrimental effects of grease contamination on GUV formation [9]. The chamber was secured with clamps at three points along the electrodes, two adjacent to the stopper and one opposite. Subsequently, the chamber was connected to a pulse generator (UTG9005C, UNI - T, Dongguan City, China or PSG 9080, Joy- IT, Neukirchen-Vluyn, Germany) and placed in an incubator set to a temperature of 60°C . Copper tape was applied to the outer edges of the electrode to enhance

wire-electrode contact. Consistent with previous experiments [17,18,25], a voltage of 2 V and a frequency of 10 Hz were maintained. The pulse generator after 2 hours was turned off, and the chamber remained in the incubator for an additional hour.

2.6. Dynamic light scattering

Dynamic light scattering (DLS) was utilized to determine the hydrodynamic diameter and polydispersity index of liposome suspensions (Litesizer 500, Anton Paar, Graz, Austria).

2.7. Fluorescence Imaging and Data Analysis

To cover the entire volume of the chamber, images were captured from 16 different regions of the sample. One hundred vesicles were then randomly selected from these images. In cases where the images did not encompass 100 vesicles, all observed vesicles were included in the analysis. Imaging was conducted utilizing a fluorescence microscope (Olympus BX51, Olympus, Tokyo, Japan), and vesicle diameters were measured using the line tool within the Fiji software [34].

Unless specifically indicated otherwise, numerical results are presented as mean \pm standard deviation. All data analysis and visualization were carried out using the R programming language [35].

3. Results

In order to further improve our newly developed protocol, we performed experiments to test the effect of using different vesicle types during lipid film deposition, the effect of spin-coating duration, and the effect of different Chol concentrations. To confirm the utility of the optimized protocol, our samples were also compared to those obtained from fully dried lipid films.

3.1. Optimization of the protocol

3.1.1. The spin-coating duration for formation of damp lipid film

We tested various coating durations ranging from 15 s to 60 s at an angular speed of 600 rpm. The deposited liquid was an aqueous suspension of LUVs obtained by extruding MLVs through a 100 nm pore diameter filter. We concluded that 30 s is the optimal duration when using the aforementioned lipid mixtures. A shorter duration of spin-coating resulted in an uneven film thickness or a film that was too wet, leading to low reproducibility of the experiment and high GUV heterogeneity within the successful samples. As shown in our previous study, for a spin-coating duration of 30 s, the lipid film remained damp, with high GUV electroformation yield [25]. Longer spin-coating durations were avoided due to the risk of excessive drying of the lipid film and inducing the Chol demixing artifact. To prevent additional drying of the film due to water evaporation, the electrode was immediately used to build an electroformation chamber.

3.1.2. Comparison of the MLVs and LUVs Deposition on the Electrode

Since the film in our protocol is kept damp, we rely on the vesicles to rupture when they come into contact with a hydrophilized electrode surface to obtain a lipid film [26,36,37]. However, how easily the vesicles rupture depends strongly on the vesicle type and size [38]. Therefore, we compared the GUVs prepared from lipid films obtained with three different types of vesicles - MLVs formed by RSE method, LUVs obtained by extrusion of MLVs through 100 nm membrane pores (LUV 100), and LUVs formed by additional extrusion of LUV 100 vesicles through 50 nm membrane pores (LUV 50). The Chol/POPC mixing ratio was maintained at 1.5 in all these experiments. DLS was used to determine the size distribution of the suspensions of each vesicle type. The average hydrodynamic diameter and polydispersity index were 761.1 nm and 0.29 for MLVs, 141.9 nm and 10.4 for LUV 100 vesicles, and 96.2 nm and 11.9 for LUV 50 vesicles.

Deposition of the lipid film from all three vesicle types resulted in a high GUV yield (Figure 2a), with average diameters and standard deviations when using MLVs, LUV 50 and LUV 100 vesicles

being $28.5 \pm 3.71 \mu\text{m}$, $33.9 \pm 5.27 \mu\text{m}$ and $24.5 \pm 1.39 \mu\text{m}$, respectively (Figure 2b). Although the average diameter of GUVs obtained from LUV 100 vesicles depositions was the smallest, this method resulted in the highest reproducibility among samples (smallest error bars in Figure 2b). In addition, the widths of sample diameter distributions were also smallest for this method, indicating the highest uniformity of GUV size compared to the other two protocols (Figure 2c). The advantage of depositing MLVs instead of LUVs is the shorter protocol duration, as the conversion to LUVs is skipped. However, samples made from MLV deposits displayed lower reproducibility (Figure 2b) and a more heterogeneous GUV size distribution (Figure 2c). This could be due to a larger average diameter and polydispersity index of MLVs compared to LUVs, as smaller vesicles rupture more easily compared to larger ones. The average diameter of GUVs was largest when LUV 50 vesicles were used. Since smaller vesicles rupture more easily, we expected reproducibility to be highest with LUV 50 vesicles, but this was not the case (Figure 2c). This is probably a side effect of the additional extrusion compared to LUV 100 vesicles. We tried to produce LUV 50 vesicles directly from MLVs, but the flow resistance was too big, especially at higher Chol concentrations. Consequently, we first produced LUV 100 vesicles, and then passed them through a 50 nm filter, as LUV 100 vesicles offered less resistance compared to the MLVs. Due to additional extrusion the duration of the protocol was even longer and causes a greater loss of final suspension volume, as part of the lipid suspension is always lost during the extrusion process due to fluid leakage. Consequently, the GUV distributions we obtained after deposition of such suspensions were less reproducible (Figure 2 b, c), as a different total amount of lipids was deposited on the electrode. We also tried extruding the vesicles through a 30 nm filter, but this only exacerbated the problem of high resistance, so we decided to exclude such suspensions from the comparison.

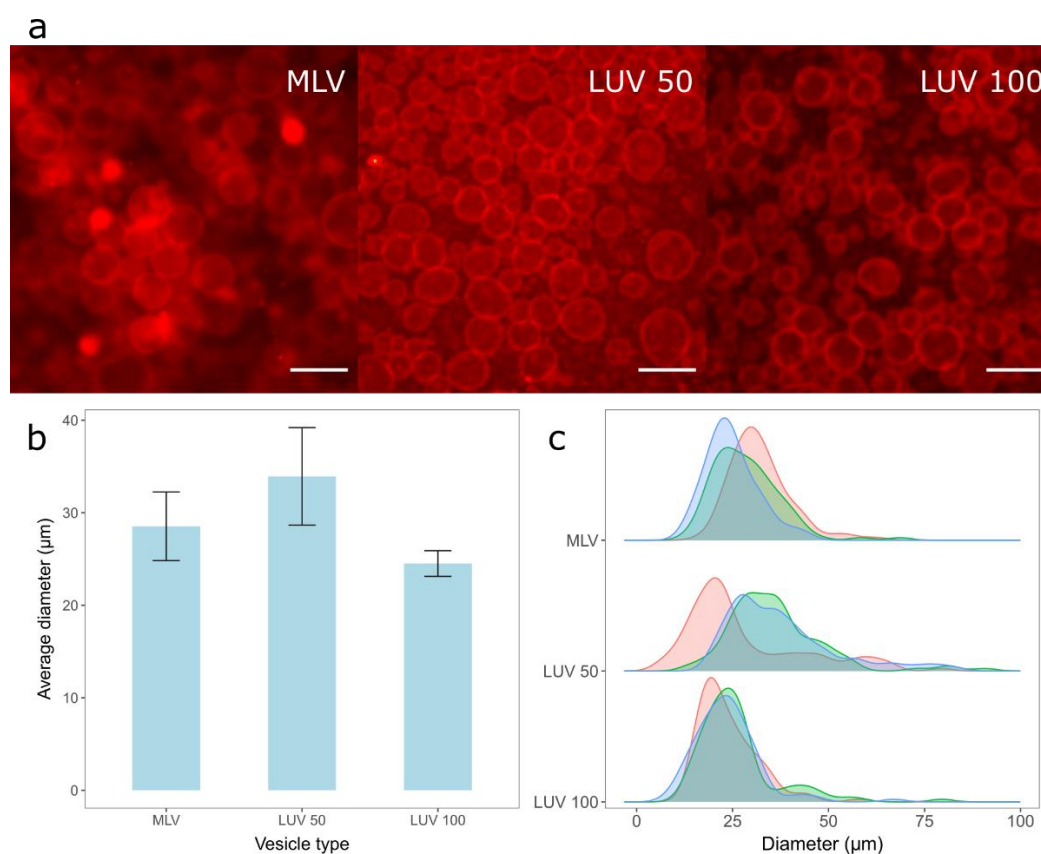


Figure 2. Electroformation of GUVs for deposition of different vesicle types. (a) Fluorescence microscopic images of GUVs formed when different vesicle types were deposited. MLV represents the deposition of MLVs on the electrode, LUV 100 represents the deposition of LUVs formed by extruding MLVs through 100 nm membrane pores, and LUV 50 represents the deposition of LUVs formed by extruding LUV 100 vesicles through 50 nm membrane pores. The scale bar represents 50 μm . (b) Comparison of the average diameter and standard deviation. Each distribution represents

300 randomly selected vesicles from three independent samples (100 vesicles per sample). (c) Size distribution densities of GUVs for deposition of different vesicle types for each sample. Each distribution density represents one independent sample (100 vesicles).

3.2. The Effect of Chol Content

After deciding on the duration of spin-coating and the vesicle type to be used for deposition, we tested the effect of Chol content on GUV electroformation. The mean diameter and standard errors of the formed GUVs were $45.5 \pm 5 \mu\text{m}$, $41.13 \pm 1.68 \mu\text{m}$, $32.67 \pm 1.28 \mu\text{m}$, $24.51 \pm 0.8 \mu\text{m}$, $29.18 \pm 0.08 \mu\text{m}$, and $26.97 \pm 2.95 \mu\text{m}$, respectively for Chol/POPC mixing ratio between 0 and 2.5 (Figure 3a). As expected, the distribution of vesicle diameters shifted to lower values with increasing Chol content (Figure 3b) [18]. That could be due to decreasing membrane elasticity and increasing rigidity when more Chol is added, making vesicle formation more difficult. For Chol/POPC mixing ratios greater than 1.5 (more than 60% of Chol in the mixture), where the GUVs may contain CBDs, a significant decrease in the average GUV diameter is measured. It is about 40% lower compared to the pure POPC bilayer.

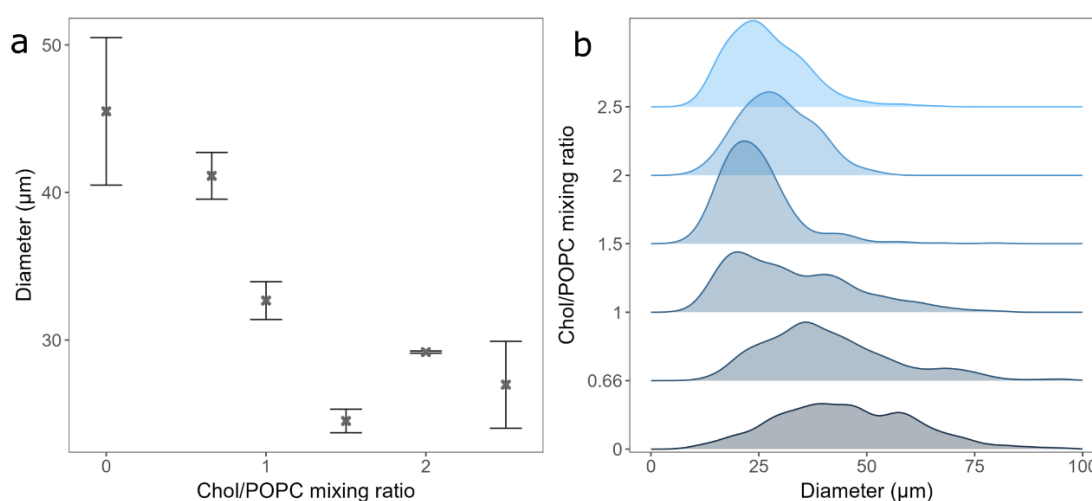


Figure 3. (a) GUVs mean diameters for different Chol/POPC mixing ratios. Points and bars represent mean values and their standard errors. (b) Size distribution densities of GUVs for different Chol/POPC mixing ratios. All experiments were performed using deposition of LUV 100 vesicles spin-coated onto electrode for 30 s.

For Chol/POPC mixing ratios of 2 and lower, we found only a couple of small anhydrous Chol crystals per sample. This is probably due to small dry patches forming on the electrode during spin-coating. However, compared to electroformation samples made using a completely dry lipid film, this amount was negligible.

3.3. Comparison with Samples Obtained from Completely Dry Lipid Films

Finally, the samples prepared with the optimized protocol were compared with those obtained from fully dried lipid films. These additional samples were made by spin-coating the aqueous lipid suspension for either 30 s or 240 s and then placing the coated electrodes in a desiccator for 30 min to ensure that any residual water was removed from the sample (Figure 4a). Drying not only increased the amount of Chol crystals, but also decreased the GUVs yield (Figure 4b, c). At a low Chol content, a negligible amount of GUVs was found in the samples. For the high Chol content, the GUVs yield was much lower compared to the equivalent damp lipid film scenario, with many defects and anhydrous Chol crystals.

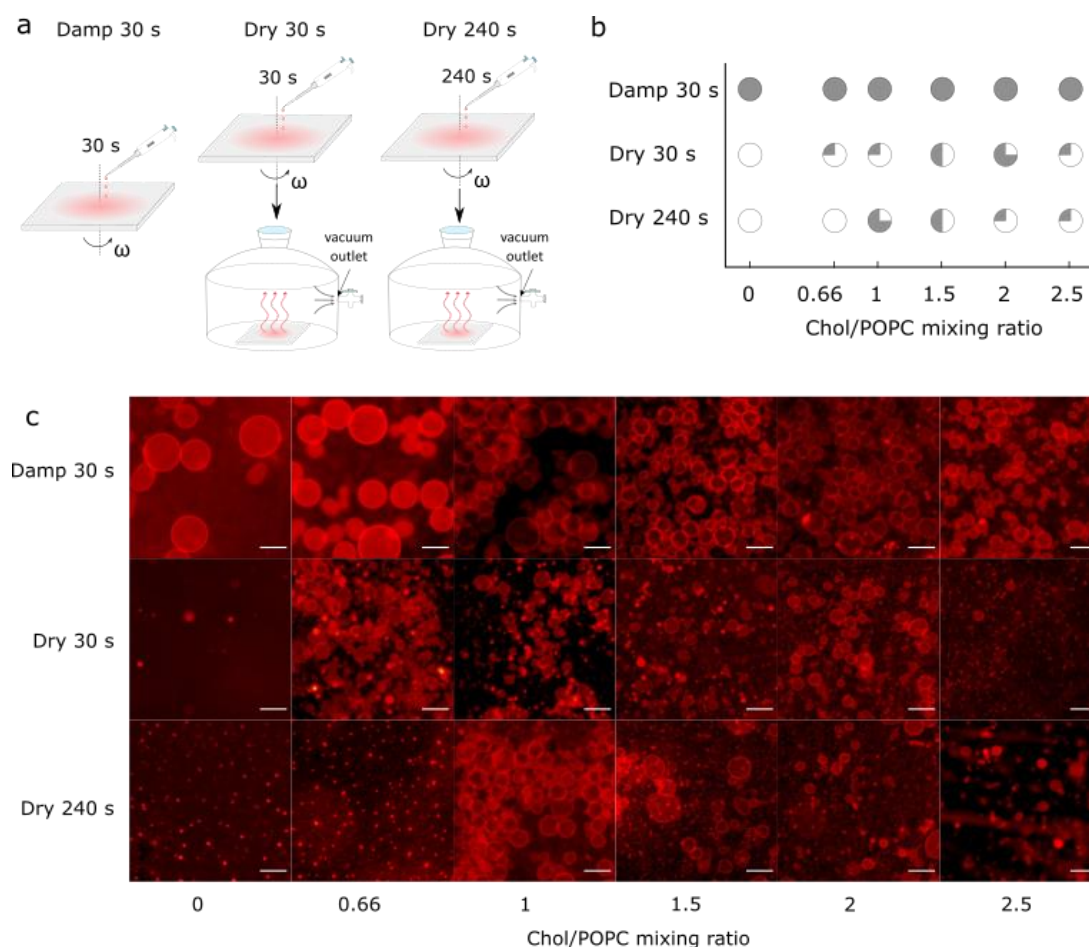


Figure 4. (a) Schematic depiction of differences in our novel protocol for three different experiments. In the first experiment damp lipid film was formed by depositing LUV 100 vesicles on electrode and spinning it for 30 s at 600 rpm angular velocity (Damp 30 s). In second experiment, Damp 30 s lipid film was put under vacuum for 30 min (Dry 30 s). In third experiment instead of spinning for 30 s, electrode was spun for 240 s, and afterwards put under vacuum for 30 min (Dry 240 s). (b) Electroformation successfulness using Damp 30 s, Dry 30 s and Dry 240 s protocols, for obtaining lipid films, depending on different Chol/POPC mixing ratios. Successfulness takes into account the population homogeneity, yield and number of defects. It is displayed through circle fullness, where a fuller circle indicates better successfulness. Empty circles denote that no GUVs were formed or their number was negligible. (c) Fluorescence microscopy images of GUVs for the different Chol/POPC mixing ratios using three different approaches for obtaining lipid films. The scale bar represents 50 μm .

5. Conclusions

In this study, we optimized an improved electroformation protocol that bypasses the dry lipid film phase of the traditional electroformation method and combines the RSE method, the plasma cleaning, and spin-coating techniques to obtain a damp lipid film. To optimize the protocol further, we conducted additional experiments to test the effect of using different vesicle types during lipid film deposition, the effect of spin-coating duration, and the effect of different Chol concentrations. In order to confirm the utility of the optimized protocol, our samples were also compared to those obtained from fully dried lipid films.

Spin-coating duration of 30 s was found to be optimal in terms of balance between electroformation successfulness and lipid film dampness. A longer duration of spin-coating would dry out the lipid film and exacerbate the Chol demixing artifact. Compared to previous protocols that used damp lipid films, such as that of Baykal-Caglar et al, our method significantly shortened the

preparation time by eliminating the 22-25 hour high-humidity drying phase and replacing it with 30 seconds of spin-coating [3].

In terms of vesicle type used for film deposition, the LUV 100 vesicles gave the best results in terms of reproducibility, even though the average diameter was smaller compared to the MLVs and LUV 50 vesicles. The advantage of depositing MLVs instead of LUVs is the shorter protocol duration. However, samples made from MLV deposits displayed lower reproducibility and a more heterogeneous GUV size distribution. This is probably due to a larger average diameter and polydispersity index of MLVs compared to LUVs, as smaller vesicles rupture more easily compared to larger ones. The average diameter of GUVs was largest when LUV 50 vesicles were used, but the reproducibility of the samples was lowest. This is probably a side effect of additional extrusion performed compared to LUV 100 vesicles. Due to high flow resistance through a 50 nm filter, we could not extrude MLVs directly. We first produced LUV 100 vesicles and then passed them through a 50 nm filter. The additional extrusion step extends the duration of the protocol even further and causes a greater loss of final suspension volume, as part of the lipid suspension is always lost through liquid leakage during the extrusion process. This problem was even greater in our case as the resistance increased with increasing Chol/POPC mixing ratio.

Finally, our samples were also compared with those obtained from fully dried lipid films. Drying increased Chol demixing artifact, where higher amount of Chol crystals were found, which proves that the lipid film should never go through a drying phase when using mixtures with a high Chol content.

This protocol allows us to successfully prepare GUVs and study the physical properties, lateral organization and domain function of lipid membranes with a very high Chol content, such as the membranes that resemble composition of the fiber cell plasma membrane of the eye lens. Furthermore, the avoidance of organic solutions and plasma cleaning of the electrode have been shown to be advantageous for the preparation of GUVs containing charged lipids and buffer solutions. Consequently, we believe that our protocol might prove successful in those cases as well. Additionally, the protocol could also be adapted for protein-membrane interaction studies because protein denaturation is reduced by avoiding the dry film phase and the absence of organic solvents [11–13].

Author Contributions: Conceptualization, I.M., M.R.; methodology, I.M., Z.B.; software, I.M., Z.B.; validation, I.M., Z.B.; formal analysis, I.M., Z.B.; investigation, I.M., Z.B.; resources, M.R.; data curation, I.M., Z.B.; writing—original draft preparation, I.M.; writing—review and editing, I.M., Z.B., M.R.; visualization, I.M., Z.B.; supervision, M.R.; project administration, M.R.; funding acquisition, M.R. All authors have read and agreed to the published version of the manuscript.

Funding: Research reported in this publication was supported by the Croatian Science Foundation (Croatia) under Grant IP-2019-04-1958.

Institutional Review Board Statement: Not applicable.

Data Availability Statement: The data presented in this study are available upon reasonable request from the corresponding author.

Acknowledgments: We thank Ante Bilušić and Lucija Krce for access to their lab equipment.

Conflicts of Interest: The authors declare no conflicts of interest.

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