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Preparation and characterization of non-equilibrium giant unilamellar vesicles.

Preparació i caracterització de vesícules unilamelars gegants fora de l'equilibri.

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"The scientist is not a person who gives the right answers, he's one who asks the right questions."

Claude Lévi-Strauss

Thanks to science. It showed me how to be critic, analytic and patient. It forced me to doubt, think beyond and anticipate facts. Try, fail, and try again. Regardless where I end personal and professional-wise, I will always be a scientist and I will always apply science to my daily life.

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REPORT

CONTENTS

1. SUMMARY

Vesicles are spherical-shaped structures formed by the self-assembly of lipids bilayers. Several types of vesicles have been described during the past years due to its potential scientific interest. Specifically, giant unilamellar vesicles or GUVs, have been deeply studied because of its similarity with cell membranes and also they have been used as a model system to study a broad number of features. Thanks to their big size and volume in comparison with other type of vesicles, GUVs attract the interest of many scientific fields such as soft matter physics, chemistry and biotechnology[1]. This last one is probably the most relevant as, in medical fields, GUVs are proposed to be drug vehicles and an important part inside drug-delivering mechanisms. GUVs can encapsule active molecules and carry them to the site of interest but their fragility and short live are clear downsides that are being investigated.

To be as accurate as possible and build a system close to real cell membranes, more than one lipid is further used to form GUVS. This aspect is extremely interesting because of the behavior of this lipids when they are altogether and when temperature changes. A segregation of two phases, also known as lipid raft, is expected and can be distinguished using fluorescence microscopy.

Hence, in this project the main goal is the pursuit of a robust protocol to form big and stable GUVs and to observe how lipids dispose in different conditions.

Keywords: Vesicles, lipid bilayer, cell membranes, unilamellar, fluorescence microscopy, lipid raft.

2. RESUM

Les vesícules són estructures esfèriques formades per auto-assemblatge de bicapes lipídiques. Diferents tipus de vesícules han sigut descrites durant els anys a causa del seu potencial i de l'interès que creen en molts camps científics. Concretament, les vesícules unilamelars gegants o GUVs, han sigut profundament estudiades per la seva semblança amb les membranes cel·lulars i també han sigut utilitzades com a model per l'estudi d'un gran nombre de propietats. Gràcies a la seu gran mida i volum en relació a altres tipus de vesícules, les GUV atrauen l'interès de molts camps dins de la ciència com poden ser la física de la matèria condensada, la química i la biotecnologia[1]. És aquesta última la que, probablement, sigui més rellevant ja que, en el camp mèdic, les GUVs es proposen com a vehicle per medicaments i com una part important del mecanisme de conducció d'aquests medicaments a zones concretes. Les GUVs poden contenir dins seu molècules actives i les poden portar fins al lloc d'interès però, la seva fragilitat i curta vida són un factor negatiu que està sent investigat.

Per assegurar que es crea un sistema realment semblant a les membranes cel·lulars, les GUVs han d'estar formades per més d'un lípid. Aquest aspecte és molt interessant degut al comportament dels lípids quan estan junts sota diferents temperatures. Es pot observar una segregació en dues fases, també conegut com dominis lipídics, i s'espera que siguin distingibles amb un microscopi de fluorescència.

Així, el principal objectiu d'aquest projecte és trobar un protocol robust per formar GUVS grans i estables i a més, observar com els dominis lipídics es disposen en diferents condicions.

Keywords: Vesícules, bicapa lipídica, membrana cel·lular, unilamelar, microscopi de fluorescència, domini lipídic.

3. INTRODUCTION

3.1. PHOSPHOLIPIDS

Phospholipids are the fundamental building component of cell membranes. They are a type of lipid composed of a phosphate group, one alcohol and two fatty acids. The head of the molecule has a polar character due to the presence of the phosphate group. This part is hydrophilic because the electric charge in the phosphate group attracts water molecules.[2]

The fatty acids are placed in the tail of the phospholipid. They have a neutral character which means that water molecules will not be attracted to them (nonpolar tail). Thus, within a phospholipid, hydrophobic and hydrophilic parts coexist. This coexistence is known as amphipathic behavior or character of a certain molecule. Figure 1 shows a schematic representation of a phospholipid

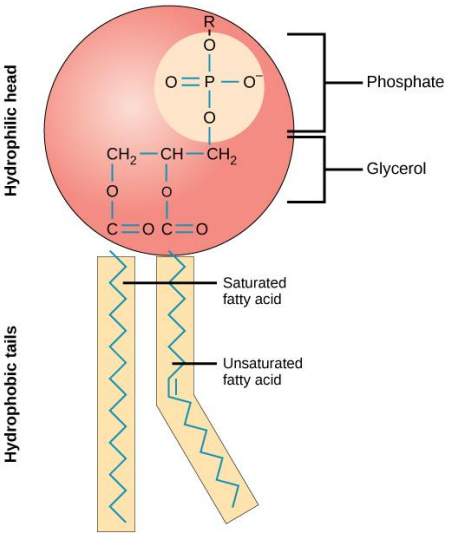

Figure 1. General structure of a phospholipid (OpenStax, 11/05/2021 via Wikimedia Commons, Creative Commons Attribution).

3.2. LIPOSOMES

Liposomes are small natural vesicles spherical-shaped consisting of one or more phospholipid bilayers (Figure 2). They are a simplified model of a cell membrane with neither chemical ornaments nor high stability. Thanks to its similarity to cells, a wide range of scientific fields are focusing their research on these types of vesicles, from soft matter physics, biophysics and chemistry to nanotechnology and medicine[1].

Liposome membranes are composed of a mixture of amphipathic lipids arranged forming layers that are also known as lipid bilayer or lamellae. Based on different features several types of vesicles can be found. Here some of them will be exposed.

Regarding its structure and number of bilayers, vesicles can be classified in multilamellar vesicles (MLV) and unilamellar vesicles (UV). The multilamellar vesicles have two or more phospholipid bilayer normally in an onion-shape distribution. On the other hand, unilamellar vesicles consist in a singular phospholipid bilayer spherical-shaped that encloses an aqueous solution. It is normal to find some unilamellar vesicles inside a big vesicle forming a multilamellar vesicle.[1]

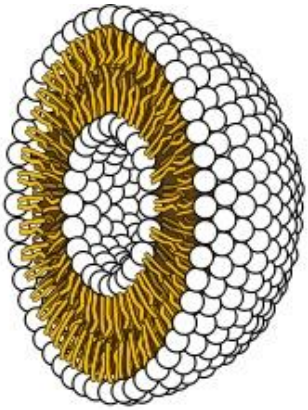

Figure 2. Disposition of phospholipid in a liposome (OpenStax, 11/05/2021 via Wikimedia Commons, Creative Commons Attribution).

In this work, unilamellar vesicles are the object of study. Regarding the size of the unilamellar vesicles, they can be classified in three groups[2]:

Small unilamellar vesicles (SUV) from 20 to 100 nm, Large unilamellar vesicles (LUV) from 100 to 1000 nm and Giant unilamellar vesicles (GUV) from 1 to 200 μm.

The object of interest in this study are Giant unilamellar vesicles due to its structural simplicity and the similarity to cells in terms of shape, mechanical, physical and chemical properties. They are widely used as cell membrane models and as a drug delivery carriers[3].

3.3. TERNARY LIPID MIXTURE

The lipid composition of the vesicles is variable and goes from one single lipid forming the vesicle to a lipid mixture. In this study, a ternary lipid mixture will be used to form the vesicles. Normally, vesicles with three components consist of one saturated lipid (with a high melting temperature), one unsaturated lipid (with low melting temperature) and cholesterol[4].

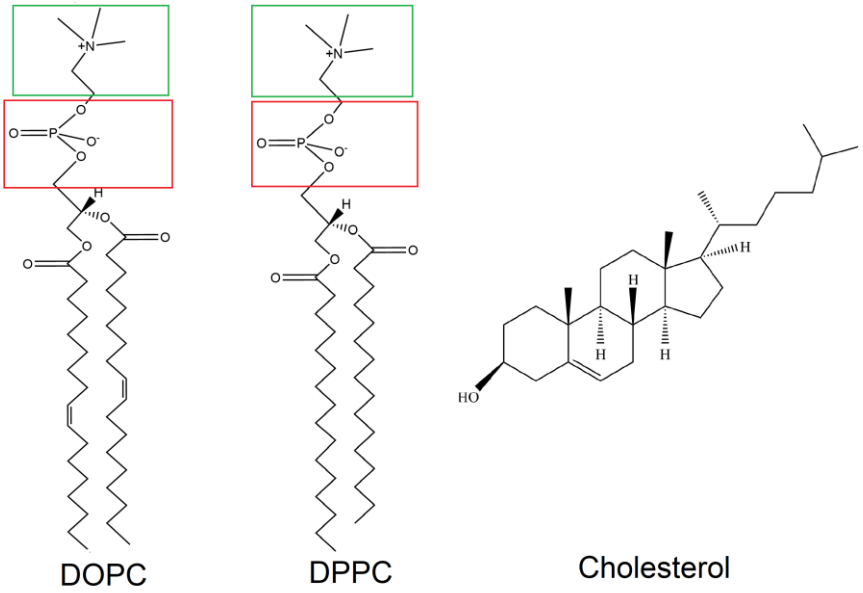

Figure 3. Structure of the three lipids used. In green, the choline group with positive charge. In red, the phosphate group with negative charge. Long chain represents the nonpolar part of the lipid.

Specifically for this study, the lipids used will be DOPC as the unsaturated lipid (low melting temperature), the DPPC as the saturated lipid (high melting temperature) and cholesterol (Figure 3). Combining different lipids one can increase the fluidity of the membrane and thus, its stability and durability as the rigidity of the structure decreases allowing the shape to adapt and vary.

It has been reported that the lipid mixture used in this work is the one that works better for the formation of vesicles with two phases[4].

3.4. LIPIDIC THERMODYNAMICS

In this section, the thermodynamic principles behind the disposition of lipids within vesicles are reviewed so to understand the mechanism of formation.

The following aspects and considerations are extracted from Shingeyuki Komura et al work[6]: The first systems studied were formed by saturated and unsaturated lipid binary mixtures and lipid-cholesterol mixtures. In these models, the presence of an unsaturated lipid shifts the gel transition temperature of the saturated lipid. Also, cholesterol affects the chain melting transition. These two aspects allow the formation of a phase diagram that shows the area of temperature and composition that exhibit the coexistence of several phases (Figure 4). It is believed that this coexistence is more interesting when both phases are liquid than when the coexistence is gel-liquid[7]. The best example is the liquid-liquid coexistence found in liquid-ordered and liquid-disordered disposition.

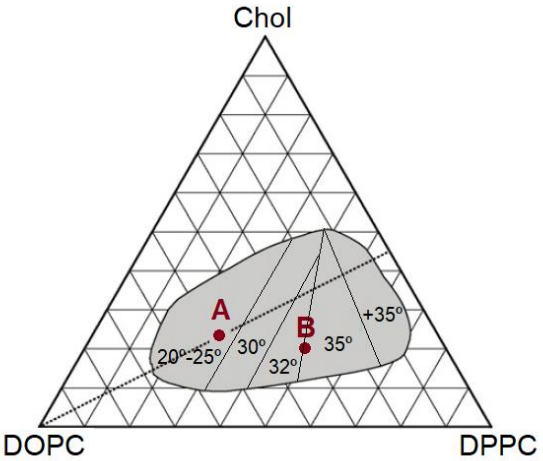

Figure 4. Ternary mixture diagram where grey region is the zone of coexistence of phases with schematic reference of the temperature ranges that cover the phase coexistence.

An important point to address at this stage is the phase transition temperature of lipids. This transition temperature, also known as melting temperature (T_m) is the point where a lipid changes from gel phase to liquid phase. This melting point is crucial for biological membrane as determines the stability and thus, the life of the cellular membrane.

This transition temperature comes along with some volume and enthalpy changes that allow a thermodynamical analysis to build relations in different systems and conditions[8].

In this study, a deep thermodynamical analysis is not carried out because it becomes not trivial. For the simple systems formed by two phospholipids it is accessible to understand using basic thermodynamic variables as Gibbs free energy change, mixing entropy and an interaction parameter as it is presented by Paulo F.F. Almeida et al[7]. As it is not the principal topic of this work, the lector is encouraged to check the bibliography. For the ternary systems, the thermodynamical analysis is slightly more complicated due to the presence of several parameters of interaction between lipids, and even more, if this third lipid is Cholesterol which can have a big impact in the geometry and features of the vesicle.

Nevertheless, melting temperature plays an important role when it comes to define the objectives of this work, as the stability of the vesicles and the coexistence of phases is examined.

It is worth to mention at this stage of the study that for the lipids used in the ternary mixture explained in Section 3.3, the determinant temperature is related with the composition of the membrane. Assuming that the mixture is represented by DOPC/DPPC/Chol, if the composition is 1:1:1, the T_m is 29 ºC, for 2:1:1 it is 30ºC and for 1:2:1 it is 34ºC, being 41ºC the highest T_m supported by the sample[4].

3.5. LIPID RAFTS

Against what is expected, the lipids are not uniformly distributed all around the membrane surface. In this section, a general view is presented. Membranes formed by a mixture of lipids can show a coexistence of two liquid phases. These are commonly known as liquid-ordered (L_o) and liquid-disordered (L_d) phases. It is a common phenomenon when two long chain phospholipids, one saturated and one unsaturated, are mixed in presence of cholesterol. Cholesterol is a critical component due to its effect in terms of stability of the membrane[5].

The coexistence of phases appears at a wide range of compositions and temperatures but, at a given temperatures, the acyl chains of the lipids in the L_o phase are more ordered than the acyl chains of the lipids in the L_d phase.

A prove that a membrane is not in a thermodynamical equilibrium is the irreversible way towards the death of a cell. Taking all this into consideration, it is normal that the proportion and composition of every lipid within a vesicle (as mentioned before, a simple and valid model of cell membranes) is not uniform and has this well-known domains or rafts. Thus, these rafts are based in the intern interaction between the lipids that form the membrane, what is to say, they look for the liked and disliked components throughout the membrane to stay with.

3.6. POLYMERSOMES

Nowadays, other structures similar to GUVs are studied to cover the same applications (mainly drug-delivering) that vesicles do. As will be explained further on, vesicles have some downsides in terms of formation and manipulation. These downsides can be overcame with polymersomes.

Differing from vesicles, that are formed by a double phospholipid layer, polymersomes consist of two layers of synthetic polymers. Indeed, these synthetic polymers and phospholipids have similar chemical features and behavior, being length the most significant difference between them. Synthetic polymers have a considerable larger size than lipids, thus polymersomes have bigger membrane thickness[9].

4. OBJECTIVES

The main scope of this study is to observe the reversible transition between phases in Giant unilamellar vesicles (GUV) under temperature control and the relation, if existing, between both. For this, two phases need to be clearly observable, vesicles have to be over a certain size, and a relation between these two aspects and the temperature has to be obtained.

The study is mainly experimental with some individual objectives enclosed:

To prepare ternary vesicles of a mixture DOPC/DPPC/Cholesterol using the electroformation method and try to optimize it.

To observe and characterize both liquid-ordered and liquid-disordered phases through fluorescence microscopy.

To try different compositions and temperatures to cover the most of the ternary mixture diagram.

To plot a direct relation between temperature and phase coexistence and prove the reversibility of the process.

After consuming most of the time that was expected to be invested in the experimental part of the study, the objectives need to be reassessed because of the impossibility to clearly see the phase transition. All the inconveniences are further discussed in Section 6.

Thus, the study is then reorientated to understand the electroswelling method and optimize it according with the technologies present in the laboratory. A robust procedure to form Giant unilamellar vesicles with low polydispersity is pursuit as well as to understand how different variables affect the vesicles formation.

Despite the fact that the purpose of the project was to find some concluding quantitative and numerical results, an exhaustive qualitative discussion is performed instead.

5. METHODOLOGY

In this section, the experimental method will be deeply discussed.

5.1. EXPERIMENTS

To obtain the vesicles, the procedure followed is the one described by Almendro Vedia et al[10]. From all the methods reported, this is the easiest to achieve in terms of the material required and thus, the budget needed. It has been proved to work well for the formation of considerable number of vesicles.

5.2. SOLUTIONS, MATERIAL AND PROCEDURE

In this work, two types of solutions need to be prepared to carry the electroformation out. One is a sucrose solution and the other is a ternary lipid solution. The sucrose (Sigma Aldrich ; S0389) solution is 200 mM in water whereas for the ternary lipid solution, three individual solutions have to be done in order to obtain the ternary lipid solution that will be used for the vesicle formation. This solution is a 1:1 solution of DOPC (Avanti Polar Lipids ; 850375P) + DPPC (Avanti Polar Lipids ; 850355P) and 30 mol% Cholesterol (Sigma Aldrich ; C8667) of 3 mg/mL each. For this, a certain quantity of lipid is dissolved in an organic and volatile solvent such as chloroform (JT Baker ; lab grade).

The material required for the electroformation process is the following: two ITO glasses, which are glasses coated with a conductor material (Indium Tin Oxide), four adhesive tapes for each of the glasses, adhesive putty, two clips and two wired that will be stuck to the ITO glass (Visiontek ; 100 ohms/sq 25mm x 75mm x 0,7mm) with a silver conductor colloid (TED PELLA INC ; 16045). All these materials are disposed as shown in Figure 5 to form the cells where electroformation will take place.

According to the reported procedure, the steps followed to build the electroformation cell are these:

- Rinse the two sheets of ITO glass with Mili-Q water and dry them with a compressed air pistol. Once is dry, check the conducting side using a multimeter (ISO-TECH IDM91E)
- Wash again the conducting side with chloroform and dry it again with the compressed air pistol. It is helpful to mark the conducting surface with a diamond tip to avoid further confusions.
- On one of the ITO glasses, stick four adhesive tapes one above the other. Do the same in the other ITO glass.
- Peel the wire and, besides the adhesive tapes, place the peeled part using one drop of the UV glue (Norland Products ; NOA81) on both edges of the wire so the contact between the ITO surface and the wire is ensured. Do the same in the other ITO glass.
- With the silver conducting colloid, wet the peeled part so the write and the ITO glass are in contact and let it dry. Check with the multimeter if there is conductivity between the ITO and the end of the wire (the one that is not attached to the glass).
- Make a cylinder with the adhesive putty forming a “U” with one end longer than the other one. Place it between the adhesive tapes and the edge of one of the ITO glasses.
- Now add two drops of 5 µL of the ternary lipid solution in the middle of the ITO glass and within the space limited by the adhesive putty.
- Join both ITO glasses one above the other and slightly press them. The separation between both glasses shouldn’t be larger than 4mm. Use two clamps to ensure the sealing of the cell.
- Then fill the cell with the 200 mM sucrose solution till it is full and bend the long end of the putty to definitely close the cell.

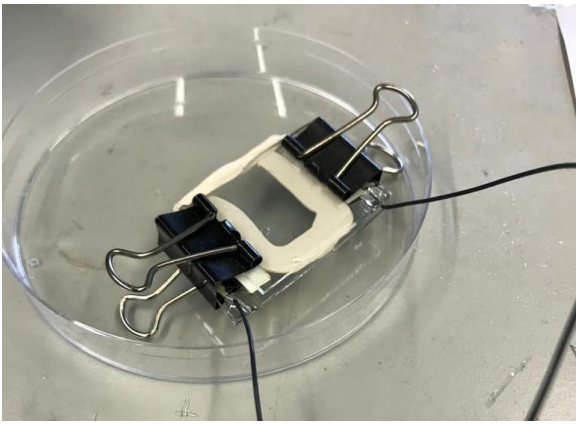

Figure 5. Hand-made electroformation cell

5.3. ELECTROFORMATION PROCESS

As firstly reported by Angelova and Dimitrov[11], the cell built according to what was described in the past section is later connected to a generator (RS PRO AFG – 21012) with an alternate current (AC). The electroformation (or electroswelling) process has been widely reported but one needs to take into account that in every study, several changes in the procedure are detected. Depending on the features that are observed, notable differences in terms of electrodes, frequencies, voltages, time of formation and temperatures are observed from each report to another. Hence, in this project, the optimization of the method according to the goals of the study is so important that will take up an individual section to be deeply discussed.

Regardless of the specific conditions, to understand what happens inside the cell one needs to look at the structure of the lipids used. As mentioned before, phospholipids are formed by a polar head and two nonpolar tails which different behavior can be foreseen. Once the lipid drops are placed in the ITO glass, the organic solvent is removed by evaporation and the lipids self-assemble into a stack of bilayers above the glass[12]. This bilayer disposition is stable so, unless some energy is applied, the bilayers will remain as they are. As shown in Figure 3 (Section 3.3), the phospholipids used for this experiment have a zwitterionic character. That means that, while having a neutral net charge, a positive charge (placed on the nitrogen in the choline group) and a negative charge (from the phosphate group) are found in the same molecule providing the phospholipid with a dipole character. So, it is expected that under AC, the dipole will change its

orientation (Figure 6) following the direction of the electric field triggering a periodic redistribution of charges all over the cell.

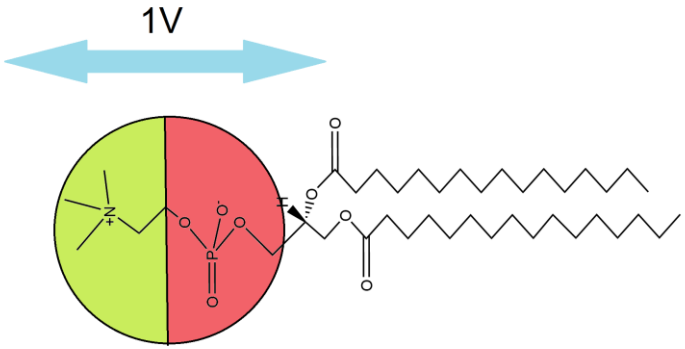

Figure 6. Movement of the dipole present in phospholipids under an electric field

In addition, the electric field applied induces weak dipole forces present in the solution. That helps to increase the water flux between the bilayer stacks and thus, enhances the separation of these bilayers once the swelling solution is added. When the phospholipids are disposed as a bilayer, the edges are free to contact with the aqueous solution directly with the tails, which are hydrophobic. This fact has an associated energy that, to be reduced, requires the decreasing of the surface of that edge. The mechanism that phospholipids follow in order to carry out this minimization is to form spherical vesicles.

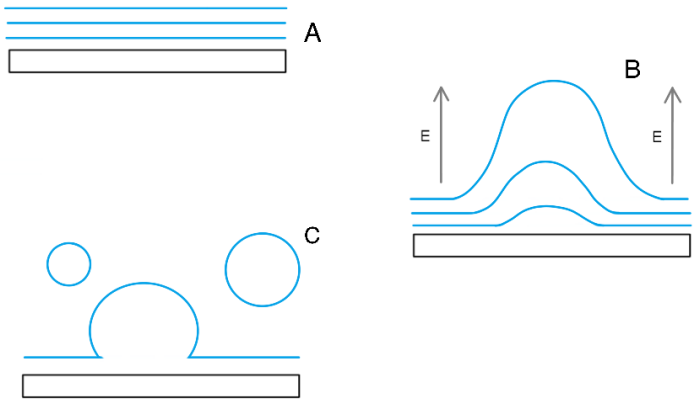

Figure 7. Simplification of the mechanism of formation of vesicles by electroformation.

When one bilayer bends to form the vesicles, a new energy shows up but this time to destabilize the system. In order to overcome the bending energy, the edges need to join and disappear closing the vesicle[12].

5.4. DEPOSITION AND OBSERVATION

Once the vesicles are formed, there is a critical step which is deposition. Vesicles are sensible to temperature changes and to hard movements and vibrations. To take them out of the cell, a bigger hole in the plastic tip of the micropipette pipe needs to be done otherwise, the friction will break them when absorbed.

The support for the vesicles deposition is a PDMS (polydimethylsiloxane) (Sylgard ; 184 elastomer kit) well with a cylinder in the middle. This well is stuck into a glass slide using UV glue. According to the chosen procedure, approximately 150 mL of a 100 mM sodium chloride (Sigma Aldrich ; S9888) are poured into the well before 30 mL of vesicles are added. The well is sealed with a glass cover in order to avoid water evaporation and consequent osmotic stress[10].

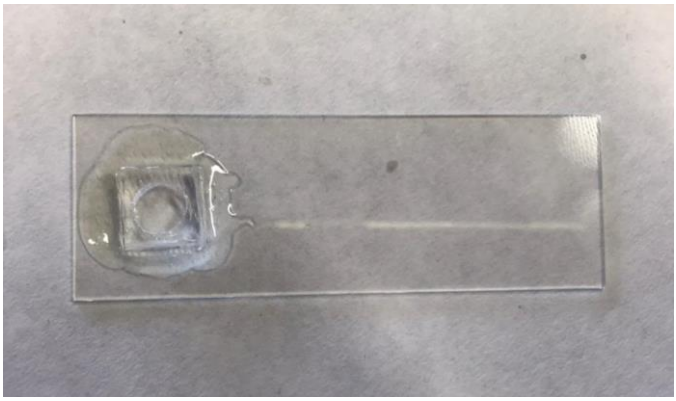

Figure 8. Picture of the well used for the deposition and observation.

This sodium chloride solution is used to bring the vesicles to the bottom of the well so that most of them can be observed in the same plane. The mechanism of this process is based in the osmolality principle and the relation between weights of the ions and molecules that form the whole mixture. The sucrose solution that is inside of the vesicles is denser than the sodium chloride one that is outside. This fact leads the deposition of the vesicles into the bottom of the

well. Same way, it is important to ensure that the overall osmolality is the same than at the beginning of the experiment, otherwise, the vesicles could easily explode or shrink.

The observation will be performed using an optical microscope (Nikon Eclipse E400POL) with a phase-contrast filter to see both phases in the vesicle. Also, the two phases will be observed with fluorescence microscopy. Image acquisition will be performed with an Andor iXon ULTRA EMCDD operated with Image J (open-source software). In the ternary lipid mixture, a fluorophore is added in a 1% to the solution. This fluorophore is NBD-DMPE (Avanti Polar Lipids ; 81043C), a phospholipid with a fluorophore group on its head.

Then, the sample will be irradiated with an intense near-monochromatic light provided by a LED that is able to emit high wavelength light.

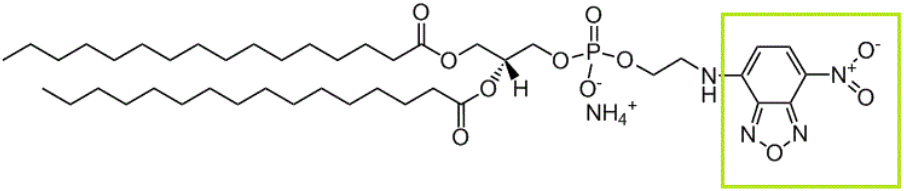

Figure 9. Structure of a molecule of NBD-DMPE with its fluorophore group distinguished.

5.5. OPTIMIZATION OF THE METHOD

After several trials following the reported method, some changes were required in order to achieve the goals of the study. Only one phase was being observed and the polydispersity was too high to perform histograms.

Hence, to see two phases, different parameters were ranged:

- **Composition:** As explained before, a ternary mixture of DOPC/DPPC/Chol is used. Fixing the quantity of cholesterol and assuming 1:1 DOPC/DPPC proportion doesn't show the two phases, a 2:1 and 1:2 DOPC/DPPC proportion are tried to optimize this part of the method.
- **Temperatures:** As several temperatures cover the coexistence of the two phases, temperature of the cell is another variable which is tested. The cell is placed into the oven at different temperatures to check if there is any impact on the formation of the

vesicles. Once the two phases are achieved, a control of temperature will be applied in order to see the reversibility of the process of coexistence of the phases.

- **Frequency:** This is the frequency of the AC at which the cell is connected. The initial 10 Hz seems to endorse the formation of a massive number of vesicles but not as big as required. Other working frequency reported is 2 Hz[13] and both results are compared in the results section. If experimental timing allows, other frequencies will be tried to compare.
- **Time:** The cell is left overnight despite the fact that in several reports[14], 2 or 3 hours are enough to obtain vesicles with the desired features.

An exhaustive discussion regarding the final optimization of the method takes places in the Section 6 of results of the experimental part of the study.

6. RESULTS AND DISCUSSION

This section shows the different strategies followed in order to achieve the goals of the work together with the difficulties faced when trying to accomplish them. A discussion and comparison between results is also deeply carried out.

The first step of this study is to learn how to prepare the electroformation cell and obtain a considerable ability on it. At the beginning, the wires were being welded which presented an obstacle as a certain knack was required. Moreover, vesicles with more than one lipid are slightly harder to obtain so, to make it easier to obtain them, just DOPC is used on the first trials. The frequency used for the firsts experiments is 10 Hz as it is reported in some of the bibliography cited before during the work. Also, no initial temperature is applied and the cell is left overnight. It is worth to clarify that from now on, all pictures were taken with the same optical magnification (x20) and with the same ratio betweem pixels and μm (0,64 pixel/μm and 100 μm in scale).

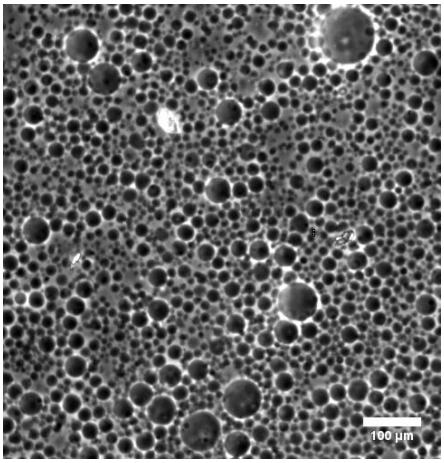

Figure 10. Vesicles of DOPC under 10 Hz

The cell is correctly done and vesicles are formed. There are only few which have size enough, while the most of them are too small to study their features. Next step is to introduce the rest of lipids (DPPC and Chol). To optimize the handy part of the formation, the welding of the wires is replaced by the silver colloid described on the procedure in Section 5.2. Other parameters remain constant to see their impact on three-lipid vesicles. All lipids are added now to the cell and the initial proportion of lipids is 1:1 for DOPC/DPPC. The results show that indeed, three-lipid vesicles of interesting size are still more difficult to obtain in comparison to one-lipid vesicles. It is also seen that electroformation produce a massive number of vesicles.

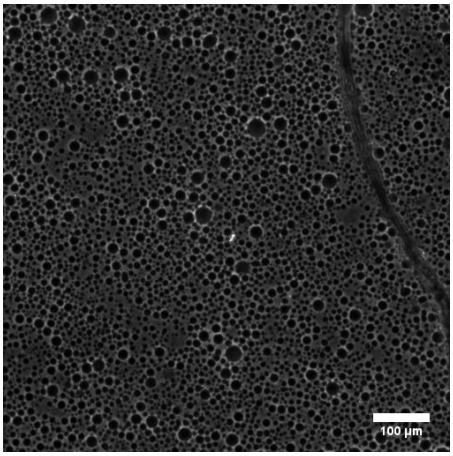

Figure 11. Ternary vesicles of DOPC/DPPC/Chol 1:1 under 10 Hz and no temperature.

Observing so many vesicles, one tries to reduce the volume of lipid introduced into the cell. 2 drops of 5 µL are initially added to the cell so 10 µL of lipid solution is present in a pretty small cell. Now, 3 and 4 µL are added to two different cells. Moreover, the cells are introduced into the oven at 38ºC as it is above the T_m of all the three lipids and it is suspected that can help the formation of vesicles with two liquid coexisting phases.

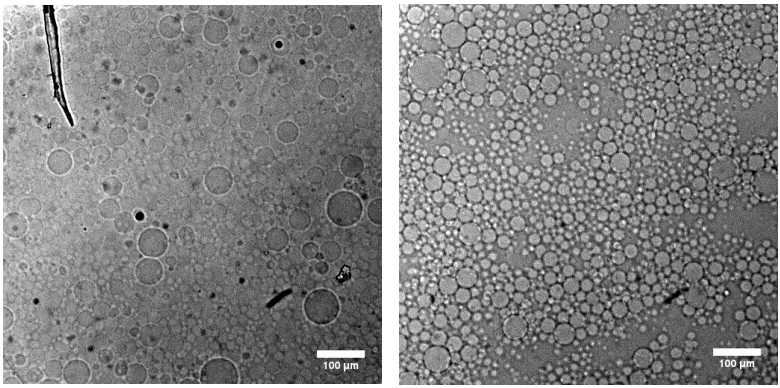

Figure 12. Ternary vesicles using (right) 3 µL and (left) 4 µL of lipid mixture under 10 Hz and 38°C.

According to these results (Figure 12), it seems that lower quantities of lipid stimulate the formation of less vesicles and, in average, bigger. Using 4 µL as a drop volume, the difference is hardly appreciable. Still two phases are not observed so there is no solid criteria to relate the temperature to coexistence of two liquid phases yet. Despite that fact, more assays applying temperature are carried out in order to see if there is any relation between the temperature of formation and the size of the vesicles.

Now, an additional step is required to decrease the number of vesicles observed in the well because it is difficult to analyze features when there are so many vesicles. To do so, a dilution is performed inside of the well adding more volume of the sucrose solution. Several dilutions are studied: 50 µL of vesicles are added to 50, 100 and 150 µL of the 200 mM sucrose solution.

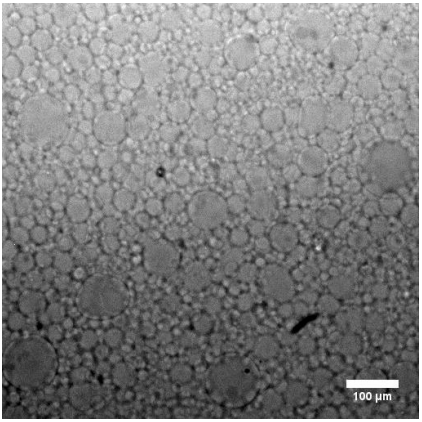

Figure 13. Ternary vesicles under 10 Hz at 38ºC overnight. (Diluted in 50 µL of 200 mM sucrose).

This additional step seems to give better results as, when diluted, larger vesicles tent to fall to the bottom of the well while in other pictures, all vesicles are in a similar plane as the size difference is not enough to shift the larger ones to the bottom. It is worth to mention that the microscope was focused on the bottom plane which can induce a blurry aspect to the image.

Also, here some "pear-shaped" vesicles are found and those are specially interesting because two-phased vesicles normally have this characteristic form. It was not possible to reuse this well but, when these mentioned vesicles show up, fluorescence microscope observation is something very useful to perform as it is the best technique to differentiate two diferent domains.

However, still too many vesicles are formed in the cell so, to overcome this inconvenience, frequency is the variable that is adjusted. What frequency does to the cell is to spin the electric field direction faster when it is higher. Hence, an electric field moving fast (and overnight) can provoke a faster reorganization of the charges present in the lipids and thus, a faster formation of small vesicles. Applying this logic, lower frequencies may allow results closer to the expected. 2 Hz is a frequency reported on several bibliographic sources consulted so it is the chosen value for the next experiments.

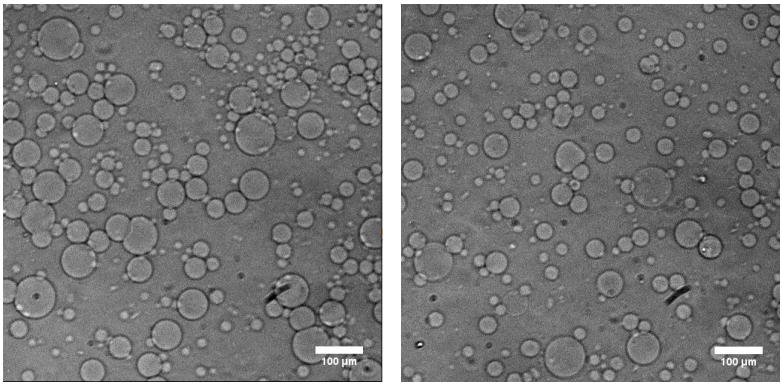

Figure 14. Ternary vesicles under 2 Hz at 38ºC overnight. (Left) Diluted in 50 µL of 200 mM sucrose solution. (Right) No diluted

According to the results shown in Figure 14, the considerations related to frequency and size of the vesicles seem to be pretty accurate. Polydispersity is lowered and the average size of the vesicles is considerably increased. In this case, dilution doesn't help to have better results because, before diluting, the number of vesicles is the desired and reducing it is not useful. Now, fluorescence microscopy is used to check if two phases are achieved in the cell of Figure 14.

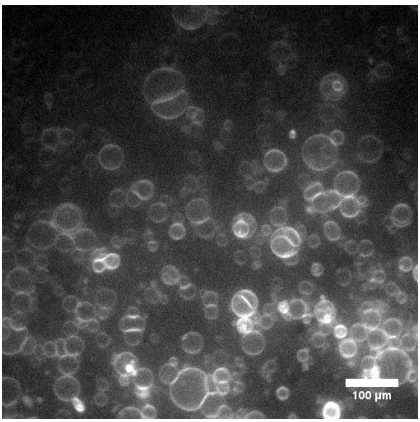

Figure 15. Ternary vesicles under 2Hz at 38 ºC and left overnight observed through a fluorescence microscope.

It is here seen that in general, vesicles have a uniform color in the surface, what shows that there is no raft formed. If formed, the part of the vesicle containing DPPC is supposed to shine

due to the attachment of the fluorophore as they are similar in structure. Some buds are observed in some vesicles, but there is no reason to assign this to a phase coexistence. It is more likely to be two vesicles together or even one inside another.

Once the formation of vesicles reached the desired size and polydispersity, the next step to focus on is to obtain phase coexistence. For this, as explained in Section 5.5, different compositions of lipids are used in order to see the impact into the rafts. The starting composition assayed is DOPC/DPPC 2:1, but the results are not good. Actually, out of 5 cells with this mixture, only in one of them vesicles were found. There are not clear evidences that could explain this failure. For the first time, the fact that lipids (before dissolving) may not be perfect conditions is taken into account. However, it also can be related to a wrong construction of the cell and many other human errors. One interesting vesicle is found in the mentioned cell because two possible phases are detected, one brighter than the other. So, despite the fact that is not big enough to clearly analyze features, a temperature ramp is applied with a temperature-control device (INSTEK mK2000) to see the behavior.

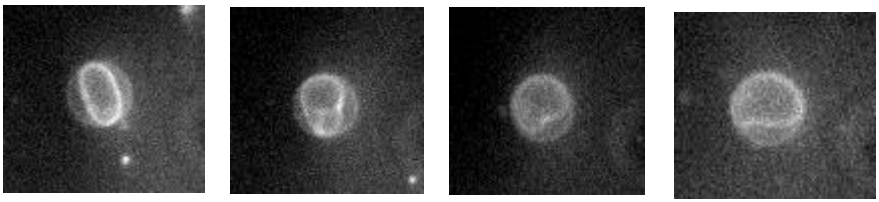

Figure 16. Ternary vesicles DOPC/DPPC 2:1 under a ramp temperature from 23 ºC to 45 ºC.

The interesting fact about rafts is that above T_m, one phase is observable and when temperature is decreased, two phases go back to the original state. Here, when temperature is increased, the volume of the inner vesicle also increases, to the point that almost forms a single one with the outer. This could be a raft, but the vesicle is so small (notice that it is augmented for the picture) that is hard to conclude.

At this stage, one experimental issue comes across and it is quite hard to solve. Once the temperature is raised, temperature fluxes move the vesicles and a blurry screen occupies all the field of view. When working at high frames (pictures taken per unit of time) it becomes difficult to focus the microscope, follow the vesicles behavior and monitor the experiment.

Regardless the technical problems, with 2:1 composition no giant vesicles are formed. To solve this, a new composition is assayed and it is 1:2, being DPPC the major component of the vesicle. The rest of parameters and condition remain constant as they are proven to give good yield. After several trials, 1:2 proportion doesn't allow the vesicles to form two phases. In this case, when fluorescence microscopy is used to observe the vesicles, small bright bulks dazzle the rest of the picture making it really hard to analyze the vesicles of the picture. The origin of this bulk remains in the excess of DPPC, that, as mentioned before, has similarity in terms of structure with the fluorophore. Thus, the probability of finding a vesicle only formed by DPPC and with the whole surface coated with the fluorophore is very high.

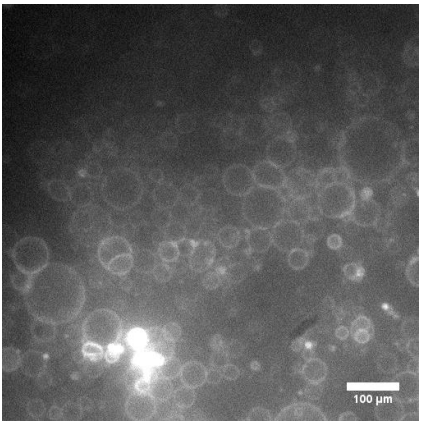

Figure 17. Ternary mixtures of DOPC/DPPC 1:2 under 2Hz, at 38 ºC overnight.

It can be sensed that giant vesicles are formed with this protocol, but it is impossible to determine whether there are lipid rafts or not. Some interesting vesicles appear in this cell but, when applying temperature, the pictures become too blurry.

Now, as the experimental time is almost expiring, several trails are performed but now changing the time of formation of the cells. As mentioned in Section 5.5, it is suggested to form vesicles within 2 or 3h[14] and also with the possibility of applying frequency ramps (from 2 Hz to 10 Hz). In addition, as it is reported by Christoph Herold et al[15] exists the possibility of activating the ITO with an annealing previous to the formation of the cell in order to "activate" the ITO coating. In one of the cells the ITO glass is left at 150 ºC during 30 minutes.

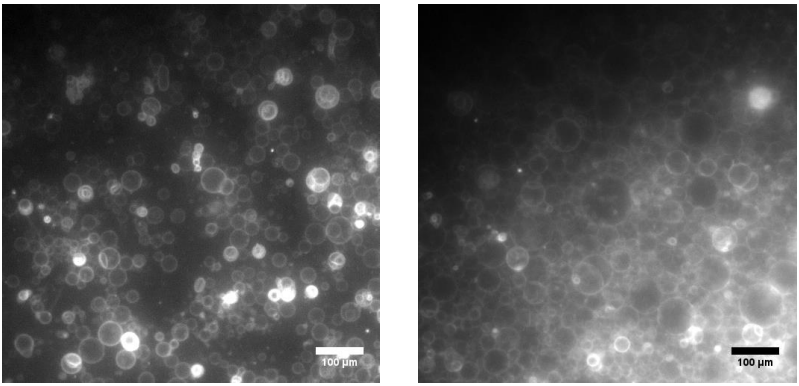

Figure 18.Ternary vesicles of DOPC/DPPC 1:2. (Left) No annealing, 2h of formation and frequency ramp (2 to 10 Hz). (Right) Annealing is applied to the ITO and the cell is under 2 Hz during 2h.

As it can be observed, annealing doesn't help the formation of giant vesicles and thus, no phase coexistence can be observed. On the other hand, the results formed in 2h are good in terms of average size but not in terms of polydispersity. Neither 2 Hz nor frequency ramps show the desired results.

At this stage of the study, no more experiments were performed and all the data was collected and, based on it, qualitative conclusions were extracted.

7. CONCLUSIONS

The conclusions extracted from this work are here enounced:

-Electroformation (or electroswelling) is a good method to form a massive number of vesicles. The mechanism of the method is understood and a considerable ability has been developed when it comes to the creation of the cell.

-GUVs have been deeply studied and reported and thus, there are plenty of specific protocols. Most of them have been followed and the results were not comparable. A lack of reproducibility is found in these works consulted.

-It seems that to accomplish the initial objectives, access to technologically advanced devices is required.

-As two phases are not properly observed, there is no solid criteria to build a relation between composition, frequency, time and phase coexistence.

-In theory, vesicles with more DOPC should be easier to form but it didn't happen. It could be due to a problem with the material used.

-An optimized method for GUVs is obtained regardless the composition. It is using 3 μL of lipid mixture, 2 Hz of frequency, overnight, under 38ºC and with no dilution.

-Despite that the outcome of the experiment was not successful, there is no doubt that GUVs can lead to new scientific developments but require a proper investment to deeply study them.

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9. ACRONYMS

GUV: Giant unilamellar vesicles

T_m: Melting temperature

L_o: Liquid ordered

L_d: Liquid disordered

DOPC: 1,2-dioleoyl-sn-glycero-3-phosphocoline

DPPC: 1,2-dipalmitoyl-sn-glyceo-3-phosphocoline

NBD-DMPE: (1-O,2-O-Dimyristoyl-L-glycero-3-phospho)-2-(7-nitro-2,1,3-benzoxadiazole-4-ylamino)ethanol

PDMS: Polydimethylsiloxane

ITO: Iridium Tin Oxide

UV: Ultraviolet

LED: Light-emitting diode

Hz: Hertz