Bachelor's degree thesis

Study of macroscopic and microscopic properties of liposomes produced using microfluidic methods

Javier Rojo González

Supervised by:

Professor David A. Weitz (Harvard University)

Luis Carlos Pardo Soto (UPC)

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Abstract

For the last decades, lipid vesicles or liposomes, vesicles formed by a bilayer of amphiphilic lipids, have been used as a toy model for studying the cell membrane and for applications in cosmetics and drug delivery. Traditional methods for producing liposomes face some problems such as the heterogeneity in size and composition of the liposomes produced. A few years ago, a novel method that produces liposomes with homogeneous size and composition was developed. This novel method is based on the use of water in oil in water ultra-thin double emulsions, with lipids dissolved in the oil phase, as templates for the liposome production. These ultra-thin double emulsions are produced using glass capillary microfluidic devices.

This new method for producing liposomes seems very promising, but since the liposomes are formed by the oil phase evaporation of the double emulsions, the doubt that some residual oil in the bilayer may alter the properties of the liposomes appears. In this work different phenomena and properties of liposomes that have been studied for the ones produced using conventional methods are studied for liposomes produced using microfluidic methods.

The microfluidic approach for the production of liposomes also allows, with a small modification of the microfluidic device, the production of asymmetric liposomes, which are liposomes with a different lipid composition in the inner and outer leaflet. Asymmetric liposomes have barely been studied due to the complexity in obtaining them using conventional methods, and for this reason the microfluidic approach is promising for studying asymmetric liposomes. The current limitations and problems of the asymmetric liposomes production using microfluidic methods are also explored in this work.

Key words

Microfluidics, glass capillary devices, ultra-thin double emulsions, lipid vesicles, liposomes, phase separation, domains, symmetrical liposomes, asymmetrical liposomes, dewetting, area compressibility modulus, atomic force microscopy, osmotic pressure, shape deformation.

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Introduction

Cells are the basic functional, structural and biological unit of live. One of the necessary structures of the cell, and common to all the different types is the cell membrane. The main function of the membrane is to act as a barrier and control the exchange of molecules between the extracellular and intracellular media, assuring adequate conditions of the intracellular media for the development of all cell functions.

The cell membrane is a complex structure with thousands of different molecules with different functions. Basically the cell membrane is formed by a phospholipid bilayer that acts as a fluid matrix where other molecules such as sterols, proteins, saccharides… lie in [1]. Phospholipids are amphiphilic molecules made of a polar hydrophilic head and two fatty acid hydrophobic tails and can arrange themselves forming a bilayer with the polar heads in contact with water and the hydrophobic tails in the inside of the bilayer. Due to the complexity of the cell membrane and in order to get a better insight on how it behaves, simpler models have been artificially made and study. The simpler model one can think of is having only a lipid bilayer. These vesicles made only of a lipid bilayer are called liposomes and have been thoroughly studied for the last decades.

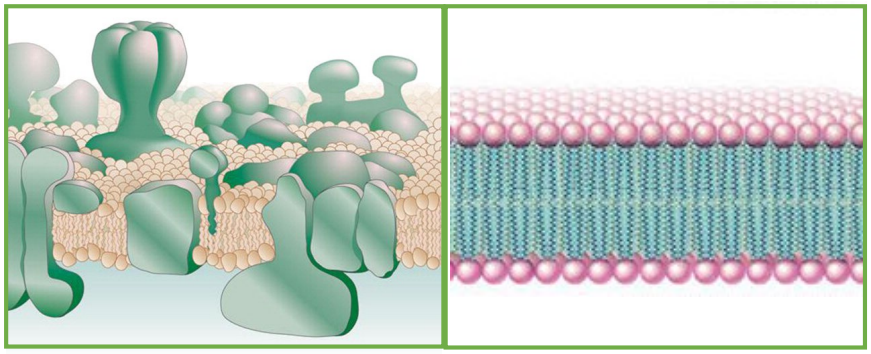

Figure 1. Left: Cell membrane schematic. Image extracted from [2]. Right: Single component liposome bilayer schematic. Image extracted from Encyclopaedia Britannica. The simplicity of the liposome bilayer in from of the cell membrane can be easily seen by the absence of the molecules that crowd the cell membrane.

Liposomes made of one single type of phospholipid present different phases ([3], [4]) depending on temperature, but the most important phases are the gel phase at low temperatures, where the diffusivity of lipids is low and the structure is ordered and the liquid disordered at high temperatures phase where the diffusivity of lipids is high and the structure is disordered. Also if some sterol molecules are added the gel phase transforms into a liquid ordered phase where the diffusivity is high because the sterol disrupts the tight packaging that limit lipid movement but the order of the structure is preserved. The transition temperature between the ordered and disordered phase depend on the chemical structure of the phospholipids, especially on the presence of double bonds in the fatty acid chains (unsaturated lipids) because each double bond produces a kink in the chain. The double bond kinks make it more difficult for the phospholipids to arrange in an ordered and packed structure so the transition temperature is much lower when double bonds are present than where there are not any (saturated lipids). Also when mixing two different types of phospholipids one with high transition temperature and the other with low transition temperature, phase separation can be observed in the liposome, with some domains

in the liquid disorder phase and other ones in the gel or liquid ordered phase depending on the presence of sterols ([5], [6], [7]).

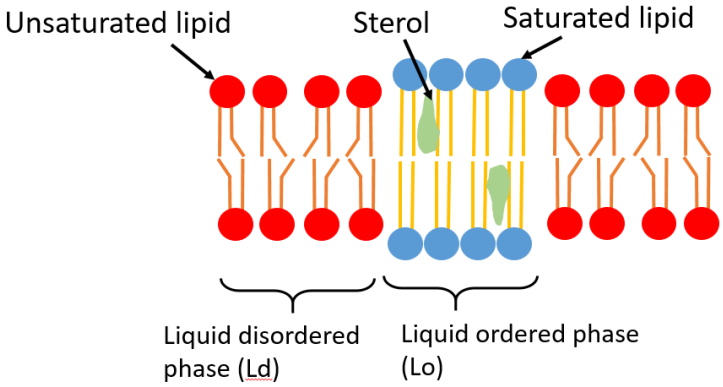

Figure 2. Phase separation schematic of a mixture of unsaturated lipid, saturated lipid and sterol.

The majority of liposome studies have been done on symmetrical liposomes, where symmetrical means that the inner leaflet of the bilayer has the same composition as the outer leaflet. But it turns out that the cell membrane is asymmetrical, so in order to understand what are the effects of this asymmetry in cells, asymmetrical liposomes must be studied. Asymmetrical liposomes have recently begun to be studied, but the problem is the difficulty of obtaining asymmetrical liposomes with conventional methods. A novel method for producing asymmetrical and symmetrical liposomes is the use of microfluidic devices for producing them.

A concern about the liposomes produced using microfluidics methods is the possibility that some residual organic solvent is present in the bilayer changing the properties of the liposomes. In this work different reported properties and behaviours of liposomes produced using conventional methods are studied for the liposomes produced using the microfluidic approach. Also the challenges and problems that arise when producing asymmetrical liposomes using microfluidics methods are studied.

A more detailed explanation of all the exposed above can be found in Annex A: Theoretical framework.

Microfluidic devices for liposome production

Given the importance of liposomes and their uses in being a model for studying the cell membrane and in cosmetic [8] and drug delivery applications [9] because of their biocompatibility and ability to carry different types of molecules in them; lots of methods for liposome production have been developed ([10], [11]).

Despite the huge variety of methods, all of them have some common steps:

1. Solving the lipids in an organic solvent.
2. Drying the organic solvent.
3. Introducing the lipids in an aqueous media.
4. Purify the liposomes and analyse the data.

The main problem that conventional methods for producing liposomes, such as rehydration and electroformation, face is that the resulting liposomes are highly heterogeneous in size and composition and when these methods are used to encapsulate some molecules or substances the encapsulation efficiency is very low. The approach used in Weitz's laboratory to generate liposomes overcomes all these problems and is the one used in this work. This approach consists on generating ultra-thin water-oil-water (W/O/W) double emulsions using glass capillary microfluidic devices. Then the collected double emulsions will dewet (process that will be explained in another section and that has been studied in Weitz's group specially for polymersomes) forming the liposomes.

The main advantages of the microfluidic approach for producing liposomes are:

1. The liposomes have a homogeneous size and this size can be tuned by controlling the parameters of the device or the flow rates of the different phases.
2. The composition of the liposomes is homogeneous and can be controlled by the composition of the organic solvent phase.
3. The encapsulation efficiency is very high since the inner phase is encapsulated in double emulsions.
4. The production of double emulsion is continuous, so a high-throughput production of liposomes could be achieved.
5. Asymmetric liposomes can be generated just changing slightly the design of the device.

Microfluidic devices can be done with many materials but usually they are made out of glass or PDMS (Polydimethylsiloxane). Glass devices are made using glass capillaries to guide the flow and they are hand-made in the laboratory. PDMS devices are made used soft lithography in a workshop specialized in that fabrication method so there is less space for error at fabrication compared to the glass capillary ones. Despite being the PDMS devices more easily fabricated, as lipids have to be dissolved in an organic solvent, due to the chemical incompatibility of organic solvents (at least the ones that are used for liposome production) with PDMS and the inability to use two different coatings in the devices this option has to be discarded. Glass capillary devices will be used.

Microfluidic devices

Different designs of the glass capillary devices are used to create double emulsions. The double emulsions produced using this kind of devices are really homogeneous in size [12] and because of that, the liposomes produced using this method are also homogeneous in size. The double W/O/W emulsions produced are used as templates for the liposomes. What happens is that the oil phase (organic solvents) has the lipids dissolved in it, so the vesicles are formed spontaneously when the oil phase evaporates. As we are using an organic solvent that has a small solubility in water the evaporation of the oil phase can take a really long time depending on the thickness of the double emulsion. Because of that, we are interested on making ultra-thin double emulsions so the dewetting process can take place in a reasonable time. Although the production of liposomes from thick double emulsions have been reported [13], ultra-thin shell double emulsion templated liposomes are formed much faster so that will be the approach used in this work.

Ultra-thin double emulsions have been used for liposome production for a few years, and several papers have studied different aspects of liposomes produced by this method ([14], [15], [16]). The glass capillary device used for the ultra-thin double emulsion production will be different depending on the type of vesicles we want to produce, symmetrical or asymmetrical.

Symmetrical liposome device

For symmetrical liposome production, we need three different phases, the inner water core phase, the organic solvents with lipids dissolved in them and the outer water phase. The glass capillary device scheme is shown below in figure 3.

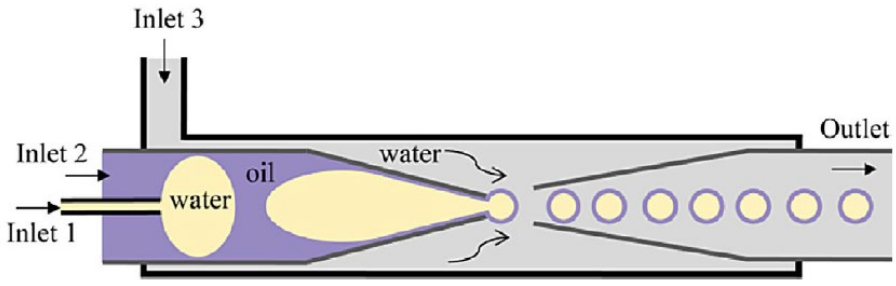

Figure 3. Microfluidic device used for ultra-thin shell double emulsions production to obtain symmetrical liposomes. Image extracted from [14].

As it can be seen in the left side of the figure, the glass capillary device consists in a small capillary (inlet 1) inserted inside a bigger capillary tapered in its end and whose diameter of the tip can be chosen during the fabrication of the device. This bigger capillary is called the injector capillary since it is the one that produces the double emulsions. On the right of the figure there is another capillary also tapered but with bigger diameter than the injector capillary. This capillary is called the collection capillary and is the one that collects the double emulsions. The injector and collector capillaries have their tips aligned facing each other and are inside another square capillary with a bigger size.

There are three inlets and only one outlet in this device. The inner water phase (the phase that will be inside the double emulsions) is injected through the inlet 1, the oil phase with lipids is injected through the inlet 2 and the outer phase is injected through the inlet 3. The outlet is the collection capillary and all the phases are pumped in the direction from left to right.

The functioning of the devices relies in two different phenomena. The first one is a basic phenomenon in microfluidics when there are two different fluids co-flowing, this means flowing in the same direction and one of the liquids flows inside the other. In this case two different behaviours can be observed [17], dripping and jetting (figure 4). Dripping and jetting can be easily understood by taking a faucet analogue (in a faucet the inner fluid would be water flowing in air).

When the water flow is small enough it can be seen that droplets of water are being formed at the tip of the faucet and when they acquire enough mass they fall down. This is the dripping regime, a regime where the capillary forces are important so drops are formed until they get so big that innertial effects are stronger than capillary ones and then the drop detaches. So in the dripping regime we get a continuous and homogeneous drop production.

When the water flow is big enough it can be seen a jet forming. It forms because the capillary effects are negligible in front of inertial effects. What can also be seen is that this jet undergoes an instability called the Rayleigh-Plateau instability, that is responsible for the appearance of undulations on the jet profile that increase in amplitude as we are getting further from the start point of the jet until the jet breaks in drops [18].

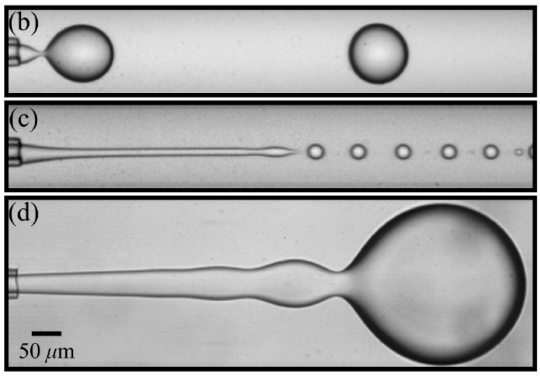

Figure 4. Image extracted from [17]. (b) Dripping regime. (c) and (d) Jetting regime.

The regime can be determined by the value of two adimensional numbers, the Capillary number of the outer phase (Ca), that accounts for the ratio between viscous stresses and surface tension, and the Weber number (W). which accounts for the ratio between the inertial forces and surface tension. If both of these numbers are below the unit, the coflowing system is in the dripping regime, while if any of these numbers are above the unit, the jetting regime can be observed.

$$Ca = \frac{\eta_{out} \cdot u_{out}}{\gamma} \qquad W = \frac{\rho_{in} \cdot d_{tip} \cdot u_{in}^2}{\gamma}$$

Where η is the viscosity, u the velocity, γ the surface tension, ρ the density and d the diameter.

In microfluidics the viscosity of the different fluids plays an important role. To make it easier for the system to work in the adequate regimes so the device operates properly the inner water phase viscosity is increased by solving polyethylene glycol (PEG) in it. This also makes the double emulsions denser than water, so when collecting double emulsions in a vial, they will sink to the bottom making it easier to observe them using microscopes.

The other important effect is due to the hydrophobic coating of the injector capillary. The inner water phase is injected through the inner capillary in the injector capillary so there is a coflow of the inner water phase inside the oil phase. The flow rates are chosen so the inner water phase is in the dripping regime and the droplets form are bigger than the injector capillary. Because the injector capillary is hydrophobic there is still a small layer of oil between the inner phase droplet and the injector capillary wall. This small layer of oil is the key to the formation of the ultra-thin double emulsions. This way in the injector capillary we can see drops of water phase separated by oil phase between them and the walls.

The inner water and oil phases in the injector capillary are also in coflow with the outer phase. In this case for a good device it doesn't matter the regime we choose, since both dripping and jetting regimes will form ultra-thin double emulsions. What happens is that when an inner water drop arrives at the tip, it is pushed against and oil phase film and when drops are formed, the oil phase surrounds completely the inner water phase forming ultra-thin double emulsions.

The collector capillary is treated with a hydrophilic coating to make sure that the outer water phase is at the walls of the collector capillary and the double emulsions don't break because of contact with the wall. To collect the double emulsions, they are directed to a store vial filled with a water solution and they sink to the bottom.

To assure the stability of the double emulsions and avoid their rupture due to high internal tensions surfactant is added to the internal and external water phase so the stability of the double emulsions is increased and they can be collected without problem. The surfactant used is polyvinyl alcohol (PVA).

Using this kind of devices one can get double emulsions with a size ranging between several tens to several hundreds of micrometres. This way the liposomes that are produced are giant liposomes.

Asymmetrical liposome device

For asymmetric liposome production, a simple variant of the device for symmetrical liposomes can be used [19]. To be able to get asymmetric liposomes two different lipid solutions in organic solvents are used so in the double emulsions the inner part of the oil phase (inner middle oil from now on) has different lipids dissolved than the outer part of the oil phase (outer middle oil from now on). Although in previous works these double emulsions have been referred as triple emulsions to make an emphasis on the two different lipid solutions, since the organic solvents used to dissolve the different lipids are the same (and because of their miscibility), technically these emulsions are double emulsions.

To get these different lipid solutions arrange properly so the lipid composition in the inner side of the oil phase is different from the lipid composition in the outer side, a small glass capillary is inserted in the free space between the injector capillary and the square capillary at a certain

distance of the tip of the injector. Through this capillary the outer middle oil phase is injected. Since the injector capillary is treated hydrophobically this oil phase will surround the injector tip and add another oil layer to the double emulsions that are produced the same way as the symmetrical so the oil phase of this "original" double emulsions is the inner middle oil phase. This arrangement can be more easily seen in figure 5.

Since for the liposome formation from these double emulsions it is really important to get ultra-thin double emulsions and we are adding another oil layer making them thicker, to get ultra-thin double emulsion the square capillary is tapered at its centre so the shear of the outer phase can be increased and this way the resultant double emulsions can be thin enough.

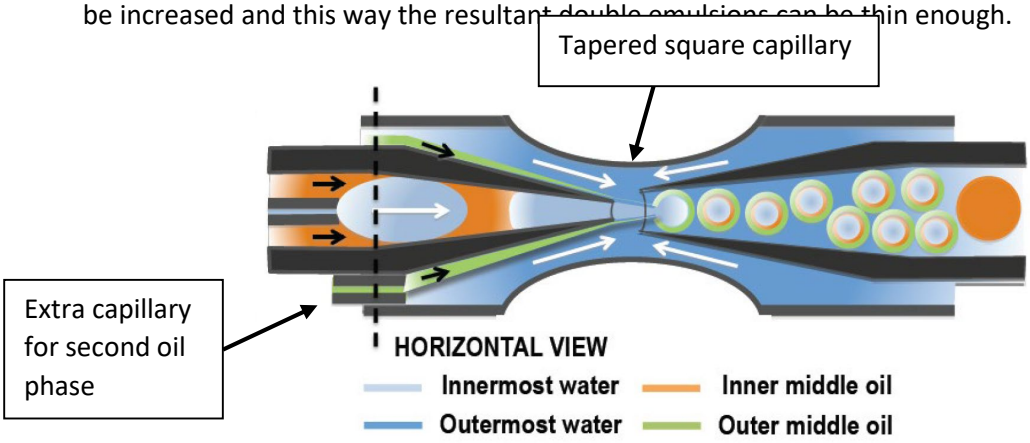

Figure 5. Scheme of the glass capillary device for asymmetrical liposome production. Differences with the symmetrical liposome device are highlighted. The different oil phases arrangement in the double emulsions can be easily seen. Image extracted from [19].

Dewetting phenomena

So far we have talked about how ultra-thin shell double emulsions are produced. Once we have collected these double emulsions the most critical step in liposome formation takes place and it is called dewetting. This dewetting process has been studied before especially in the case of polymersomes ([20], [21], [22]). Although the dewetting phenomena for the formation of liposomes is not completely understood, since the polymer molecules used for the formation of polymersomes are amphiphilic molecules as the lipids but larger, the physics of both of them should not differ very much and the dewetting phenomena of polymersomes can give some insight on how the dewetting phenomena of liposomes occurs. For this reason, the dewetting phenomena for polymersomes is explained in this section.

For the dewetting phenomena to take place it is necessary that the oil phase is made up of two different organic solvents. One of them is a good solvent for the polymers and has a good solubility in water and the other one is a poor solvent for the polymers and has a really low solubility in water. Here, we consider good solubility when the good solvent present in the double emulsions can be dissolved completely in the water where the double emulsions are stored. Another condition for the dewetting phenomena to take place in an effective way for polymersome production is that the inner core osmolality is 10-20 mOsm higher than the collection solution osmolality.

When the double emulsions are formed the good solvent for the polymers is progressively dissolved in the water of the vial. We end up this way with double emulsions rich in poor solvent with polymers in it. Since it is a poor solvent for the polymers, they rearrange forming a layer in the inner interface and another layer in the outer interface. This polymer layers have their hydrophilic part in the water side of the interface and their hydrophobic part in the poor solvent shell as shown in the left side of the left image of figure 6.

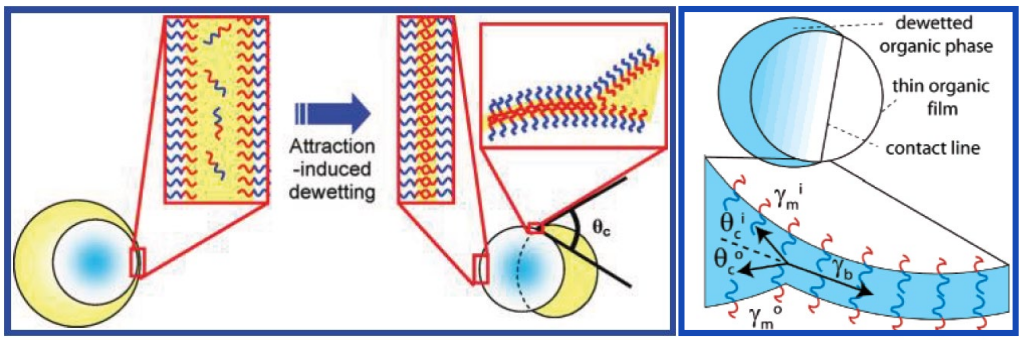

Figure 6. Left image extracted from [20] and right image extracted from [21]. Left image: Scheme of the dewetting process showing the behaviour of amphiphilic molecules. Right image: Scheme of line tensions in a dewetted double emulsion.

As it can be easily understood there is an energetically favourable tendency of the polymers forming the interfacial layers to get together, due to the hydrophobic attraction of their tails. The energy released in the process of attraction of the polymer layers forming an oil solvated bilayer is called energy of adhesion and it accounts for the tendency of the layers to come together; the higher, the stronger will be the attraction between layers. This energy of adhesion depends on polymer concentration and is higher when the concentration is higher, so the evaporation of the poor organic solvent that increases polymer concentration also increases the energy of adhesion.

The energy of adhesion is the responsible for the lipid layers coming together displacing the poor solvent that at the end forms an oil pocket attached to the double emulsion, this is a region where the poor solvent is concentrated as show in figure 6. This oil pocket is stabilized forming a certain angle (θ_c) with the double emulsion and this angle is determined by a balance of surface tensions. To understand this better, let's make a brief definition of surface tension.

Surface tension of an interface/surface is defined as the force per unit length needed to divide the surface in two other surfaces. Another definition of surface tension is the energy per unit of increased area needed to increase the surface area. Because if this, surface tension is also called surface energy. As it is a force per unit length a force equilibrium can be derived from surface tension, but as it is also energy per unit area this can make us relate energy of adhesion with surface tension too.

In our system with the oil pocket, at the frontier of the oil pocket and the oil solvated bilayer we find three different surface tensions as shown in the right image of figure 6. γ_b is the surface tension of the oil solvated bilayer film, γ_m^i and γ_m^o are the surface tensions of the inner and outer

water-oil interfaces with adsorbed lipids. If we attend to the force balance (taking into account that θ_c^i=0), the next equation equality should be fulfilled:

$$\gamma_b = \gamma_m^i + \gamma_m^o \cdot \cos(\theta_c^o)$$

Taking into account that cosine function is always smaller or equal to 1 and that 0° ≤ θ_c^o ≤ 180° a condition that must be fulfilled for the dewetting process to happen is:

$$S = \gamma_m^i + \gamma_m^o - \gamma_b < 0$$

Where S is called the spreading coefficient and is a measure of the tendency of a fluid to spread in a surface between two other fluids. If it is positive it means that the fluid spreads in the surface while if it is negative the fluid tends to not spread in the surface forming drops over the surface, just like the oil pocket. A common example of a negative spreading coefficient system is the drew drops that forms in the surface of leaves. These drops don't spread on the surface but form drops that lie on the surface with a small contact area.

After the oil pockets are formed the final step for the formation of liposomes is the evaporation of the poor solvent oil pocket. Evaporation of the oil pocket is a slow process due to the low solubility of the poor solvent in water and it can take from days to weeks depending on the evaporation rate that depends on external and internal factors. At the end of the process we get the polymersomes formed.

Surface tensions are critical for the dewetting process to happen. For the dewetting phenomena to happen we should be sure that the negative spread coefficient condition is fulfilled. Since the surfactant used in the solutions when the double emulsions are made lowers γ_m^i and γ_m^o this can lead to a positive spread coefficient so the dewetting process never happens and leads to stable double emulsions. This way the concentration of surfactant should be controlled and kept below some threshold to guarantee a negative spread coefficient.

Once this dewetting process for polymersomes has been explained, it has to be noted that liposomes are much more instable than polymersomes [23]. Their stability depends strongly on different parameters such storing conditions, surface tension, composition, adhesion of molecules in the surface… The consistent production of liposomes using microfluidic methods is then not a straightforward task.

Mechanical properties of liposomes

The thickness of liposomes usually lies around 5 and 10 nm with the exact value depending on the lipids that forms it. Since the liposomes that are made using glass capillary devices have a diameter that varies from tens to hundreds of micrometres, from a continuum mechanics perspective these liposomes can be studied as 2-dimensional systems [24]. As the liposomes we are interested in are in liquid phase, at equilibrium, no shear stress is present in the liposome, so the study of the system can be simplified. If we study this 2-dimensional system in the linear regime two magnitudes of mechanical interest can be defined:

1. Area compressibility modulus

The area compressibility modulus relates the membrane tension, which is the attractive force per unit length between the molecules of the liposome, with the increase in area (area dilation). The formula that relates these quantities is the following:

$$\sigma = K_A \frac{\Delta A}{A_0}$$

Where σ is the membrane tension, K_A the area compressibility modulus, ΔA the area increase and A_0 the initial area.

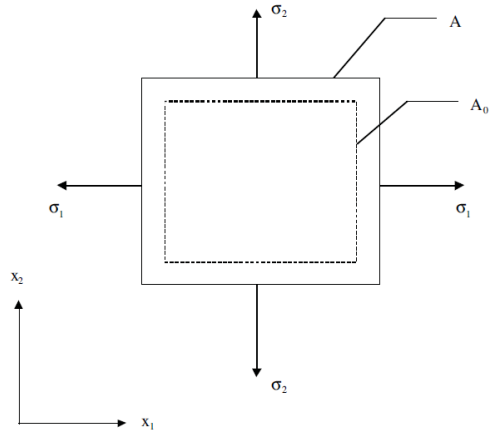

Figure 7. A membrane, initially of area A_0, subjected to a uniform extensional stress along its edges, $\sigma_1 = \sigma_2$, causing an increase in area to A. Image extracted from [24]

2. Bending modulus

The bending modulus relates the torque acting on the membrane with the curvature through the following equation.

$$M_\alpha = K_b \frac{\partial u_3}{\partial x_\alpha}$$

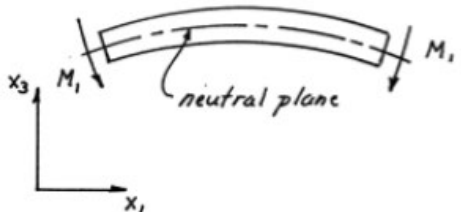

Figure 8. Plate bending subjected to M_1 moment. Image extracted from [24]

Where M_α is the torque in the membrane plane perpendicular to α direction, K_b is the bending modulus, u_3 is the displacement in the direction perpendicular to the membrane and x_α is the coordinate of the α direction that lies in the membrane.

The bending modulus of liposomes have been measured for different composition and sizes ([25], [26], [27]) and so has been the area expansion modulus ([28], [29], [30]). A comparison on the energy needed to deform a liposome shows that the energy needed to expand the area of the liposome is order of magnitudes greater than the energy needed to give it extra bending [31]. Because of this the most relevant mechanical property of liposomes is the area compressibility modulus and the bending and shear modulus can be neglected when studying the deformation of liposomes under some force.

Atomic force microscopy (AFM) measurement

There are several methods for measuring liposome mechanical properties. In this work the method used for measuring the mechanical properties of liposomes is the atomic force microscopy. Atomic force microscopy is mainly used with three different objectives: Force measurement, imaging and manipulation. As our interest lies in measuring the mechanical properties of liposomes the force measurement capability of AFM will be used. The working principle of this method is simple, a cantilever with a tip is pushed against the sample and a force versus displacement curve is obtained (figure 9). The force is obtained by knowing the cantilever bending and this is known by measuring the deflection of a laser that reflects on the cantilever. Using the cantilever spring constant given by the manufacturer the force can be calculated and the displacement is computer controlled.

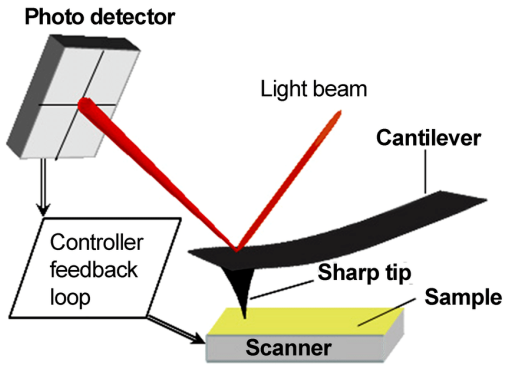

Figure 9. Atomic force microscopy scheme of working principle.

To extract the area compressibility modulus of the liposome from the force displacement curve a model is needed so by fitting the model equation the mechanical parameters can be obtained. The model used (not yet published) is based the one developed by Edith Schäfer [31]. The model of Edith Schäfer accounts for the deformation of liposomes when compressed between two parallel plates (figure 10) so for the measurement instead of the typical sharp tip in the AFM cantilever a plane tip is used. For the development of the model two basic and well known equations are used:

$$\sigma = \sigma_0 + K_A \frac{\Delta A}{A_0}$$

Where σ is the membrane tension, σ_0 the pretension or tension intrinsic to the membrane when it forms, K_A the area compressibility modulus, ΔA the area increase and A_0 the initial area. This first equation relates the area expansion to the membrane tension.

$$\Delta P = \sigma\left(\frac{1}{\rho_1} + \frac{1}{\rho_2}\right)$$

Where ΔP is the pressure difference between the inside and outside of the liposome, σ is the membrane tension and $1/\rho_1$ and $1/\rho_2$ are the main curvatures. This second equation is called the Young-Laplace equation, and relates the difference of pressure with the membrane tension and liposome geometry.

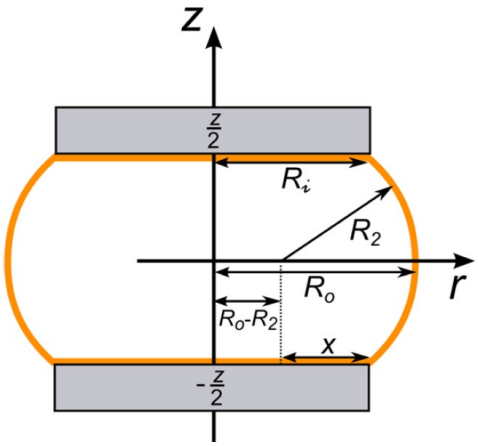

Figure 10. Scheme of liposome compression between two parallel plates and the main parameters used in the model. Image extracted from [31].

Some assumptions are made so the geometry of the liposome when compressed between two parallel plates can be obtained and with this geometry the force-displacement curve can be obtained. The main assumptions of this model are:

- The pressure is constant inside and outside the liposome
- The membrane tension is constant
- The volume of the liposome is constant (low water permeability).
- The contact area is the same for both plates
- The radius R_2 is constant for the part of the liposome not in contact with the plates
- Axisymmetry about the z axis.

With these assumptions an equation can be extracted that can model the force-displacement curve.

$$F = \frac{2\pi R_o R_i^{\,2}\left[\sigma_0 + K_A \frac{\Delta A}{A_0}\right]}{R_o^{\,2} - R_i^{\,2}}$$

Where R_o and R_i can be obtained as a function of the cantilever displacement using another three equations obtained by applying some of the assumptions.

By fitting this equation to the force-displacement experimental curve the values for the prestress (σ_0) and the area compressibility modulus (K_A) can be obtained.

Materials and methods

Microfluidic device fabrication

Microfluidic device for symmetrical liposomes

The steps and procedures used to fabricate the microfluidic glass capillary devices used for making double emulsions (which later will become symmetrical liposomes) are:

1. First of all, we take a borosilicate square capillary (figure 11B) with inner side of 1.05 mm and we cut it to the length we want. To do that, a line is carved with a diamond tip pen perpendicular to the capillary axis and then the capillary is manually broken, since the carve will induce the fracture to propagate at that point.

2. This square capillary is fixed to a glass slide using DEVCON® 5 Minute® Epoxy amber [1:1] (figure 11A). This adhesive works by mixing epoxy resin with epoxy hardener in a proportion 1:1. The right proportion is guaranteed by the applicator (Devtube). To use the epoxy, the mix is located in hexagonal plastic cup and mixed with a wooden stick (figure 11C) until it starts to cure. Finally, it is applied with the wooden stick or toothpicks if we need more precision (figure 11D).

3. In order to get the injector and collection capillaries, a borosilicate round capillary of outer diameter 1.00 mm is used (figure 11E). We use a flaming/brown micropipette puller model P-97 (figure 11F) with a custom program to taper the capillary to a final diameter of around 20 µm (figure 11G).

4. To get the injector capillary and collector capillary to the right diameters sandpaper of 2500 grit is used (figure 11H). To avoid breaking and cracking of the capillary tip the sandpaper is hold in the edge of the table with some of it been outside the table. Then, the capillary tip is rubbed gently against the part outside the table, sliding it only in one direction. From time to time, to avoid getting the tip dirty with glass dust an air compressed gun is used to blow it away from time to time. To check that we have the right tip diameter a microscope is used to check by eye. Depending on the size of the double emulsions we want to produce different sizes of injector and collector tips must be used, but as a general rule the injector tip diameter is sanded to 20-80 µm and the collector tip is 2 to 3 times bigger than the injector tip.
   Since the injector tip has to be free of cracks in order to get a better performance once it is sanded down to the right diameter, a blow torch is used to heat it up a bit so the cracks can be cured. In this last procedure one has to be careful since an excess on heat can lead to the clogging of the tip because the glass would melt and close it.

5. The injector tips are immersed in n-octadecyl-trimethoxy silane for the hydrophobic coating and the collector tips are immersed in 2-[methoxy (polyethyleneoxy)propys] trimethoxy silane for the hydrophilic coating (around 0.5 mL both cases). The vials are kept horizontal around 1 hour (figure 11I) so thanks to capillarity effects the coating substances can get everywhere inside the capillaries. After one hour the capillaries are

taken out of the solutions and the excess of coating solution is removed with the air compressed gun. It has been seen that the hydrophilic coating is easier to remove with the air compressed than the hydrophobic coating, and that at the end there are always some drops inside the capillaries but this is no problem since later they will be removed when the device is checked to see if there are any leaks.

6. To make the inner capillary, a round capillary of outer diameter 1.00 mm is stretched using a blowtorch. This step is delicate since you have to heat the capillary enough so it can be plastically deformed but not too much since it will melt, and pull it with the right force so the stretched region is thin enough to fit inside a round capillary and not too thin that it gets clogged (figure 11J). At the end, using a diamond tip pen the stretched region is cut so we finally get the inner capillary (figure 11K).

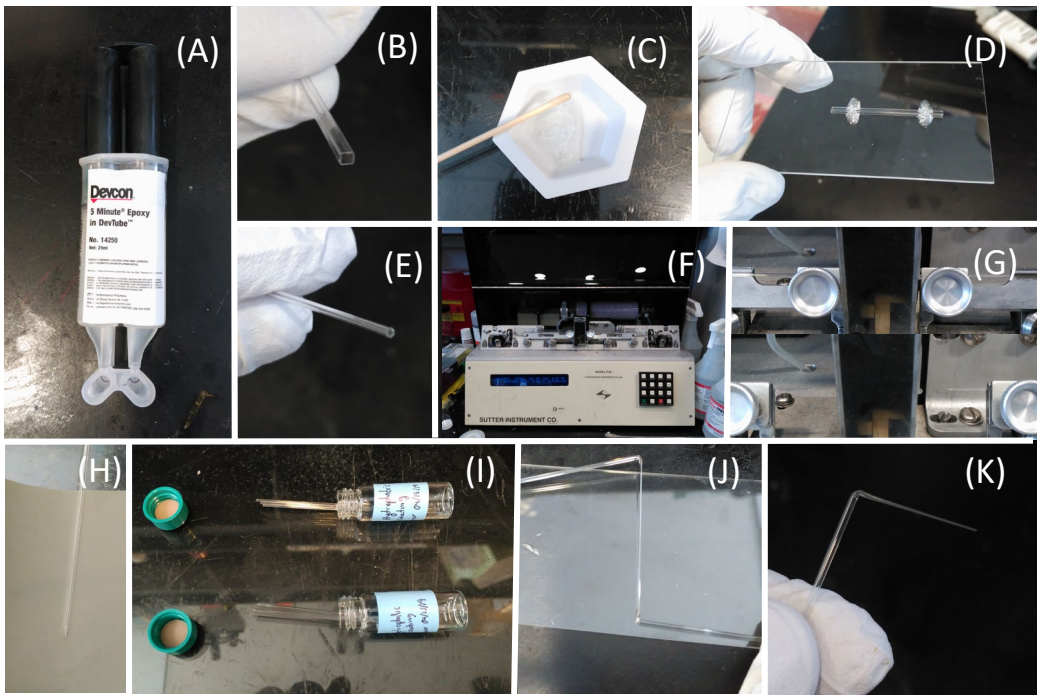

Figure 11. (A) Epoxy adhesive. (B) Square capillary. (C) Adhesive mixing. (D) Square capillary fixed in glass slide. (E) Round capillary. (F) Micropipette puller. (G) Capillary tapered to ≈10μm. (H) Sand paper of 2500 grit. (I) Coating of capillaries. (J) Inner capillary stretched. (K) Inner capillary

7. Now it is time to assemble everything. First the injector and collector capillaries are inserted inside the square capillary fixed in the glass slide through opposite sides. Then a little bit of epoxy is added to the injector and collector to fix them, and while it is curing both capillaries are aligned using a microscope. The alignment is critical for the correct operation of the device and one must be very careful to make sure that the injector and collector capillaries are aligned also in the direction perpendicular to the glass slide. A bad alignment of the two capillaries would result in the device not working. The distance between the tips of the injector and collector capillary for the device to work correctly is from 1 to 2 times the injector diameter.
   Due to the small difference between the inner length of the square capillary and the outer diameter of the collector and injector capillaries it is not hard to align them. To make this work even easier, especially for the alignment in the direction perpendicular

to the glass slide, the free ends of the injector and collector capillaries are inserted in square capillaries and the alignment is done (figure 12A).
Once they are aligned the inner capillary is inserted in the injector one and fixed with epoxy (figure 12B).

8. The next step is to fix some needle tips to every entrance of the device so the liquids can be pumped in. For that we take some needles tips and cut the base of them (figure 12C). This is not strictly necessary but is useful for two main reasons: The first one is an economic question, cutting the base means reducing the lost volume that is kept there, so when working with expensive solutions it allows us to safe some solution. The second one is to avoid air bubbles inside the base of the needles, when cutting them, the air bubbles are less significant so the device works better (although there are ways to get rid of air bubbles before operating the device). On the economic side, another way to reduce loss volume is to use smaller needle tips so the volume lost in the tubing is also smaller.
   Then we cut with a razor some triangles in the base where the capillaries will pass (figure 12D) and we use epoxy to fix the needle tips to the different entrances. To do so, we mix the epoxy and when the mix starts to cure we use the wooden stick to drip some epoxy drops on the borders of the tip to fix it and seal it. It is important that the epoxy has started to cure and is viscous so it doesn't enter the tip and clog the device. When we finish and wait long enough for the epoxy to finish curing the device is done (figure 12E) and one can see from the other side of the slide the device structure (figure 12F).

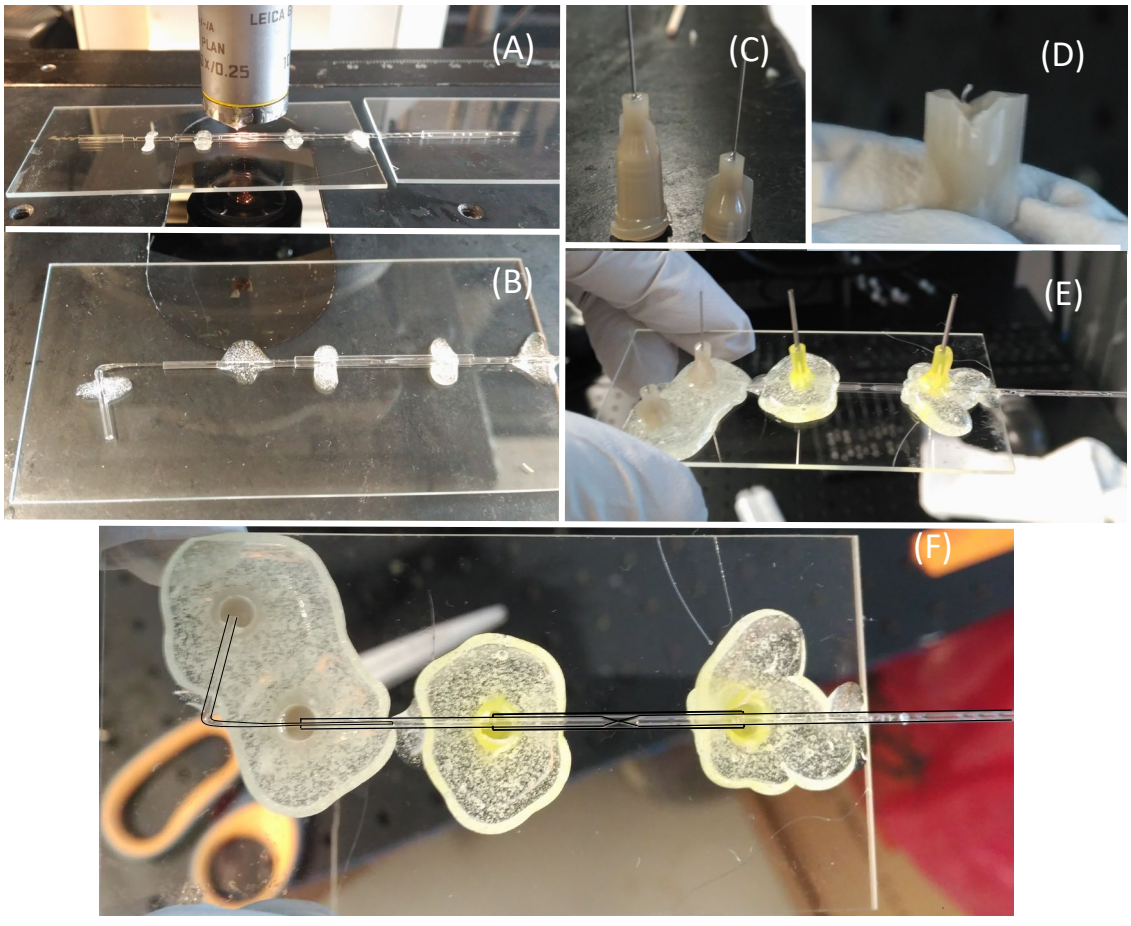

Figure 12. (A) Injector and collector alignment. (B) Inner capillary fixed. (C) Needle tip base cut. (D) Triangles cut where the capillaries will pass. (E) Device done. (F) Device from below. Channel structure.

9. The next step is to check that the device is not clogged and that there are no leaks. For that we use a water syringe to inject water to each tip using the suitable tubing and check that all the possible paths of the water are not clogged. To check all the paths, we can use our fingers to close some channels to check that the water flows to the other channels. If we detect a leak the solution is to add epoxy to the leaking point closing it. Sometimes when fixing the needle tips we may get some epoxy inside the tip because the epoxy fell onto the tip. This can be fixed by cutting the tip using some scissors.

10. The collection capillary can also be extended adding a glass slide with epoxy to the main one and extending the collection capillary using a square capillary and another round capillary. Since we work with organic solvents we might want to reduce the contact of them to plastic, so we substitute the plastic tubing that has to be added at the end to collect the double emulsions with a glass capillary extension. Different variations of the device can be made as shown in figure 13.

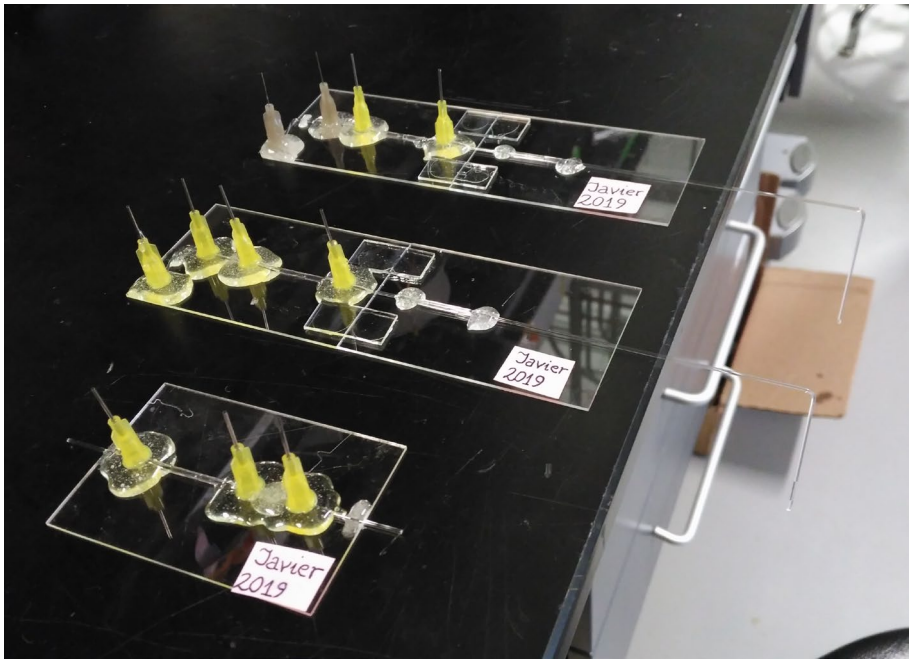

Figure 13. From bottom to top: Device with inner capillary entrance and collector exit free. Device with inner capillary entrance with a tip and collector exit extended. Device with inner capillary and injector entrance with smaller tip and collector exit extended.

As the glass capillary microfluidic devices are handcrafted, their making involves several steps that can be critical for the proper working of the device. For this reason, it takes some time to learn how to make these devices consistently, and even when having learnt that, some random and non-controllable factors such as the quality of the coating can make the device dysfunctional. These devices can keep working from several weeks to several months. The main reason for them to stop working is that the epoxy creates some tension on the capillaries when it cures and they end up misaligning so the device stops working properly. Also the continuous exposure of epoxy to organic solvents forms leakages with time that have to be fixed by adding more epoxy to the leakage spot.

Microfluidic device for asymmetrical liposomes

The fabrication of microfluidic devices to produce asymmetrical liposomes is very similar to the fabrication of the devices for symmetrical liposomes with few differences in the following steps.

1. The square capillary is tapered in its centre using the micropipette puller before fixing it to a glass slide (figure 14A and 14B).

6. Using the same technique used for making a thin inner capillary, another thin capillary is made so it can be inserted between the injector and square capillary.

7. When assembling everything, first the thin capillary that goes between the injector and square capillary is inserted in the square capillary, then the injector and collector are inserted, with care not to break the thin capillary, and aligned at the tapered region of the square capillary (figure 14C and 14D). Then the inner capillary is inserted in the injector.

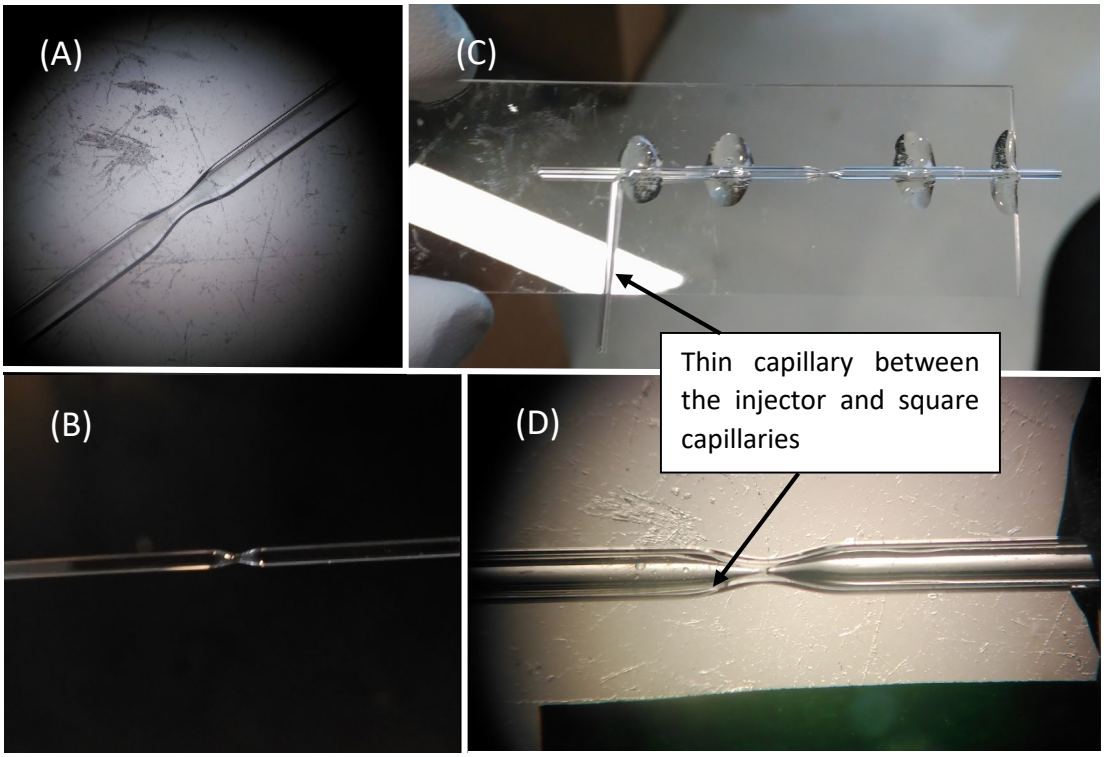

Figure 14. (A) View of square capillary tapered on a microscope. (B) View of square capillary tapered as seen by eye. (C) Assembly of injector, collector and thin capillary. (D) Microscope view of the injector and collector capillaries aligned with the thin capillary between the injector and square capillary.

Double emulsion production

Once the device is fabricated it is time to use it for ultra-thin double emulsion production. The working principles of the glass capillary devices have been explained in the previous section Microfluidic devices. The experimental setup for the production of ultra-thin double emulsions is common for the two types of devices we use during the development of the work (the ones for symmetrical liposomes production and the ones for asymmetrical liposomes production).

For injecting the different phases Harvard pumps (figure 15A) are used. This pumps are designed for injecting fluids at a constant flow rate that can be stablished by the user. The flow rate is controlled by controlling the speed of a moving wall that presses a syringe with the fluid to be injected (figure 15B). To determine the wall speed from the flow rate the diameter of the syringe must be introduced by the user too. The syringes used are BD plastic ones for the water phases and gas tight glass ones for the organic solvents (plastic ones can't be used with the organic solvent since they would be dissolved by the solvent as time passes contaminating the oil phase).

To connect the syringes to the device needle tips as the ones used for making the devices are added to the syringes. For the plastic syringes first an acrodisc from PALL company is added to the syringe so the solution is filtrated from dust and any other kind of dirtiness before entering the device and at the end of the acrodisc the tip is added (figure 15B). Then the tips of the syringes are connected to the needles of the devices (figure 15C) using polyethylene (PE) tubing from Scientific Commodities (figure 15D), Inc. The yellow needles and the glass round capillaries are connected using PE-5 tubing (0.86 mm of inner diameter) and the grey needles are connected using PE-2 tubing (0.38 mm of inner diameter).

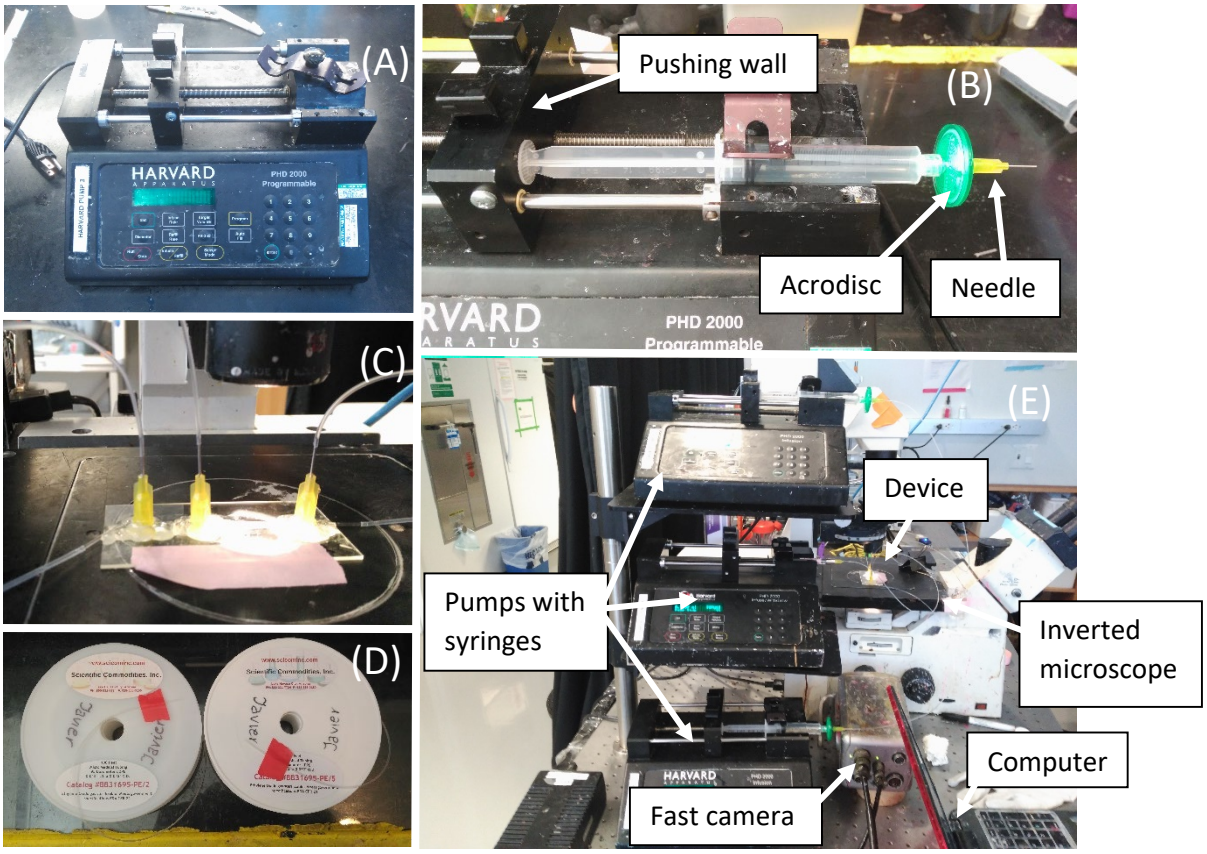

Figure 15. (A) Harvard pump. (B) Syringe with acrodisc and needle in Harvard pump. (C) Device connected using tubing. (D) Polyethylene tubing. (E) Setup for ultra-thin double emulsion production.

To observe the device working an inverted microscope setup with a Phantom V9.0 fast camera attached and connected to a computer is used. The complete setup for producing ultra-thin double emulsions is shown in figure 15E. The difference between using a device for producing symmetrical or asymmetrical liposomes lies in the number of syringes (phases) needed and the connections made.

Double emulsions for symmetrical liposomes

In the case of using a microfluidic device for symmetrical liposome production, three different phases are injected in the device:

- Inner water phase: Solution made of 9 weight percentage (wt%) of polyethylene glycol (PEG) 6000 Da number average molecular weight and 1 wt% of polyvinyl alcohol (PVA) in deionized (DI) water.
- Oil phase: Mixture of good lipid organic solvent, bad lipid organic solvent, lipids and fluorescence dyes (if needed). There are several recipes that can be used and will be discussed with more detail in the next sections.
- Outer water phase: Solution made of 6 wt% PVA in DI water.

The different phases are injected in the device as shown in figure 16.

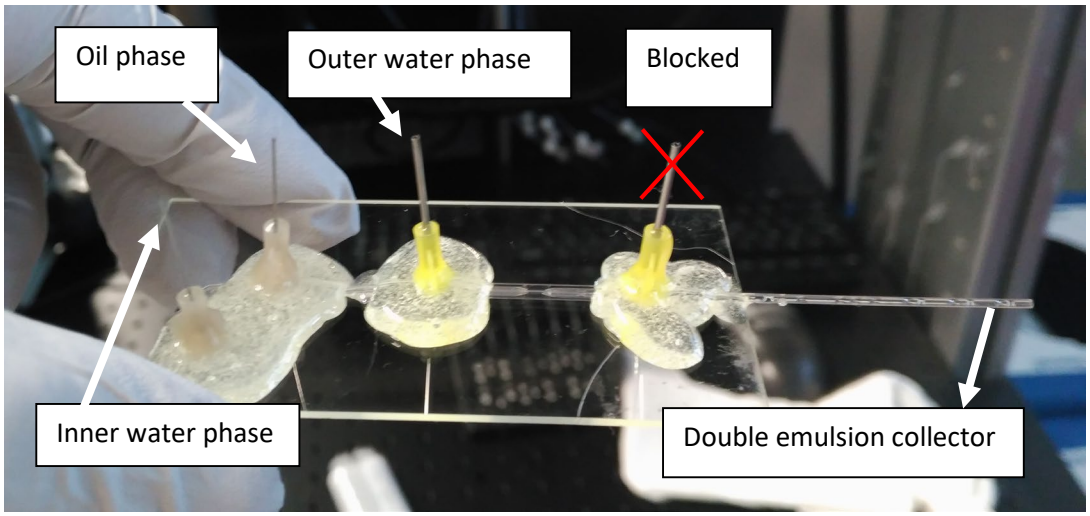

Figure 16. Different phases inputs and outputs indicated in a microfluidic device for symmetrical liposome production

When thinking about the flow rates of each phase no universal values can be given. This is because each new device works better with different flow rates and also a good device will work good in a wide range of flow rates and depending on what one wants to achieve some flow rates are more convenient than others (for example if we want double emulsions as thin as possible we will want to reduce the oil phase flow rate and increase the inner water flow rate or if we want to produce a high amount of double emulsions we will increase the oil phase and inner water phase flow rates). Some normal values for a correct working of the device are:

- Inner phase flow rate: ~ 800 µL/h
- Oil phase flow rate: ~ 500 µL/h
- Outer water flow rate: ~ 3000 µL/h

When everything is set and the microfluidic device starts to work the phenomenology described in Double emulsions for symmetrical liposomes can be observed. Images and videos showing the production of ultra-thin double emulsion using our handmade devices were taken and the most relevant features are shown in figure 17.

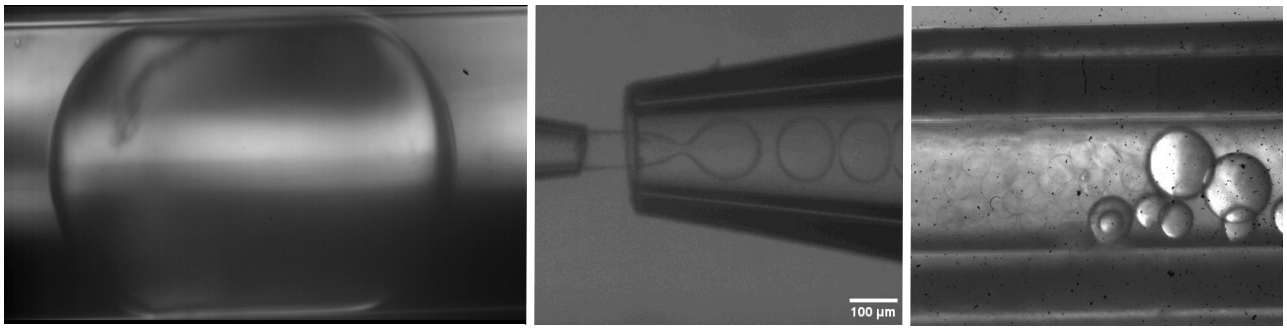

Figure 17. Left: Water drop surrounded by oil in the injector capillary. Middle: Ultra-thin double emulsion production in the dripping regime. Right: Oil drops followed by double emulsions in the collector capillary.

Double emulsions for asymmetrical liposomes

In the case of using a microfluidic device for asymmetrical liposome production, four different phases are injected in the device, the inner water phase, two oil phases with different lipid composition dissolved in them and an outer water phase. While in the symmetric case the outer water phase was injected in coflow with the other phases, in the asymmetrical case in order to increase the shear stress produced by the outer water flow producing thinner double emulsions the outer water phase is injected in coflow between the injector and square capillaries and in counterflow between the collector and square capillaries. The different phases are injected in the device as shown in figure 18.

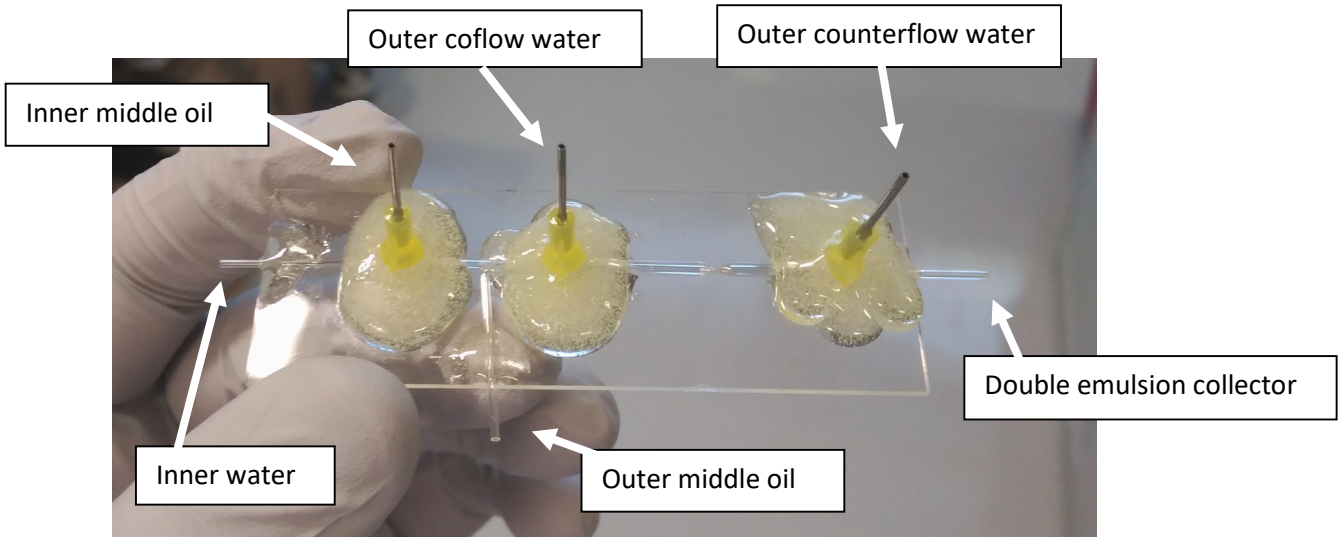

Figure 18. Different inputs and outputs of the glass microfluidic device for the production of asymmetrical liposomes.

When producing the double emulsions for asymmetrical liposomes two different production regimes were observed depending on the flow rates used. In these two regimes the flow rate of

the inner water and oil phases is the same and around 600 µL/h. The difference comes in the value of the flow rates of the coflow and counterflow outer water phase.

When these two flow rates are similar (in our experiments they were set at 3000 µL/h) the outer middle oil getting out of the small capillary inserted between the injector and square capillary is in the dripping regime, so when a drop forms it wets the injector tip and when double emulsions are produced from the injector tip the outer middle oil phase forms a layer that surrounds the double emulsions, forming the asymmetric double emulsions.

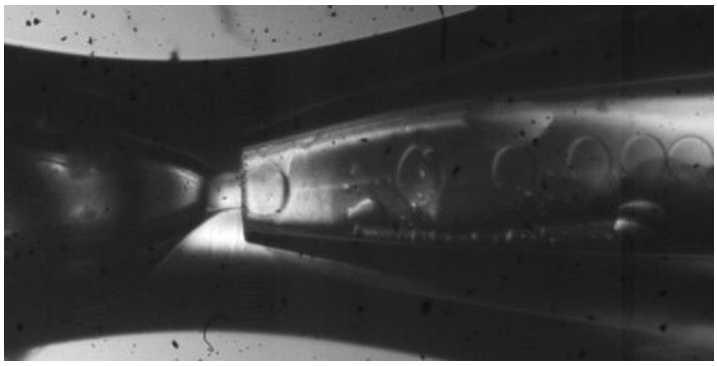

Figure 19. Double emulsion production for asymmetrical liposomes. It can be seen the outer middle oil surrounding the tip and forming and outer oil layer in the double emulsions formed.

When the counterflow outer phase flow rate is much higher than the coflow outer phase flow (in our experiments the first one is set to 9000 µL/h while the second one is set to 1000 µL/h) and some of the outer middle oil is accumulated between the injector and square capillary in a way that the right interphase of the accumulated outer middle oil is flat and bigger than the tip. When this conditions are met, when an outer middle oil drop is formed from the small capillary (the outer middle oil is in the dripping regime) the interphase moves to the right completely covering the tip, but after that, since the counterflow outer phase flowrate is higher than the coflow one, the interphase retreats back wetting the tip of the injector capillary, so when double emulsions are formed and outer middle oil phase layer surrounds the double emulsions, forming the asymmetric double emulsions.

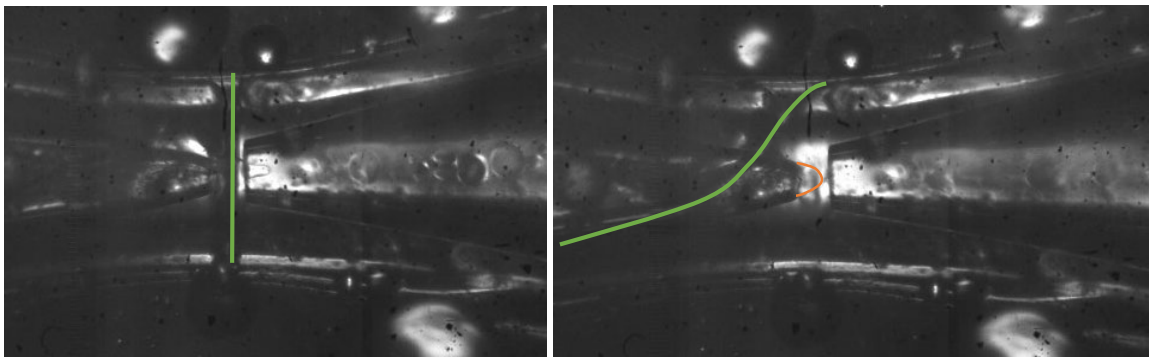

Figure 20. Double emulsion production for asymmetrical liposomes The green line shows the outer middle oil interphase and the orange line the thin double emulsion contour. Left: The outer middle oil interphase is flat and covers completely the injector tip. Some thick double emulsions are produced, but these double emulsions are very unlikely to survive. Right: The outer middle oil interphase has moved to the left of the tip leaving a thin outer middle oil layer that surrounds the ultra-thin double emulsions that are produced.

The road towards symmetrical liposomes production

The theory of symmetrical liposomes production using ultra-thin double emulsions as templates for their formation seems really straightforward, but as it usually happens, the experimental counterpart faces different problems that can complicate the realization of the theory in the lab. In the production of double emulsions using glass capillary devices, the solutions used were:

- **Inner water phase:** Solution of PEG 6000 and PVA in DI water.
- **Oil phase:** In the oil phase, chloroform or dichloromethane were used as good lipid solvents and toluene or pentane were used as bad lipid solvents. As lipids 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) was used for the single component liposomes and a mixture of DOPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and cholesterol is used for the phase separation multicomponent liposomes. In order to be able to see the different phases in the liposomes that phase separate 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt), also known as DOPE-Rhodamine, is used as the dye for the liquid disordered phase and naphtho[2,3-a]pyrene is used as the dye for the liquid ordered phase.
- **Outer water phase:** Solution of PVA in DI water.
- **Collection solution:** As the collection solution a phosphate buffer solution was diluted in water to an osmolarity around 10-20 mOsm below the osmolarity of the inner water phase. The osmolarity of the solutions is measured using The Advanced® Micro-OsmometerModel 3300.

In the first experiment the lipids were dissolved in chloroform:pentane at a ratio 1:1.8. The inner water phase is made of 8% PEG 6000 and 2% PVA and the outer water phase is made of 10% PVA. Since the double emulsions that were collected in this first experiment did not transform into vesicles but burst, more experiments were done introducing small changes to the solutions composition and different parameters of the double emulsion production. A detailed explanation of the different experiments made can be found in Annex B: The road towards symmetrical liposomes production. The different changes made in the experiments until the first liposomes were obtained are:

1. Reduction of PVA concentration in the outer water phase from 10 to 6 wt%. The reason behind this change is that PVA as a surfactant adheres to the double emulsion surface and can induce some surface tensions that lead to the instability and breaking of the double emulsion. This change is maintained in the rest of experiments.
   Also different volume ratios of chloroform:pentane were tested. This way having less pentane left when the chloroform evaporates will lead to a bigger attraction between leaflets in the double emulsion so the dewetting phenomena can begin more easily. The chloroform:pentane ratio used normally is 1:1.8.

2. Substitute pentane by toluene and decrease the ratio chloroform:toluene to the minimum possible. Since the solubility of toluene in water is higher than the solubility of pentane, the objective of this change was to make the poor solvent dissolve faster in water so the dewetting phenomena can trigger in less time.

3. Inner water solution changed from 8% PEG 6000 and 2% PVA to 9% PEG 6000 and 1% PVA. This change is maintained in the rest of experiments.
   Collection vials with larger surface area to increase evaporation rate and fasten dewetting.
   Change flow rates to get double emulsions as thin as possible, so less oil has to be dissolved in the buffer.

4. Lipid and sterol concentration increased from 5 mg/mL to 7 mg/mL. This is done to increase the attraction between leaflets so the dewetting phenomena is triggered easier.

5. Substitute chloroform by dichloromethane which is much more volatile and soluble in water, so it will disappear faster from the double emulsion. The dichloromethane: pentane ratio used is 1:0.9.
   Apart from normal vial, use vials with hydrophobic treated bottoms so this favours that the oil leaves the double emulsions. This can be seen by the organic solvent bubbles that forms at the bottom when collecting the double emulsions (figure X).
   To make this special vials we take 20 mL normal vials and scratch the curved area that joins the bottom with the walls with a diamond tip pen. Then we hit the bottom with the other side of the pen and due to fracture propagation in the zones with most defects (that is where the glass of the glass is curved) the bottom of the vial is separated from the rest. Glass slides of 25x25 mm are immersed in a solution of 0.1 wt% of Trichloro(1H,1H,2H,2H-perfluorooctyl)sylane in hydrofluoroether (HFE) 7500 and left there overnight so they get hydrophobic coating. Then the slides are taken out of the solution, and the HFE is washed out using Isopropanol. Finally, the glass slides are glued to the bottom of the vials we removed the bottoms from with epoxy adhesive (figure 21).

6. Use glass syringes to pick the organic solvents for making the oil phase instead of using plastic tip pipettes. The hypothesis behind this change is that the organic solvent dissolved some of the tip plastic and these molecules prevented the double emulsions from becoming liposomes.

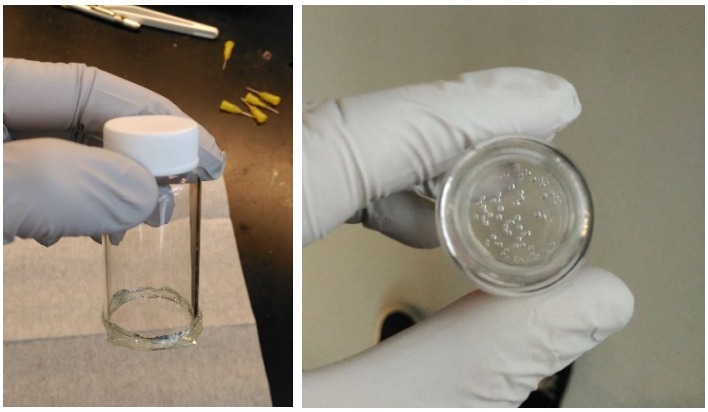

Figure 21. Left: Vial with hydrophobic glass slide substituting its bottom. Right: Oil bubbles formed in the hydrophobic bottom when double emulsions are collected in this

After this last change, liposomes were obtained with the dichloromethane-pentane and chloroform-pentane recipe in treated and not-treated vials. The conclusion of this experiments is that probably it was picking the organic solvents with plastic tip pipettes what prevented the double emulsions from becoming liposomes, probably because some plastic of the tips was dissolved when picking the organic solvent. Also, some changes made before may change the stability of the liposomes obtained. For example, decreasing the evaporation rate of the oil could be beneficial for obtaining a larger number of liposomes, since a slower evaporation means smaller tensions in the double emulsions and more stability.

Results and Discussion

Symmetrical liposomes

Single component

The single component liposomes studied are made of DOPC, which is phospholipid with a low melting temperature (-17 °C), so the resulting liposomes are in the liquid disordered phase. After collecting ultra-thin double emulsions with this lipid dissolved in organic solvents and waiting until the dewetting process finishes, the DOPC single component liposomes were obtained.

We can know if the double emulsions have become liposomes just by checking them by eye on a microscope. When the double emulsions dewet into liposomes they become more transparent to bright field microscopes since when the oil evaporates the double emulsion becomes thinner and thinner until it becomes a liposome, with a thickness of around 10 nm. Also they grow in size. The growth in size happens because the liposome membrane is impermeable to the big polymer molecules in the inner solution (PEG 6000 molecules) because of their size, while they are permeable to water [32] and the other molecules and ions ([33], [34]) present in the different solutions. This way, to balance the osmolarity of the inner and outer solutions, as the different molecules except PEG 6000 can cross the membrane, at the end, their concentrations inside and outside the liposome is the same. But to balance the osmotic pressure due to the difference in PEG 6000 concentration, water enters the liposome diluting the PEG 6000 solution.

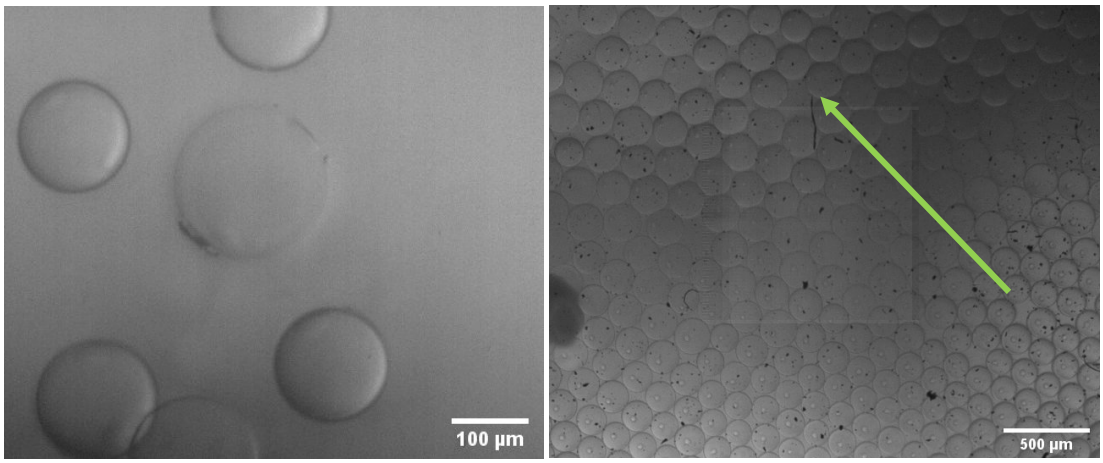

Figure 22. Left: Liposome and double emulsions in the same image. This image shows the increase in transparency and size when the double emulsions become liposomes. Right: Size gradient of double emulsions due to different oil evaporation rates.

The growth in size is possible not only because of the osmolarity difference and permeability characteristics of the membrane but because of the excess of lipid on the solution. By lipid excess, it is meant that the number of lipid molecules in the double emulsion is bigger than the number needed to make a single bilayer with the size of the double emulsion. This way, the liposome can grow until it becomes unilamellar, so there is no more lipid excess, and then the osmotic pressure is balanced by the membrane tension.

In the right image of figure 22, a gradient in size can be observed. In this case as we go in the direction where we see the increasing of size we will reach the border of the double emulsion aggrupation, while the smallest double emulsions are in the centre of the aggrupation. This fact can be explained easily taking into account that when we have a lot of double emulsions together the water around the double emulsions is saturated with oil so the evaporation rate is decreased. It is in the borders of the aggrupation where the evaporation rate is faster since the water does not get saturated so easily and there are less double emulsions per water unit volume. Due to this faster evaporation rate in the borders, the double emulsions at the borders dewet faster than the ones in the middle and their increase in size is produced before, leading to the size gradient shown.

Mechanical properties

Despite the increase of transparency and size of the double emulsions when the oil evaporates and the liposomes are formed, there is no way to know just by looking at the liposomes if there is still some of the organic solvent in the bilayer. One way to test if the liposomes made are like the ones that has been produced using other methods is by testing some of their properties. One property that could be affected by the presence of residual organic solvent is the area compressibility modulus due to the fluidization of the membrane produced by the solvent. The experimental method and model used to obtain this mechanical property of a liposome is described in Atomic force microscopy (AFM) measurement.

The measurement of the area compressibility modulus of DOPC liposomes using AFM techniques was possible thanks to the Micro/Nano Bio-mechanical characterization lab in Northeastern University. The force-displacement curves were obtained thanks to the equipment and help of this laboratory and the curves were analysed by using a custom program made by this laboratory that fits the curves to the model to obtain the area compressibility modulus.

When doing the experiment, the force displacement curves of different liposomes were measured. As shown in figure 23, three different types of force-displacement curves were obtained in the AFM measurements.

The first one is a curve that continuously grows and the slope of the force increases when increasing the displacement. This is how the force displacement curve should look.

The second one is a curve that at the beginning it starts like the first kind of curve but at a certain displacement the force falls down abruptly. This sudden fall of the value of force can be easily understood when the liposome that was compressed was observed during the experiment. During the experiment one could see that at a certain point, the liposome slipped all of a sudden, so the liposome was released from the pressure that was applied to it and because of that the force acting on the liposome drops. This probably happened because the cantilever was not perfectly parallel to the ground and/or the point where the pressure was applied was not exactly aligned with the centre of the liposome.

The third type of curve is one that changes smoothly and has an inflection point changing from concave to convex. This happens because the liposome slips progressively in a smooth way, and so it does the force acting on it.

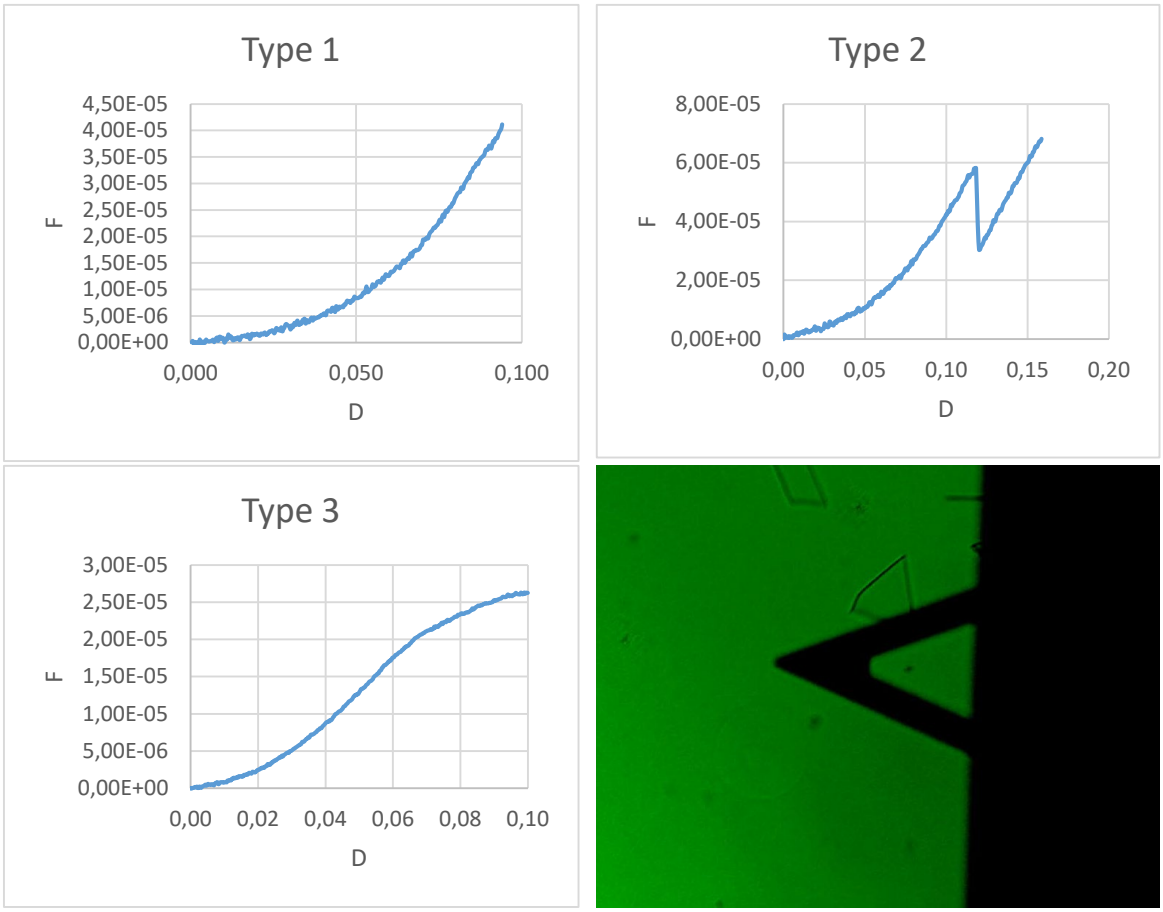

Figure 23. Different types of force-displacement curves obtained during the AFM experiments. The values of the axis are normalized for the analysis of the data. Bottom left: Image of the AFM probe seen from above and a liposome next to it.

When making the data analysis, the third type curves will not be taken into account, since the progressive slipping affects the shape of the force-displacement curve and so it will lead to a wrong result. The second type curves can still be used to obtain the mechanical properties if it is analysed only the part of the curve before the sudden force drop since the liposome is static in this part and because of that the data is valid. Also sometimes instead of getting the second or third type we get a mixture of them. In this case, the liposome is slowly slipping until some displacement point where it suddenly slips much faster, producing a sudden decrease in force. This curves won't be taken into account for the same reason as the third type curves.

After the data analysis we obtain the following results for the area compressibility modulus of different liposomes:

Sample number	Diameter [µm]	KA [N/m]
2	90,34	0,5
3	91,03	0,26
4	46,41	0,31
5	90,07	0,29
7	62,41	0,29
8	55,51	0,14
9	68,48	0,5

$$K_A = 0.327 \pm 0.121 \ \frac{N}{m}$$

This result is in agreement with the area compressibility modulus obtained by Edith Schäfer using this same method on DOPC liposomes obtained by electroformation [31]: K_A= 0.280 ± 0.120, and with previous work where the compressibility modulus was measured by micropipette aspiration [35]. This means that the liposomes produced using microfluidic methods have the same mechanical properties than the ones made using conventional methods, so if there is some residual organic solvent, it has no significant effect on the mechanical properties of the bilayer.

Ternary mixture

Phase separation

Another well studied feature of liposomes is phase separation. It has been shown than liposomes made of a mixture of a high melting temperature phospholipid, a low melting temperature phospholipid and a sterol undergo phase separation of liquid ordered and liquid disordered phases when mixed in adequate proportions (see Annex A: Theoretical framework).

In our experiments the high melting temperature phospholipid used is DPPC, the low melting temperature phospholipid is DOPC and the sterol is cholesterol. They are mixed in a molar proportion of 40% DOPC, 40% DPPC and 20% Cholesterol. In order to be able to observe phase separation two fluorescent probes are used. One of them (DOPE_Rhodamine) has a preference for the liquid disordered phase (this happens because it is basically an unsaturated phospholipid with the same fatty acid tails as DOPC and the Rhodamine fluorescence molecule covalently bond to the polar head), and the other (Naphthopyrene) has a preference for the liquid ordered domains [36]. This two fluorescence probes have different excitation and emission wavelengths, so they can be observed simultaneously. One problem with using naphthopyrene as the liquid ordered phase dye is that it bleaches out easily, so after some time passes the fluorescence of this dye is lost. Because of this in some images only the Rhodamine dye (red channel) is observed.

When adding the dyes to the mixture, DOPE_Rhodamine molecules are considered to behave as DOPC molecules, so the 40% of DOPC molecules that was stated before would correspond to 39% of actual DOPC molecules and 1% DOPE_Rhodamine molecules. The same is done with the naphthopyrene and DPPC molecules, so in the final mixture we will have 37% of DPPC molecules and 3% of naphthopyrene molecules.

The fluorescence is observed using confocal microscopy, so the probes are excited with the correct wavelength laser and the fluorescence is observed through three different channels. Two of them capture only the wavelengths around the emissions wavelengths of the fluorescence probes, so each one shows the fluorescence of a different probe. The other channel just shows the bright field image.

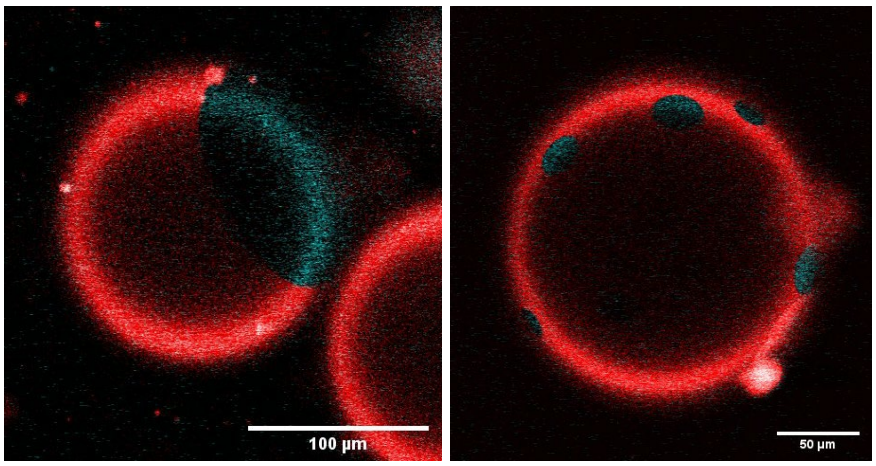

Figure 24. Phase separation images taken on confocal microscopy with channels superposed. Red channel corresponds to the liquid disordered phase (Rhodamine probe). Blue channel corresponds to the liquid ordered phase (Naphthopyrene probe). Left image: One big liquid ordered domain can be seen. Right image: Several small liquid ordered domains can be seen.

This way the phase separation can be observed. When observing different liposomes with phase separation it can be seen that some of them have only a one big domains while other present a lot of small domains as shown in figure 24. This is explained because the domains start forming randomly in different places, so at the beginning of phase separation a lot of small domain are present, but as time passes the domains grow and merge. This merging is due to the movement of the domains on the fluid membrane, so with time they have the change to come together and merge in a bigger domain. At the end, the growth and merging of the different domains results in one big domain. This way depending on how much time has passed since the starting of the phase separation it can be observed several small domains in the early stages or only one big domains in the stationary state.

Another phenomenon related to phase separation has been observed in one of the vials with liposomes that undergo phase separation. This phenomenon consists on the adhesion of liposomes and the adhesion region in the majority of the cases is in the liquid ordered phase. In the vial it was observed the adhesion of five liposomes between each other and all the adhesion regions were in the liquid ordered phase as is shown in figure 25.

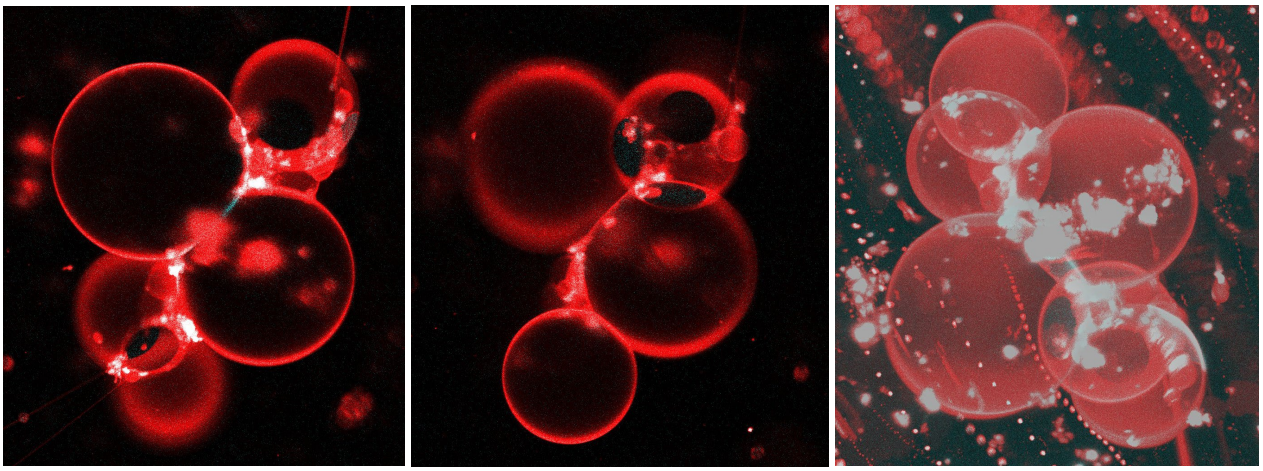

Figure 25. Left and middle: Different z planes of the five adhered liposomes. Blue and red channel superposed. It can be seen that the different adhesion regions between liposomes are in the liquid ordered phase. Right: 3D reconstruction of the adhered liposomes. For making the reconstruction a z-stack of 200 planes was taken using confocal and Fiji was used to make the reconstruction. In the reconstruction some adhesion regions in the liquid ordered phase can be seen.

This same phenomenon was also reported by Anqi Chen, another Weitz's lab member who works on phase separated liposomes. In her experiments she shows that there is no fusion of the two membranes but the two bilayers are just adhered to each other by some attracting mechanism. This adhesion has been studied before in liposomes formed by electroformation [37] where it was shown that when given the conditions for the domain formation in adhered liposomes, the liquid ordered domains appear in the adhesion regions.

The theory that explains the adhesion of liquid ordered domains was developed by E.A. Evans and V.A. Pargesian [38], and they predicted this adhesion in the discussion section: "stable adhesion is predicted to be promoted by membrane rigidification". So as the liquid ordered domains are more rigid than the liquid disordered domains, the adhesion is more stable when the adhered region are in the liquid order state. This stability is a result of entropic effects, since the thermal fluctuations of membranes acts as a repulsive force preventing adhesion. This way, the more rigid a membrane is, the thermal fluctuations will be smaller and the repulsive force by this fluctuation smaller, so the adhesion is more stable.

Temperature effects on domains

One way to verify if the domains observed in the phase separated liposomes made using microfluidic methods behave like the domains observed in liposomes made using conventional methods is varying the temperature. When increasing the temperature, the thermal energy of molecules is increased and the entropic effects become more and more relevant until the entropic effects become more important than the enthalpic effects and the domains disappear leaving a liposome with homogeneous composition. The temperature where the entropic effects are equal to the enthalpic effects is called critical temperature and depends on the composition of the liposome (see Annex A: Theoretical framework).

To test if the domains in our microfluidics made liposomes behave the same way as the domains of the liposomes produced with conventional methods the temperature is first risen to 40 °C, slightly below the melting temperature of DPPC, the high melting point phospholipid (41 °C) and above the miscibility transitions temperatures mapped in previous works [6]. At this temperature the domains should disappear and the composition of the liposome should become homogeneous. The observation of the domain disappearance is made using confocal microscopy taking videos when changing the temperature. The temperature is controlled using an Okolab cage incubator mounted on the confocal microscope. As shown in figure 26 the disappearance of the domains was observed.

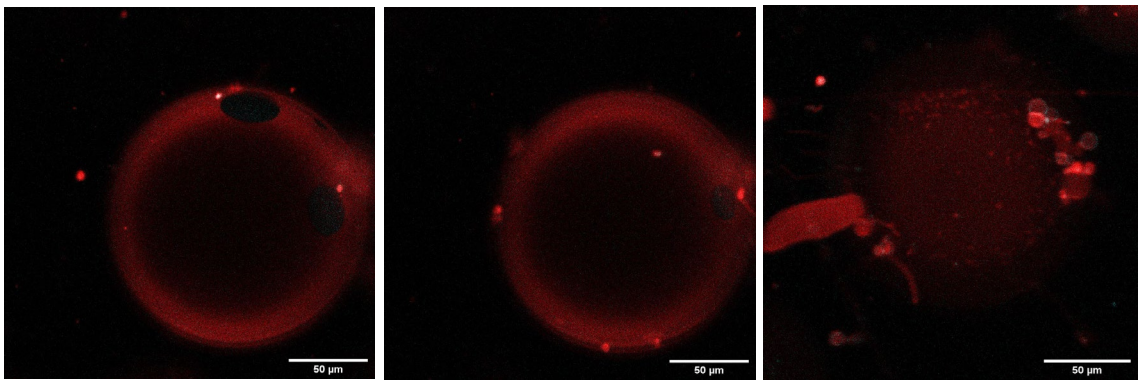

Figure 26. Images of a phase separated liposome at 40 °C as times passes. The time difference between images is around 10 minutes. It can be seen the reduction in size of domains (left and centre image) and their final disappearance (right image).

Then the temperature is lowered again to 27 °C (below the miscibility transition temperature at this composition) and the appearance of small domains that grow and merge as time passes is observed (figure 27).

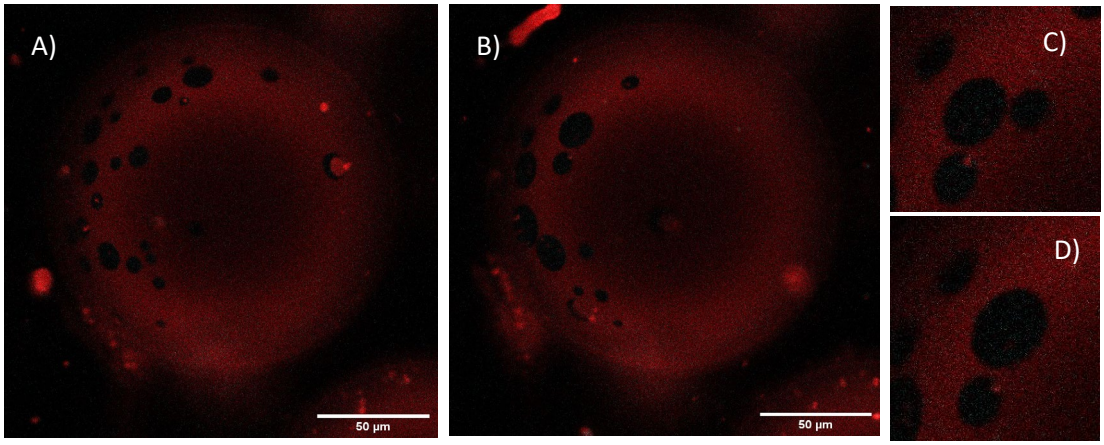

Figure 27. A) Many small domains that appear after reducing the temperature to 27 °C. B) As the time passes the domains grow and merge together, so bigger and fewer domains can be seen in the liposome. C) Two domains very close to each other before merging. D) A bigger domain result of the merging of the two closest domains in C).

After checking that the domains disappear above some temperature and appear again when decreasing the temperature, the next step was trying to find the miscibility transition temperature for one liposome. To do that, starting at 27 °C (temperature we know that is below the critical temperature) the temperature was increased by steps so for each new temperature, after the chamber temperature is the one desired a video is taken for 30-60 minutes. During this time the liposome stabilizes to the new temperature, as can be observed in the fact that at the end of the video no significant changes in the domains can be observed.

One problem of using an incubator to change the temperature of the liposomes is that the incubator is designed to keep biological samples at a certain temperature, so despite being pretty stable the changes in temperature happen slowly. Because of that only five temperature points could be explored in this experiment. Figure 28 shows images of the phase separated liposomes at different temperatures in the stationary regime.

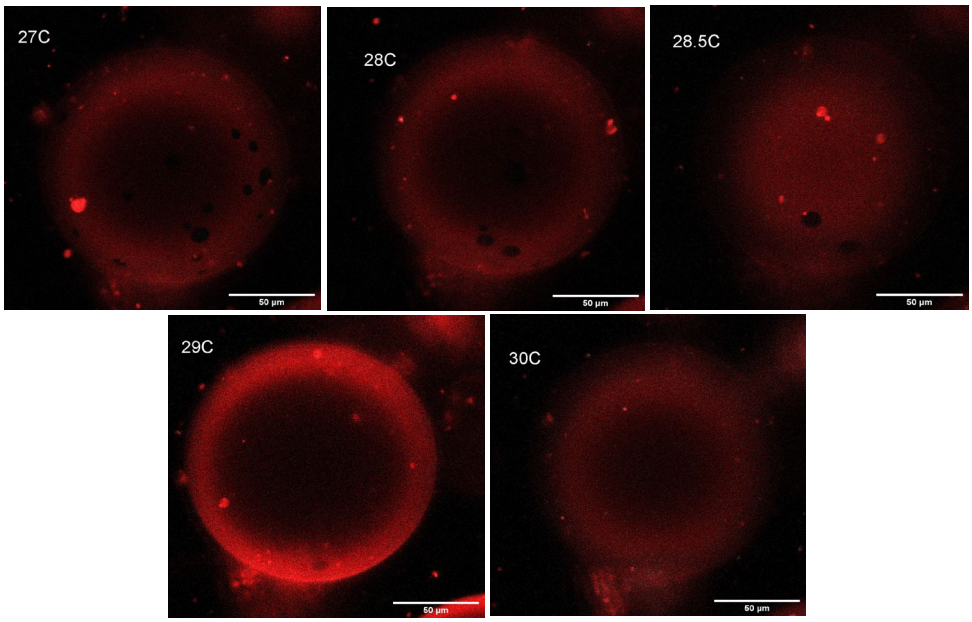

Figure 28. Phase separated liposome at different temperatures until the domains disappears.

At the start of this experiment at 27 °C several small domains can be observed. This is because this experiment was done after reducing the temperature from above the miscibility transition temperature, and not enough time was waited for the merging and growing of domains. When increasing the temperature what can be observed is that the size of the domains remains constant while their number decrease. If we had started with one big domains instead of a lot of small domains probably what we would have observed would be a decrease in the size of this domain.

At 29 °C only one domain can hardly be seen and at 30°C no domains can be observed. The miscibility transition temperature for this liposome is between 29 and 30 °C. We can now check in the miscibility transition temperature diagram the theoretical transition temperature for the composition used (40% DOPC, 40% DPPC, 20% Cholesterol) and study its consistency with the miscibility transition temperature obtained.

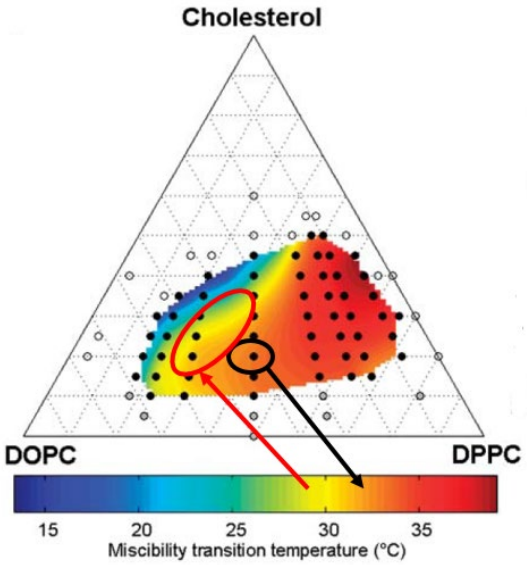

Figure 29. Miscibility transition temperature experimental diagram. The black circle corresponds to the point of the theoretical concentration of the liposomes prepared (40% DOPC, 40% DPPC and 20% Cholesterol) and the black arrow points it's miscibility transition temperature. The red circle indicates the region of concentrations for the miscibility transition temperature found in experiments. The errors of this diagram are of ± 1°C. Diagram extracted from [7].

As can be seen in figure 29, the theoretical miscibility transition temperature of our liposomes attending to their composition should lie between 31 and 33 °C. Experimentally, the miscibility transition temperature has been found to be between 29 and 30 °C. The theoretical and experimental miscibility transition regions are pretty close from each other (also it has to be taken into account that the miscibility transition diagram has an error of ± 1 °C).

One possible explanation for this difference is that the exact composition of the liposomes prepared is 39% DOPC, 1% DOPE_Rhodamine, 37% DPPC, 3% naphthopyrene and 20% Cholesterol. While DOPE_Rhodamine is a phospholipid with a fluorescence Rhodamine molecule in its head and the same fatty acid tails than DOPC, naphthopyrene molecular structure is very different from the phospholipids one and it is more similar to the cholesterol structure. Because of this, maybe the naphthopyrene molecules behave more like cholesterol and could be taken into account as cholesterol molecules for practical effects. Taking this into account the

“effective” composition of our liposomes would be of 40% DOPC, 37% DPPC and 23% cholesterol. Attending to the diagram, the miscibility transition temperature of this composition is smaller than the one for the 40% DOPC, 40% DPPC, 20% Cholesterol composition, more consistent with experimental results. Also there is always some error when preparing the solutions so the real composition of the liposomes differs a little bit from the one planned.

Because of this, the experimental miscibility transition obtained for these liposomes made using microfluidic methods is consistent with the results obtained for liposomes made using conventional methods.

Shape deformation

For the more stable multicomponent liposomes produced (see Increasing the stability of liposomes), it was observed that several days after the formation of domains, the shape of the liposomes changed. The new shape adopted by the liposomes is a snowman like shape where each of the parts has a different lipid composition (figure 30), so one part is rich in DOPC and is in the liquid disordered phase and the other part is rich in DPPC and cholesterol and is in the liquid ordered phase.

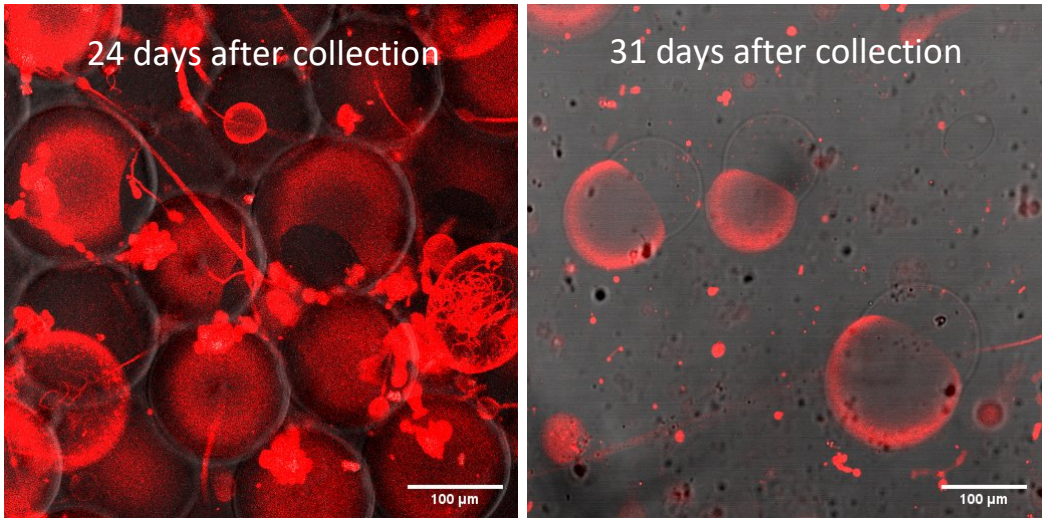

Figure 30. Left: Spherical liposomes with domains after 24 days. Right: Snowman shape liposomes after 31 days. By this time the naphthopyrene dye has already bleached and only the Rhodamine can be seen (red channel) The shape of the liposomes can be seen by the bright field channel and the Rhodamine is only present in one of the halves of the liposome. Because of this we can induce that one half is rich in DOPC (liquid ordered phase), while the other is rich in DPPC and cholesterol (liquid ordered phase)

This shape deformation has been already observed and studied experimentally for the liposomes produced using conventional methods ([39], [40], [6]). This results are also backgrounded by theory that explains that this shape is actually the stable shape for this phase separated liposomes ([41], [42]). The main mechanism responsible for this shape change is the line tension σ of the domain border. The energy contribution of this line tension is not negligible to the free energy, and because of that to minimize the free energy the length of the domain border decreases forming the neck that gives the snowman shape. The difference in mechanical properties of the liquid ordered and disordered phases plays also a role in the final shape of the liposome.

Recent experimental and theoretical work ([43], [44]) shows that when forcing the shape of a multicomponent liposome, the curvature of the shape has an effect on the composition and localization of domains. This way, the shape of the liposome has effect on the domain formation and composition, so the shape can increase the stability of domains.

Expecting to observe the transition from the snowman shape to a spherical shape by the disappearance of domains induced by the mixing of lipids when the temperature is increased, the snowman liposomes were left overnight in confocal microscope at a temperature of 44 °C (temperature which correspond to the highest temperature that can be set in our incubator and is above the melting temperature of DPPC) and a video was taken (figure 31).

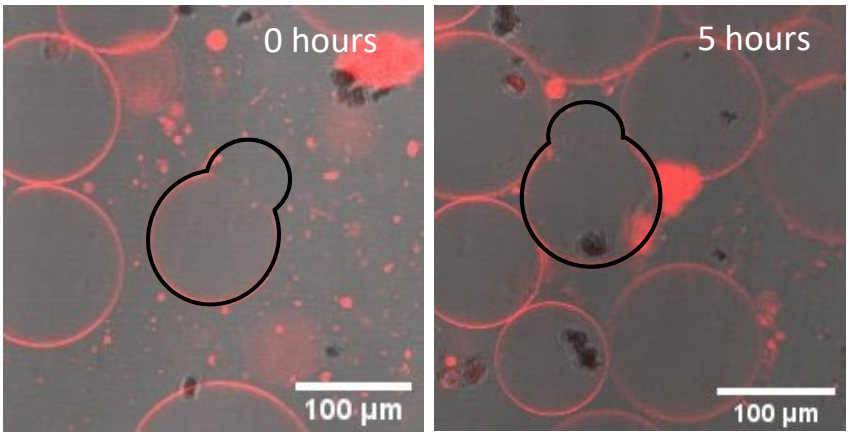

Figure 31. Snowman shape liposome at 44 °C at the start of the experiment and after 5 hours have passed. The shape is highlighted in black. No significant difference can be seen in the shape. The possible difference in shape can be a result of the slightly different orientation of the small sphere of the liposome in both images and the pressure of the surrounding liposomes.

Contrary to expected no domain disappearance could be observed in the 5 hours that the same shaped liposome was observed (after that time it broke) and no significant changes in the shape were seen. Since in previous experiments with temperature the domains disappeared in less than half an hour at 40 °C it can be stated that the domains of the shaped liposomes are much more stable than the ones observed before.

What may be happening is that when the liposomes phase separate, after some time, the line tension and difference in the mechanical properties of the different phases drives the shape deformation to a snowman shape. This shape is then the most stable for the composition of the liposome. Then, this shape increases the domain stability in such a way that even after going up the melting point of DPPC they are stable and the domains do not disappear.

The conclusion of this experiment is that for our liposomes the phase separation drives the shape deformation, and the shape deformation is responsible for the enhanced stability of domains. This way there is a feedback between the domains (inhomogeneity in composition) and the shape deformation so each one stabilizes the other.

Osmotic effects

Another phenomenon worth studying in phase separated liposomes is the effect of increasing the osmolarity of the outer medium in phase separated liposomes. This osmolarity increase increases the outside pressure on the liposomes, and this pressure is slowly reduced as the inside water gets out through the membrane. In our case we also can try to observe the effects of increasing the outer medium osmolarity on snowman shape liposomes.

Previous studies have shown the complete budding of the liquid ordered domain to the inside or outside of the liposome when the osmolarity is increased [45]. Also, studies on the osmolarity effect on symmetrical liposomes that do not phase separate have been recently made [46].

To increase the osmolarity 200 µL of solution with liposomes is picked up from the collection vials using a pipette and it is put in a smaller handmade recipient. The osmolarity of the original collection solution is around 100 mOsm. Then a 200 g/L solution of glucose in water (1110 mOsm) is added in 100 µL doses. The liposomes are then imaged using confocal microscopy.

The first interesting phenomenon is the presence of invaginations or smaller spheres attached at the inner side of the liposome as reported before in liposomes that do not phase separate [46]. These invaginations have been observed in liposomes with and without phase separation (figure 32).

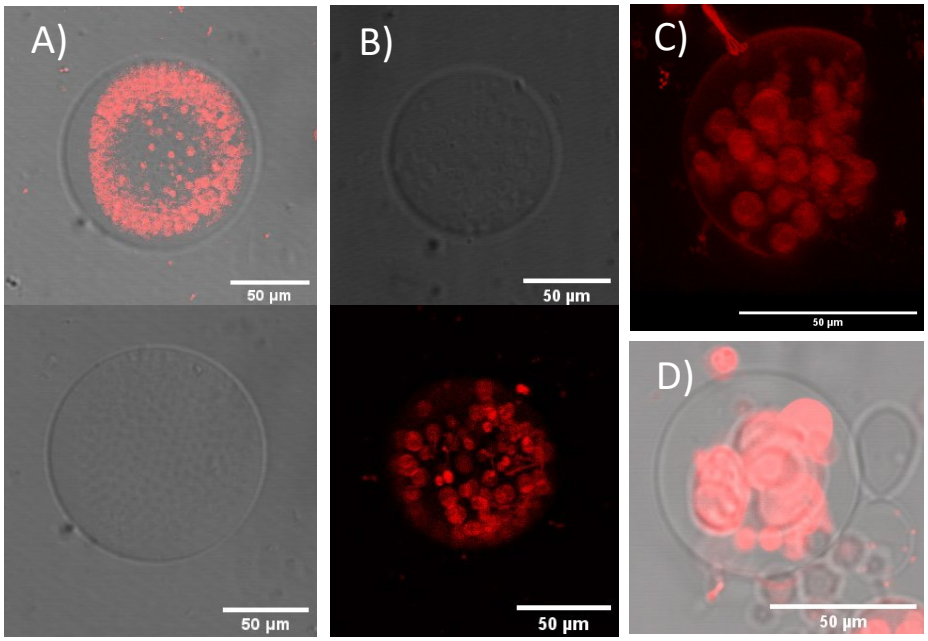

Figure 32. A) Phase separated liposome. The invaginations are present in the liquid disordered phase and the liquid ordered phase is smooth in the bright field image. B) Invaginations in a liposome that has no phase separation. C) Image taken from a 3D reconstruction of a phase separated liposome with invaginations. Through this reconstruction one can see that this spheres are in the inside of the liposomes and so they are invaginations of the membrane. D) Extreme case where all the liquid disordered phase has budded inside the liposome, so the liposome is in the liquid ordered phase (no red fluorescence). The osmolarity of the outer medium is around 430 mOsm for A) and around 600 mOsm for B), C) and D).

What is interesting and new is that for the phase separated liposomes, the invaginations are only present in the liquid disordered phase. This can be noticed because in the bright field image in figure 32A the invaginations can be seen by eye as small circles, but in the right part of the liposome the surface is free from this circles and seems to be smooth. Also, when superposing

the red channel, where the fluorescence of the Rhodamine is observed, to the bright field channel it can be seen that no red fluorescence can be seen in the smooth part. This indicates that the smooth part is the liquid ordered domain. This difference in the behaviour of the phases is understandable since the liquid disordered phase has a lower area compressibility modulus, so it's easier to deform the liquid disordered domain than the liquid ordered one. This way, the domain that deforms until equilibrium is reached is the liquid disordered on

An extreme case of this invaginations is observed when increasing the outer osmolarity to around 600 mOsm and is that all the liquid disordered phase buds inside the liposome, so at the end we get a DPPC liquid ordered liposome with DOPC liquid disordered structures inside, as shown in figure 32D.

The second phenomenon observed is the loss of spherical shape of some liposomes. When the osmolarity of the outer medium is increased water starts to get out of the liposomes slowly. This way while the area of the liposome remains constant the volume decreases, so the shape stops being spherical. The liposomes observed without spherical shape (figure 33) are not phase separated and their size is about as half as the original liposomes. The reason of this might be that with the osmotic shock some liposomes break forming smaller ones, or the liquid ordered phase buds out leaving only the liquid disordered part of the liposome (as will be shown later), and then the inside water of these smaller liposomes starts getting out of them. Also the formation of invaginations can be observed in these non-spherical liposomes.

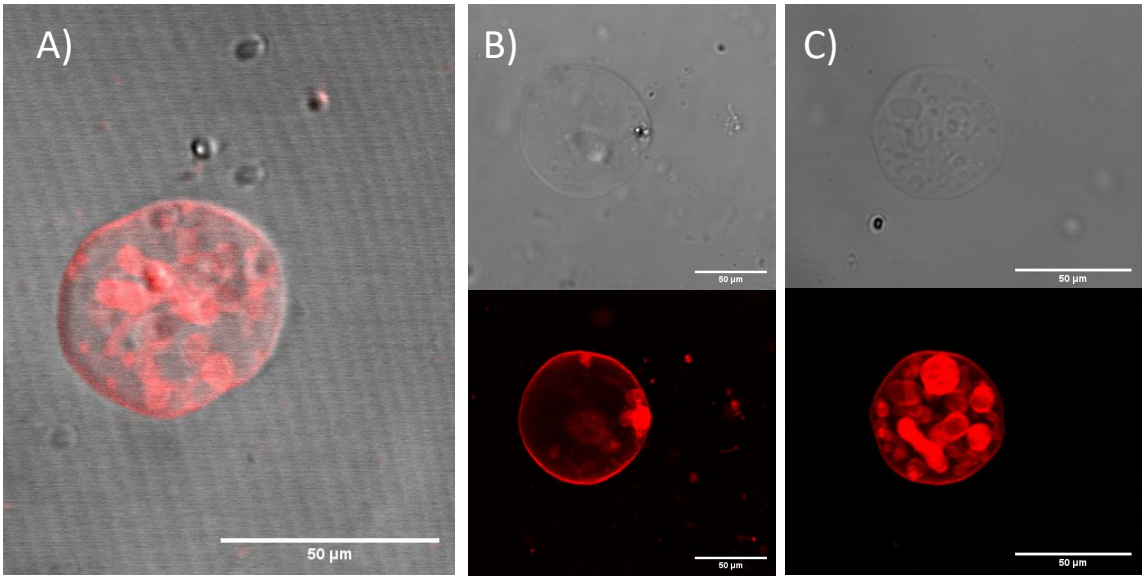

Figure 33. Images of non-spherical liposomes with invaginations of their membrane. Since the red fluorescence is all around the liposome there is no phase separation in these liposomes. Probably they were formed from the liquid disordered domain of bigger ones when budding the liquid ordered phase or breaking because of the osmotic shock. In all the images the osmolarity of the outer medium is around 600 mOsm.

The third phenomenon observed is the budding of the liquid ordered phase in snowman shape liposomes (figure 34). For observing this, after the osmotic pressure was increased one snowman shape liposome was localized and images were taken over time. It was not possible to record a video because when adding the solution, the liposomes started to float around, and when the snowman shape liposome was found and the budding phenomenon observed, this liposome was sinking to the bottom of the vial. The induction of the budding phenomena when

the osmolarity of the outer medium is increased was predicted theoretically several decades ago [41]. The reason for this budding is that under this osmotic pressure water slowly comes out of the liposome, reducing its volume. The reduction of volume favours the reduction of the domain boundary decreasing this way the line tension and finally ending up in the budding of the liquid ordered domain. The budding process takes place in the seconds to minutes time scale.

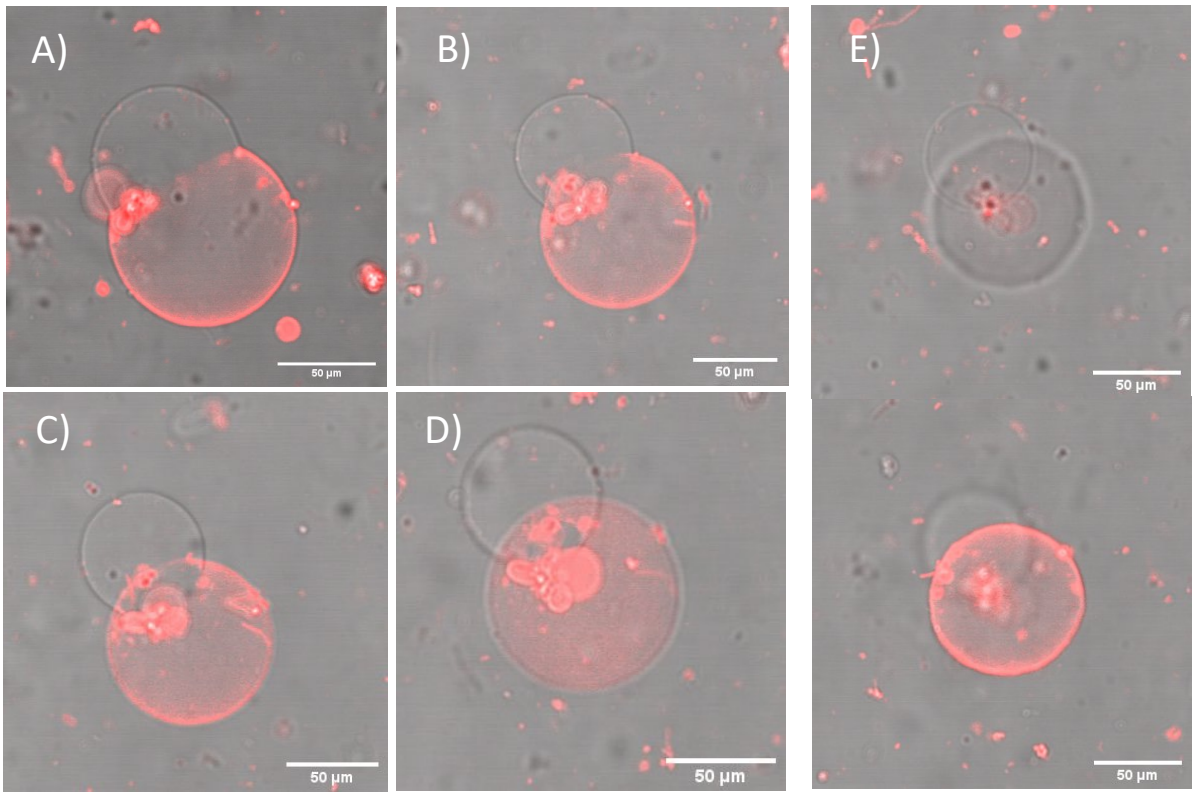

Figure 34. From A) to E) images taken of the budding process when the outer medium osmolarity was increased to around 430 mOsm. Bright field channel and red channel (liquid disordered phase dye) are superposed. From A) to D) the reduction in size of the neck of the snowman shape can be clearly seen as the budding process occurs. E) Two different z-planes images taken after the budding process is finished. The upper one shows the budded liquid ordered domain that forms a new liposome and no fluorescence is seen. The bottom one show the liposome resulting after the liquid ordered phase buds. The composition of this new liposome is the same of the liquid disordered phase of the snowman shape original one.

This phenomenon could be observed on another snowman shape liposome, but surprisingly in this case, after the liquid ordered phase budded, both new liposomes merged again, forming at the end a spherical phase separated liposome (figure 5). What could explain this merging after budding would be that after budding the two liposomes are not completely separated but in contact by a small neck and some of the lipid structures formed by invagination of the liquid disordered phase keeps them together (figure 35A). Also during the budding process some membrane area could have been lost forming these inner lipid structures, decreasing the area to volume ratio (the budding process is triggered by the increase in the area to volume ratio), so the equilibrium configuration after this area loss would be the spherical one again. Because of this, the merging is produced so the equilibrium configuration is stablished.

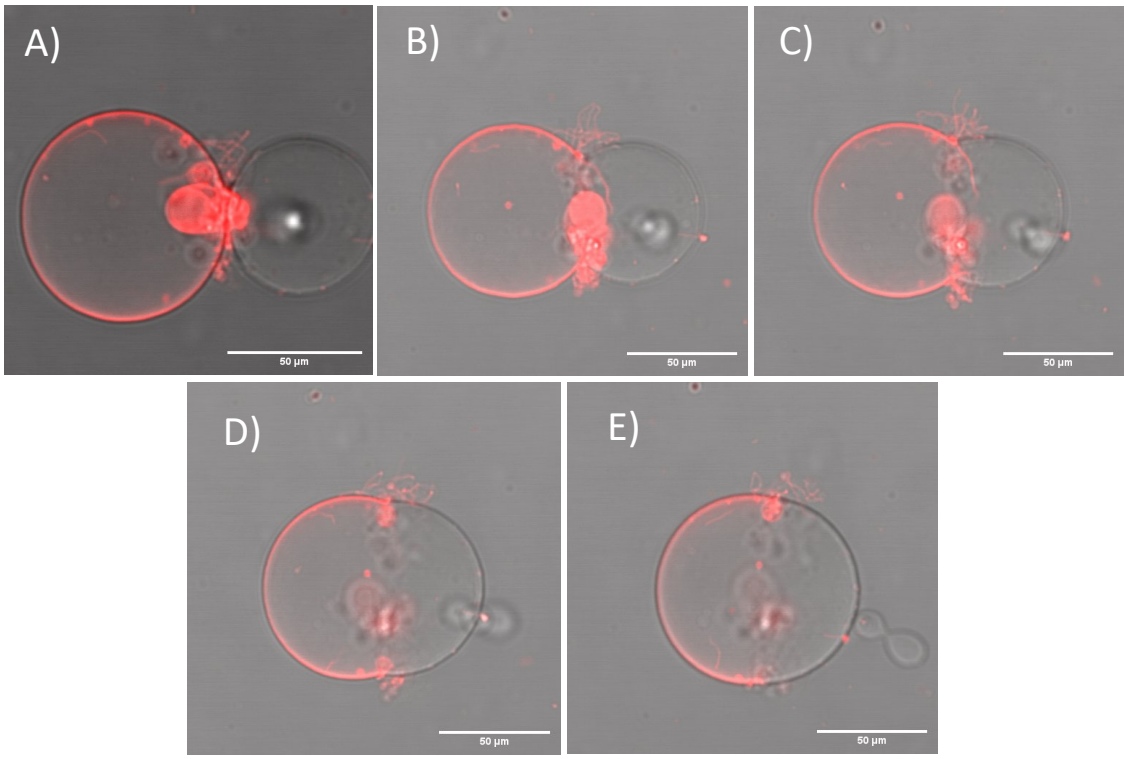

Figure 35. Images of the merging process after the budding of a snowman shape liposome was observed. The osmolarity of the outer medium is around 430 mOsm. Bright field and red channel are superposed in the images. A) The budding process result. It is notable the fluorescent structure that connects both liposomes. B) to E) Merging process forming a spherical phase separated liposome.

Increasing the stability of liposomes

After obtaining the first liposomes some problems aroused. While the liposomes could be obtained after one or two days after collecting, or even after several hours when vials with hydrophobically treated bottoms and the dichloromethane pentane was used, only a small amount of the double emulsions transformed into liposomes and these liposomes were not very stable, so when trying to pick them up with a pipette and transfer them to another recipient they didn't survive. Also these liposomes have a small lifetime of about one or two days.

While this is okay for observing phase separation and some microscopic features of the liposomes, when trying to measure mechanical properties or osmotic effects the small amount of liposomes formed and their instability is an important drawback.

To overcome these problems, more and more stable liposomes are necessary. To get them, a larger amount of double emulsions was collected (at the end having more than one layer of double emulsions at the bottom) and the organic solvent evaporation rate is reduced as much as possible. The evaporation rate is reduced by two means, first the larger amount of double emulsions produces the faster saturation of the collection solution, and this saturation reduces the evaporation rate, and second the vials are kept closed so the air in the vial also saturates with organic solvent reducing the evaporation of organic solvent from the collection solution to the air. Also to test if the collection solution had some effect on the stability two different collection solutions were tested, a phosphate buffer solution (PBS) that keeps the pH of the solution constant, and a glucose solution, which doesn't have ions dissolved in it.

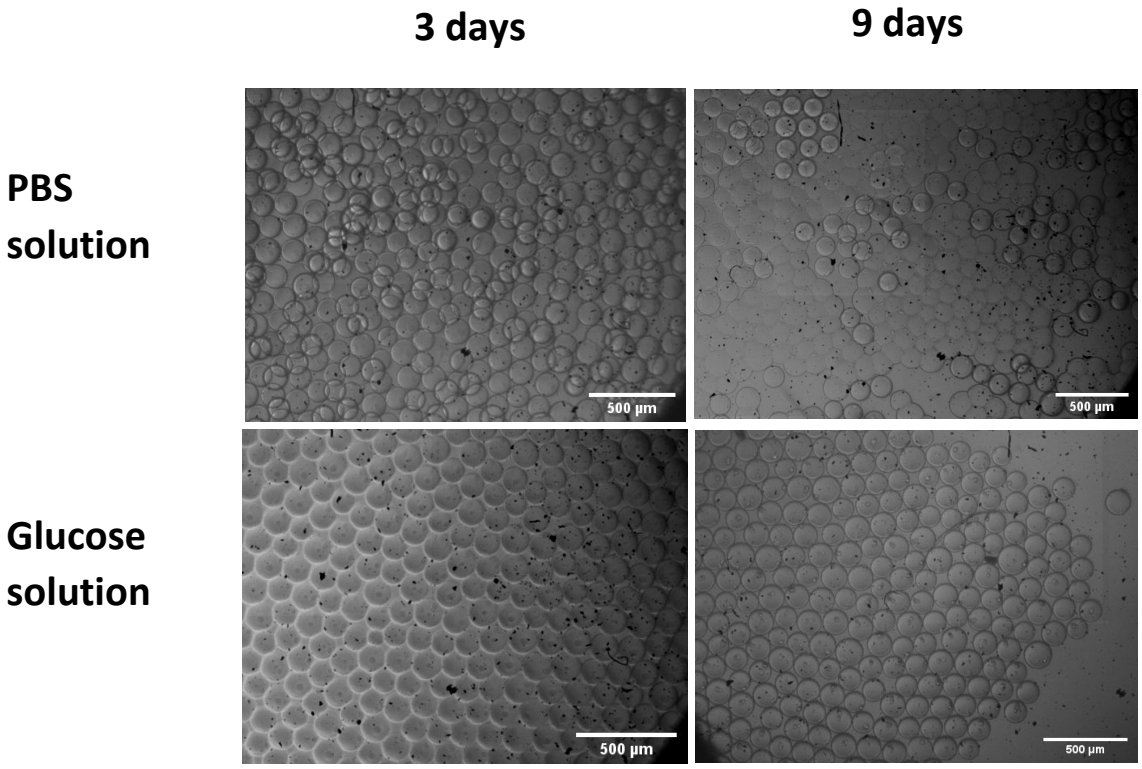

Figure 36. Double emulsions with phase separation composition lipids collected for the same amount of time with the same flowrates in glucose solution and PBS solution. It can be seen that while the double emulsions in the PBS solution after 9 days have adhered to each other, in the glucose solution they do not adhere.

When collecting a large amount of double emulsions in PBS it was observed that the double emulsions adhered together arranging themselves in a crystal like structure as shown in figure 36. This adhesion was not observed in the double emulsions in glucose solution. The hypothesis for this adhesion phenomenon is that the PBS solution, unlike the glucose solution, has a lot of ions and charged molecules in it. These ions screen the electric repulsion between the surfactant and lipid molecules in the surface of the double emulsions and even helps to the adhesion. Also the growth in size of the double emulsions may help in the process. The adhesion of double emulsions has been observed for all the vials with a large amount of double emulsions in PBS solution, especially for the double emulsions with the phase separation lipid composition which adhered more easily to each other.

In the vials collected for longer time, after three weeks since the collection, the first liposomes started to form. As shown in figure 37 a significant amount of liposomes could be obtained after waiting enough time. Also these liposomes are very stable in comparison to the first ones made. This stability can be seen in the fact that they could be transported to another recipient using a pipette and that they survived as liposomes for more than one week. With these stable liposomes the mechanical and osmotic test were made and also this stable phase separated liposomes were the ones to shape deform into the snowman shape.

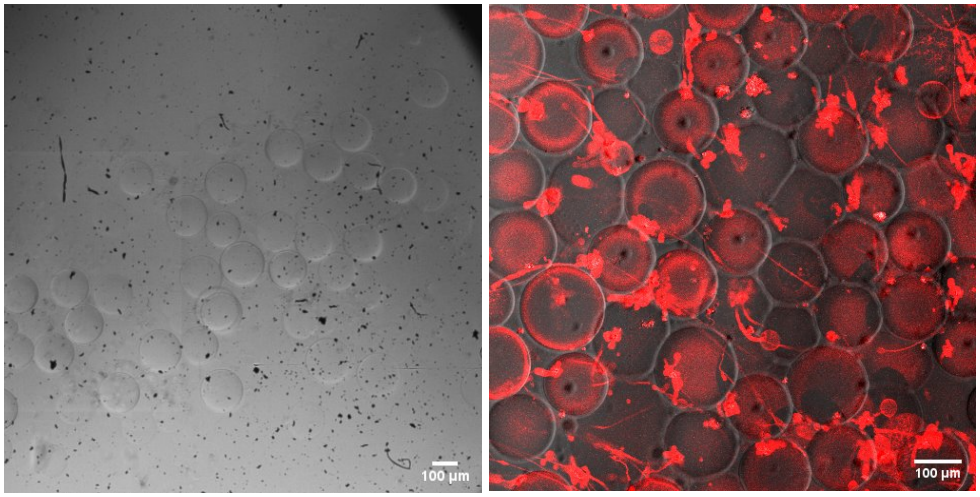

Figure 37. Stable liposomes after more than three weeks since the double emulsions were collected. This images shows that a significant amount of liposomes was obtained. Left: DOPC liposomes. Right: Phase separated liposomes.

As a conclusion, in order to obtain stable liposomes a large amount of double emulsions has to be collected and the evaporation rate of the organic solvents reduced. As the collection solution, glucose solution should be used since PBS solution produces the adhesion of double emulsions and no liposomes can be obtained from this adhered structures that break with time.

The drawback of obtaining more stable liposomes is that they take a long time to form (in our experiments they took around a month to form). There is then a compromise between the time it takes for the liposomes to form and the stability of the final liposomes.

Size limit of liposomes produced by glass capillary microfluidic methods

The size of the liposomes studied so far is in the order of magnitude of hundreds of microns (typically around 100 µm). The size of the different type of cells is very heterogeneous and can vary from around 1 µm in the case of bacteria to around 100 µm in the case of plant cells. Typically, the animal cell size varies from 10 to 30 µm. In order to study better the cell membrane using liposomes is interesting to produce liposomes with a size around the typical cell size.

This matter arises the question of what is the smallest liposome size that can be produced using glass capillary microfluidics methods. The size of the double emulsions produced in a microfluidic glass capillary devices depends basically on the flow rates and size of the injector capillary. By modifying the flow rates on already built devices, increasing the outer phase flow rate so double emulsions are produced in the jetting regime and this jet size is as small as possible the smallest double emulsion size achieved is of around 20 µm.

We can even go lower in size than 20 µm by reducing the size of the injector capillary. To do that, after a round capillary is tapered, the tip is sanded to make sure it is perpendicular to the capillary axis and smooth. Then the capillary is exposed to fire for a small amount of time so the capillary tip starts to close making the tip diameter as small as possible. In a consistent way, the capillary tip can be reduced to a size of 10 to 20 µm, three times smaller than the usual injector tip size (figure 38A). The device is assembled and using high outer flow rates so the smallest size of double emulsions produced consistently in the thin jet regime is around 10-15 µm, which is the size of small animal cells.

When producing these small double emulsions, the microscope objective magnification was not big enough so these double emulsions could not be distinguished. For making sure that we produced double emulsions a fluorescent dye (Dextran) was added to the inner water phase and the double emulsions were collected in a vial. Then they were checked at a confocal microscope where the size was measured and by seeing the fluorescence of the inner water we can be sure that double emulsions were produced (figure 38B). If the "bubbles" seen in confocal microscopy were single emulsions no fluorescence could be observed since no inner water would be present.

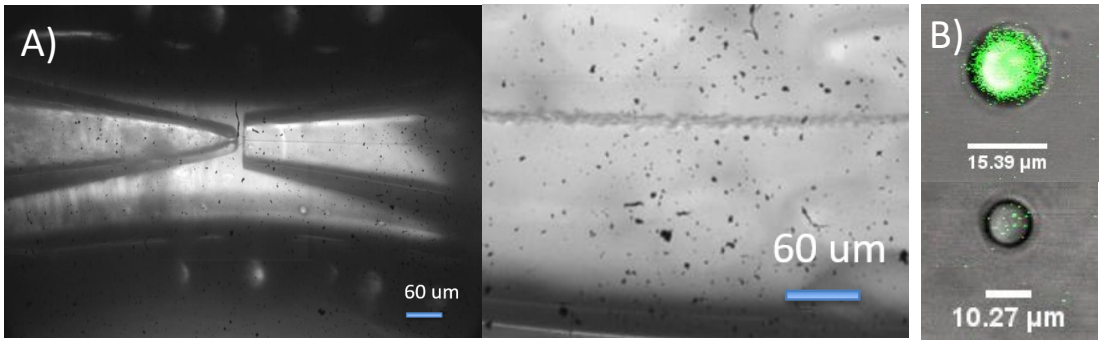

Figure 38. A) Glass capillary device with small size tip producing small double emulsions. The double emulsions are too small to be seen clearly with the objective of the microscope. B) Double emulsions observed in confocal microscopy after collection. The fluorescence intensity in the inside is higher than in the outside of the double emulsions.

Asymmetrical liposomes

Liposome production test

First of all, after making a device for the production of asymmetrical liposomes, the production of potentially asymmetrical liposomes is tested. Double emulsions are produced with an oil composition of chloroform and pentane in a ratio of 1:1.8. The inner middle oil has a concentration of 5 mg/mL of 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC), which is a phospholipid with a saturated and an unsaturated fatty acid, dissolved in it. The outer model oil has a concentration of 5 mg/mL of DOPC dissolved in it.

After collecting double emulsions and waiting for several days some potentially asymmetrical liposomes could be observed (figure 39). After successfully testing the production of liposomes with the asymmetric device more experiments on asymmetrical liposomes could be done.

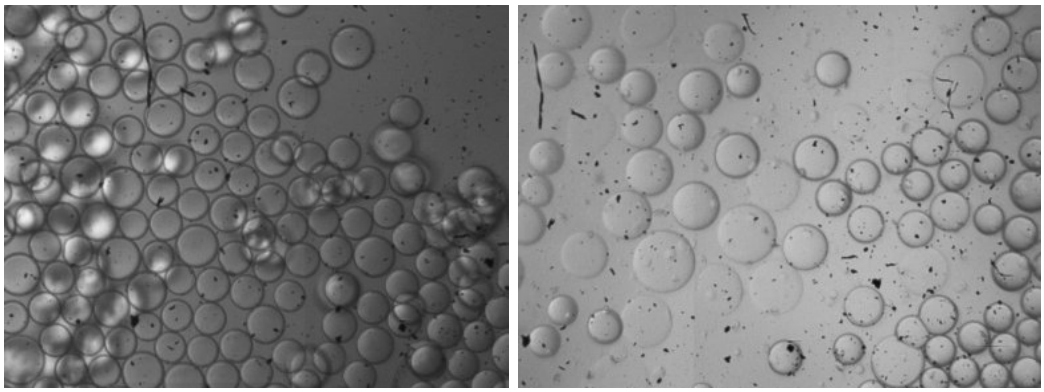

Figure 39. Left: Double emulsions from the asymmetrical device 42 hour after collection. Right: Double emulsions and liposomes formed after dewetting from the asymmetrical device 8 days after collection.

Problems and limitations

One of the most interesting phenomena observed for the asymmetrical liposomes is domain induction ([47], [48]). In asymmetrical liposomes, the domain induction is produced when only one of the layers of the liposome has an adequate composition to phase separate and this phase separation induces phase separation in the other layer just by the hydrophobic tail interactions between the layers. In order to be able to study this phenomenon further a good control over the composition of the layers is required.

Although in the symmetrical production of liposome using microfluidic methods the composition of this liposomes can be controlled very easily, in the case of asymmetrical liposomes, since both oil layers are miscible there is some diffusion of molecules from an oil layer to the other. This diffusion of lipids from one layer to the other has been studied and it has been estimated that 30% of the lipids of one layer diffuses to the other [19].

If we want to observe the domain induction, this diffusion limits the compositions that we can use in each layer so one of them will have a phase separation composition while the other don't. To study which composition to use in each oil phase the following assumptions are made:

- The number of lipids in each layer is similar
- The percentage of lipids that diffuse to the other layer is 30%

Because of this diffusion if we wanted that one layer did not phase separate because the concentration of DOPC is too high the final concentration of both layers would be very close, so any difference in the diffusion rate or concentration of the solutions prepared would result in a phase separation or not phase separation composition in both layers. To solve this problem, the concentration of DOPC and DPPC will be the same in both layers and the difference between layers would be the concentration in cholesterol, so one of them shouldn't phase separate because of the high concentration of cholesterol. The composition of the two oil phases is then set to:

- Inner middle oil: 8.75% DOPC, 8.75% DPPC and 82.5% Cholesterol
- Outer middle oil: 46.25% DOPC, 46.25% DPPC and 7.5% Cholesterol

With this composition of the two oil phases and the assumptions taken the final concentrations of the layer should be:

- Inner layer (does not phase separate): 20% DOPC, 20% DPPC and 60% Cholesterol
- Outer layer (phase separates): 35% DOPC, 35% DPPC and 30% Cholesterol

Also to be able to see the phase separation in confocal, of the 8.75% of DOPC in the inner middle oil, 1% is substituted by the fluorescent dye attached to an unsaturated phospholipid DOPE_Rhodamine and of the 46.25% of DPPC in the outer middle oil, 2% is substituted by 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) also called DPPE-NBD which is a fluorescent dye attached to a saturated phospholipid that quenches when sodium dithionite is added, so this will be useful to estimate the asymmetry of the liposomes.

One thing that was not taken into account when calculating the compositions is that unlike the phospholipid, cholesterol molecules do jump from layer to layer in liposomes [49] (this is called flip-flop) so if we now assume that at the end, the number of molecules of cholesterol will be the same in both layers, the inner layer will have a 53% of cholesterol and the outer layer will have a 39% of cholesterol so still one of the layer will spontaneously phase separate while the other will not.

When observing the double emulsions produced, the fluorescence of the two different dyes could be seen, so as each dye was in a different oil phase this means that at least both oil phases where present in the double emulsions produced. One of the assumptions made when calculating the composition was that the number of molecules of each oil layer is the same, but when looking at the double emulsion using the confocal it can be seen that the fluorescence varies between double emulsions and that some of the double emulsions have a high NBD fluorescence and low Rhodamine fluorescence while in other double emulsions the opposite can be seen (figure 40).

This difference in the number of molecules of each layer between double emulsion probably is a result of the microfluidic production of double emulsions. As double emulsions are produced, the volume outer middle oil volume that surrounds the tip is being reduced, so while the first double emulsions have a higher volume of the outer oil, the last ones have a lower volume of

outer oil. This inhomogeneity in the number of molecules of each layer invalid our calculations and makes it really difficult to estimate the composition of each layer, so although we could be able to observe the domain formation there won't be any way to know if there is domain induction or not.

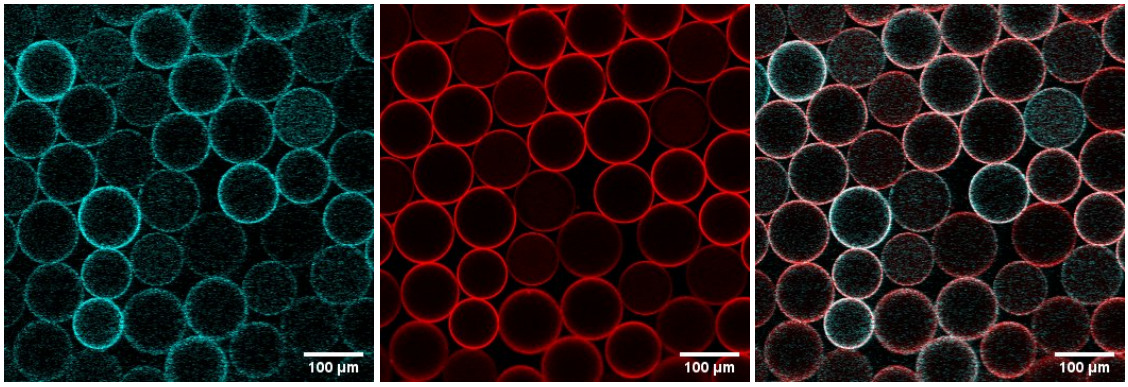

Figure 40. Left: NBD fluorescence channel. Middle: Rhodamine fluorescence channel. Right: NBD and Rhodamine channel superposed. The difference in red and blue fluorescence ratio can be seen in the colour heterogeneity when the channels are merged. This fluorescence ratio heterogeneity means different proportions of red and blue dye, and that means that the ratio of the lipid number of each oil layer varies between double emulsions.

The last thing to do with these double emulsions was to check the asymmetry. For studying the asymmetry, the idea is to quench the NBD fluorescence molecules by adding a solution of sodium dithionite which acts as a reducing agent. Since the time for finishing this work was running out and the double emulsions had not dewetted into liposomes, the quenching assay was done to double emulsions. The problem of doing this assay with double emulsions is that a reduction on the fluorescence was observed when sodium dithionite was added and when not. The explanation for this is that when the double emulsions are transferred to a smaller recipient to be observed, the evaporation rate of the oil is faster and an oil pocket that accumulate the lipid molecules dissolved in the oil layer is formed. This way, the fluorescence in the equatorial plane (the one that was observed) is reduced (figure 41).

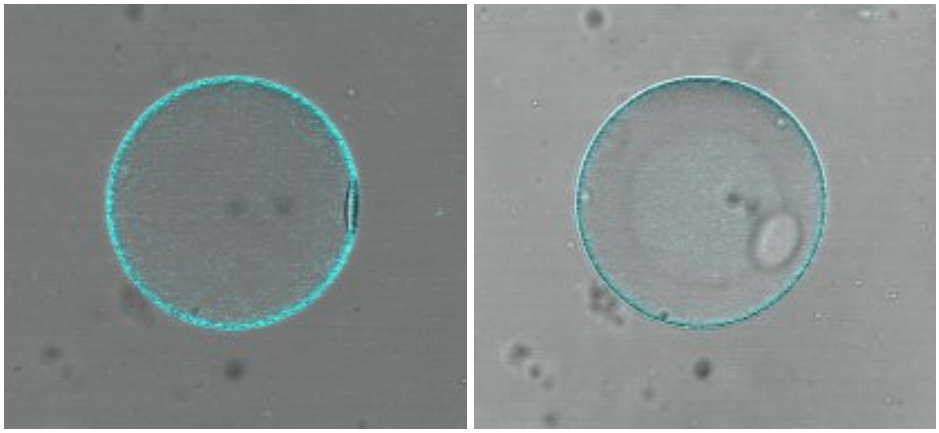

Figure 41. Left: Fluorescence when the double emulsion is located in a smaller recipient. Right: Decreasing of the fluorescence right after a fluorescence oil pocket is formed. The oil pocket contour can be seen inside the double emulsion.

Also, the double emulsions were not stable in the new recipient for a long time, so waiting until the oil pocket is formed and then adding the quenching solution is not easy to perform. Because of these problems the asymmetry of the double emulsions could not be studied.

Microfluidic production of liposomes is then a promising tool for producing asymmetric liposomes with a controlled composition of both leaflets, but to get there, some problems as the diffusion of lipids between layers and the heterogeneity in the volume ratio of the two layers must be solved first.

Conclusions

The use of microfluidic methods is a novel tool for producing liposomes that offers some advantages with the conventional methods, especially in the homogeneity in size and composition and the possibility of obtaining asymmetrical liposomes, which are hard to obtain by using conventional methods, by slightly modifying the microfluidic device design. Since this method relies on the evaporation of organic solvent from double emulsions that act as a template so the lipids arrange forming the liposomes, some concerns may arise with the possibility that some remaining organic solvent in the bilayer may affect the liposome properties.

In this work different properties and behaviours of symmetric liposomes produced using microfluidic methods have been studied and compared to the results that have been obtained for liposomes produced using conventional methods. It has been studied the mechanical properties of DOPC liposomes, and the miscibility transition temperature, shape deformation and osmotic effect on ternary mixture phase separation liposomes. Since the properties and behaviours observed are consistent with the published literature, the liposomes produced using microfluidic methods are as valid as the ones obtained using conventional methods and can be used without problem for further research.

The use of microfluidic methods for producing asymmetrical liposomes is a promising method for obtaining a good control in the composition of each leaflet, and is also explored in this work. Since the current method is based on the use of double emulsions with two oil layers with different lipid composition in the oil phase, some problems arise in controlling the composition. The two main problems exposed in this work are the diffusion of lipid molecules from one layer to the other and the inhomogeneity in the different oil layer volumes ratio, so the numbers of lipid molecules that each oil layer contribute is different and varies from double emulsion to double emulsion.

The microfluidic approach for producing liposomes is a promising tool for studying liposomes with a better control on the different parameters, such as composition and size, but there are still problems to be solved in the case of asymmetric liposomes and improvements to be made to improve the liposome production.

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Annex A: Theoretical framework

Cell membrane

Cells are the basic structural, functional and biological unit of all living organisms. Since they were studied for the first time with the development of optical microscopy, a lot of scientific and technical advancements allowed a more profound study of them in terms of structure, components, metabolic processes etc. Despite the enormous heterogeneity in cells, there is one structural element common in all of them and of vital importance: the cell membrane.

The cell membrane is the structure whose main function is to separate the interior of the cell from the outer environment and to guarantee that the conditions inside the cell are the correct ones so all the cell functions can be developed. To do this, the cell membrane is capable of controlling the flux of different substances inside and outside the cell. Another important function of the membrane is to be able to detect external signals so the cell can react to different stimuli.

The cell membrane is composed of three different types of molecules: lipids, proteins and oligosaccharides. Although the composition and the type of lipids, proteins and oligosaccharides varies between different types of cells there is a common structure for all of them: the fluid mosaic model [1]. According to this model (figure 42), the cell membrane is a bilayer of phospholipids, that are molecules with an amphiphilic behaviour (they have a hydrophilic polar head and a hydrophobic fatty acid tail). Due to their amphiphilic nature, in order to minimize the energy, the phospholipids arrange in a bilayer so that the hydrophilic polar heads are in contact with water on the interfaces of the bilayer and the hydrophobic tails are in the inside of the bilayer. There are also another two types of lipids present:

- Glycolipids, that are lipids attached to a carbohydrate as the polar head so they can be part of the lipid bilayer.
- Cholesterol, whose function is to increase the fluidity of the membrane and to maintain the membrane integrity.

As the name of the model states, and thanks to cholesterol this lipid bilayer behaves as a 2-dimensional fluid matrix with proteins in it. The possible movements of the lipids in the bilayer are rotational, translational and transbilayer (or “flip-flop”). The later one, that refers to the jump that lipids make from one leaflet to the other is the rarer one (it has been proven that under some restricted conditions it occurs) due to the high energy barrier they have to cross in order to make it. Due to this restricted movements, cell membranes have been found to be asymmetric, in the sense that the lipid composition of the inner leaflet is different from the composition of the outer leaflet.

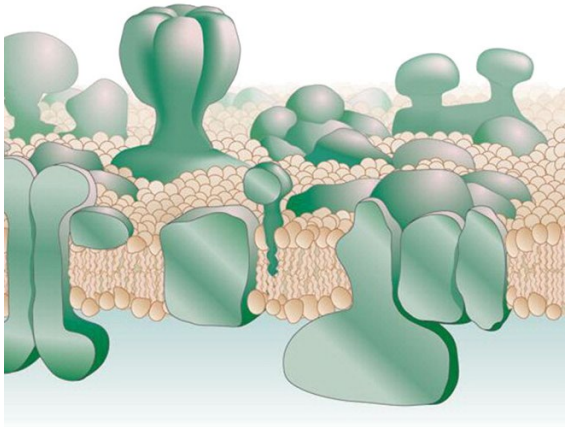

Figure 42. Fluid mosaic model of the cell membrane. Image extracted from [2].

As it has been stated previously, there are proteins associated to the phospholipid bilayer matrix. We can distinguish two type of proteins depending on how they attach to the lipid bilayer:

- Peripheral proteins, that are attach to the polar heads of the bilayer through non-covalent bonds.
- Integral proteins, that have part of them embedded in the bilayer.

It is important to remark that proteins constitute a 20% of the cell membrane components so actually the lipid matrix is crowded with proteins, and these proteins can have many different functions like controlling the flux of different molecules in and out the cell (also call channels), acting as receptor of external and internal signals and behaving as cell actuators.

Since the mosaic model was first introduced, some features, resulting from decades of research, have been incorporated to this model [2]. The most interesting features are:

- Different lipid phases have been found to be of physiological interest.
- Lateral heterogeneity has been observed. This means that there are some domains in the cells (with diameters ranging from 0.1 to 1 µm) enriched in certain lipids and proteins. This can also be seen as the coexistence of different phases in the cell membrane. What's even more interesting is that these domains are functional in the cell.

For the last decades, with the aim of getting a better knowledge of the cell membrane as a system, lipids bilayers have been artificially made and studied. Their study has led to a really good understanding of these lipid bilayer systems, also called liposomes.

Liposomes can be classified depending on the number of bilayers (unilamellar or multilamellar) and their size. Attending to the size they are classified into small (20 nm -100 nm), large (100 nm - 1000 nm) and giant (> 1 µm).

Liposomes

To understand better the lipid bilayer system, it is necessary to introduce the chemical structure of phospholipids. Basically phospholipids generally are composed of a phosphate group and two fatty acids all three of them attached to a different carbon of a glycerol molecule (figure 43). The phosphate group with the glycerol constitute the polar (hydrophilic) head and the fatty acids the hydrophobic region. There are a lot of different phospholipids that differ on the phosphate group and fatty acids that forms them.

The fatty acid tails are characterized for being a hydrocarbon chain completely non polar. Any fatty acid is characterized by the length of its hydrocarbon chain and the number of double bonds between carbons. The presence of double bonds has a great impact on the shape of the fatty acid. While a fatty acid without double bonds (also called saturated) have a linear shape,

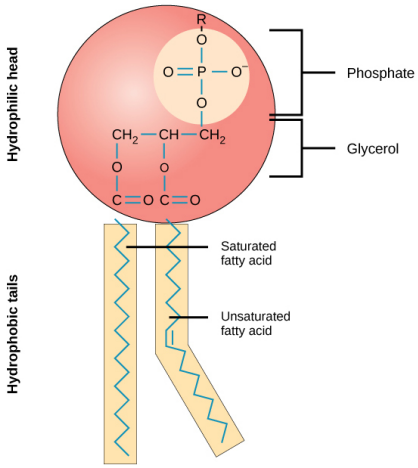

Figure 43. Phospholipid structure scheme. Image extracted from "Biology for Majors I" online course from lumen learning.

the double bonds causes the chain to bend (as in the majority of fatty acids found in nature) as shown in figure 43. A fatty acid with double bonds in its chain is called unsaturated. The type of fatty acids of the phospholipids of a liposome have a great impact on some important physical properties as we will see in the following sections.

Single component liposomes

The first systems to be studied were the lipid bilayers made of only one kind of phospholipid. The most relevant feature of this system is the existence of different phases ([3], [4]):

- Liquid disordered phase (L_d): This phase is represented by an irregular packing of the lipids. In this phase the lipids can move easily, unhindered, meaning that the diffusion coefficient of the lipids is big. In this phase the fatty acid chains are not straight, they are subjected to thermal fluctuations that changes their shape. Because of this the bilayer in the liquid disordered phase is thinner.

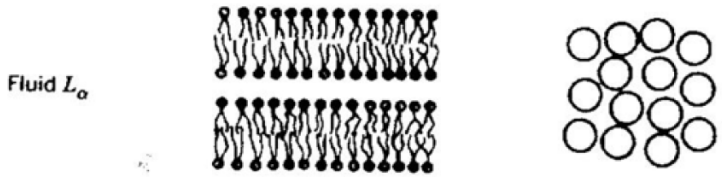

Figure 44. Depiction of the liquid disordered state. Image extracted from [3].

- Gel or solid phase (L_β): In this phase the lipids are arranged in a solid-like structure in a hexagonal packing. In this phase the mobility of the lipids is limited as they are stuck in the well packed structure, so the diffusion coefficient of the lipids is small. Also, in this state, thermal fluctuations are not relevant and the fatty acid chains are in their minimum energy conformation, which is straight for saturated fatty acids and straight with bends for unsaturated fatty acids. Because of this, the bilayer in the liquid ordered phase is thicker.

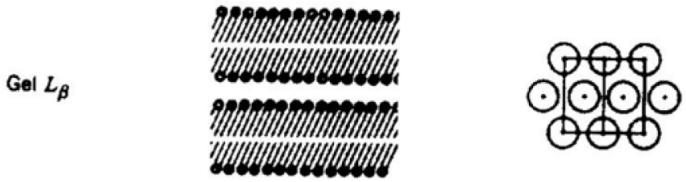

Figure 45. Depiction of the gel state. Image extracted from [3].

- Liquid ordered phase (L_o): This phase appears when adding sterol molecules (for example cholesterol) to the lipid bilayer in the gel phase. In this case, the sterol molecule disrupts the order of the phase allowing the lipids to have a higher mobility, increasing their diffusion coefficient, and in general the fluidity of the lipid

bilayer. But on the other hand the lipid bilayer conserves a good packing so the properties of the gel phase are conserved changing the lateral diffusivity of lipids and orientation of fatty acid chains.

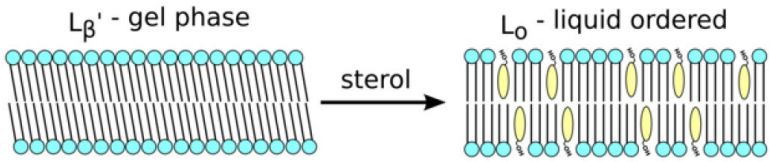

Figure 46. Depiction of the liquid ordered state. Image extracted from [3].

- Ripple phase (P_b): In the mean path between the liquid disordered phase and the gel phase the ripple phase can be observed. This phase a chain packing similar to the gel phase but the bilayer form ripples [50]. The existence of this phase depends on the type of lipid that forms the liposome, and it is present at intermediate temperatures between the ordered and disordered phase.

Figure 47. Depiction of the ripple phase. Image extracted from [3].

- Pseudocrystalline phase (L_c): This phase presents a higher order and tighter packaging compared to the gel phase. It's considered a highly ordered superlattice.

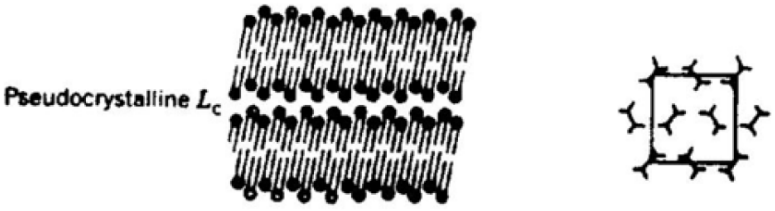

Figure 48. Depiction of the pseudocrystalline phase. Image extracted from [3].

The phase of a liposome depends mainly on temperature. The lower the temperature is, the higher order and better package the phase is. From what has been stated from lower to higher temperatures the phases will be ordered as follows: Pseudocrystalline, Liquid ordered or gel phase depending on the presence of sterols, Ripple phase when the type of lipid presents this phase and Liquid disordered.

There is an important parameter for each kind of lipid which is the melting temperature, from now on T_m, and it's define as the temperature where the liquid disordered phase transition occurs. This temperature depends on different factors:

- Saturation of the fatty acids: As stated previously the presence of double bonds creates a bend in the fatty acid tail. This bend is responsible for a worst packaging of the phospholipids, and because of that less thermal energy is needed to disrupt the ordered phase. This way, the presence of double bonds is responsible for a lower T_m, while in saturated phospholipid as more thermal energy is needed to disrupt the ordered phase the T_m is higher.

- Chain length: The longer the chain of the fatty acid is, the stronger the hydrophobic interaction between tails is and more energy is needed to disrupt the ordered phase. This way T_m increases as the chain length increases.

Multicomponent liposomes

Real cell membranes are not composed of a single kind of phospholipid but of several kinds, so the study of multicomponent liposomes becomes relevant. From now on we will centre in the study of ternary systems made of a phospholipid with low T_m (from now on T_{ml}), a phospholipid with high T_m (from now on T_{mh}) and cholesterol (which is a sterol).

This specific system is really interesting since the formation of domains has been observed and thoroughly studied ([5], [6], [7]). This domain formation is actually a phase separation phenomena and can be understood really easily taking into account the different phases that have been exposed before. Imagine we have a mixture of phospholipids and cholesterol at a temperature between T_{ml} and T_{mh}. At this temperature, the high melting point phospholipid will be more stable at a liquid ordered phase (due to the presence of cholesterol), while the low melting point phospholipid will be more stable at a liquid disordered phase. In order to minimize the energy (since the domain formation is an enthalpic-driven process [51]) the high melting temperature phospholipid will tend to join together to form the liquid ordered phase until equilibrium is reached. This way we will have domains rich in high melting point phospholipid in a liquid ordered phase coexisting with a liquid disordered phase rich in low melting point phospholipid. This can be observed labelling each phospholipid with a different fluorescence and observing them using fluorescence microscopy (figure 49).

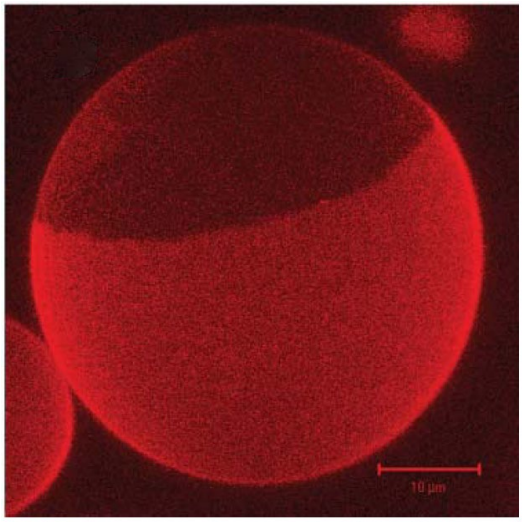

Figure 49. Phase separation for the ternary DOPC/DPPC (0.5/0.5 molar ratio) and Cholesterol (20 mol %) mixture observed using fluorescence imaging. Image extracted from [5].

Of course depending on the molar ratio of the different components and the temperature conditions, we can observe no phase separation or phase separation with different types of phases. One important parameter is the miscibility transition temperature which is the critical temperature for phase separation at a given composition. Below this critical temperature the domains will spontaneously form while above this temperature the domains will disappear and

the composition of the liposome is homogeneous. The miscibility transition temperature depends on the composition and this can be easily understood since, for example, when the molar percentage of the low melting temperature lipid is increased it's harder for the high melting temperature lipids to get together and form spontaneously a domain since the molecules will be in average more far away, and because of that in order to form domains the temperature has to be lowered so this interaction between high melting temperature lipid molecules is favoured.

This fluid bilayer ternary system belongs can be studied from the statistical mechanics' point of view as belonging to the 2-dimensional Ising model class [52]. This universality class are formed basically by 2-dimensional systems where the space can be divided into a small mesh and in each space of the mess we can have to different state with different energy and they interact energetically with their closest neighbours. In our system of study, the two different state would correspond to the presence of a high melting point temperature lipid or a low melting point temperature lipid. The 2-dimensional Ising model is characterize by the presence of a critical temperature, so below this temperature spatial correlations start to appear and some order parameter can be defined (in our case these spatial correlations correspond to the domains), and above this temperature there is no order and the system is homogeneous.

This phase separation and miscibility transition temperatures has been studied and phase diagrams for different ternary mixtures have been developed. In our case, we will be interested in DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine), DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine) and cholesterol mixture. In this mixture, DOPC is an unsaturated phospholipid, with a melting temperature of -17 °C, DPPC is a saturated phospholipid with a melting temperature of 41 °C [53] and cholesterol is a sterol. For this mixture the phase diagram has been studied ([6], [54]) and it is shown in figure 50.

This ternary mixture system has been studied experimentally, theoretically and numerically and there is a deep understanding on the behaviour and characteristics of these systems.

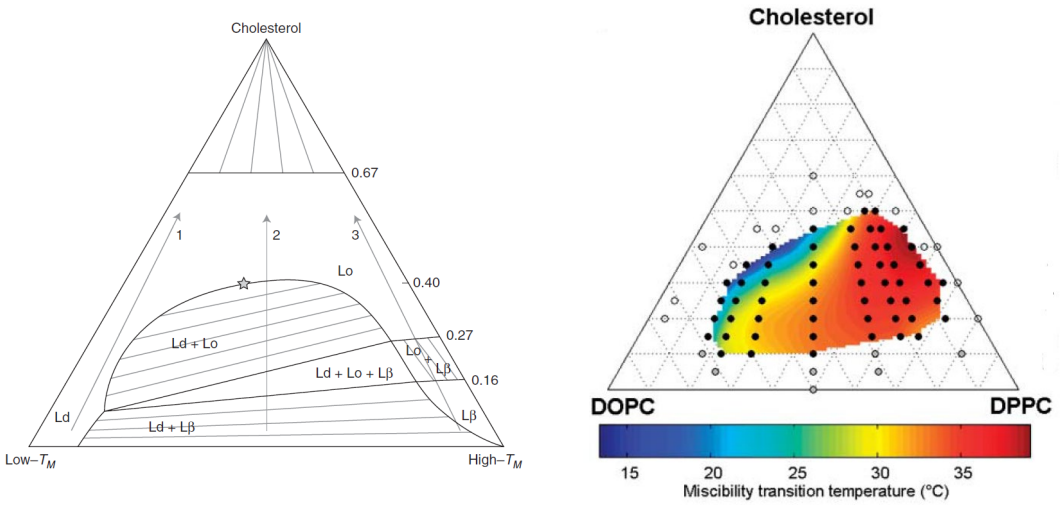

Figure 50. Left: Phase diagram scheme of a ternary mix of low melting point, high melting point phospholipids and cholesterol. Image extracted from [7]. Right: Real phase diagram for a DOPC/DPPC/Cholesterol mixture with miscibility transition temperature map. Image extracted from [6]

Asymmetrical liposomes

As it has been stated, the cell membrane is actually asymmetrical. This means, that the lipid composition is different for each leaflet (figure 51). This asymmetry is relevant for some cell process, as for example the cell apoptosis (controlled cell death) is characterized by a loss in the asymmetry of the membrane [55].

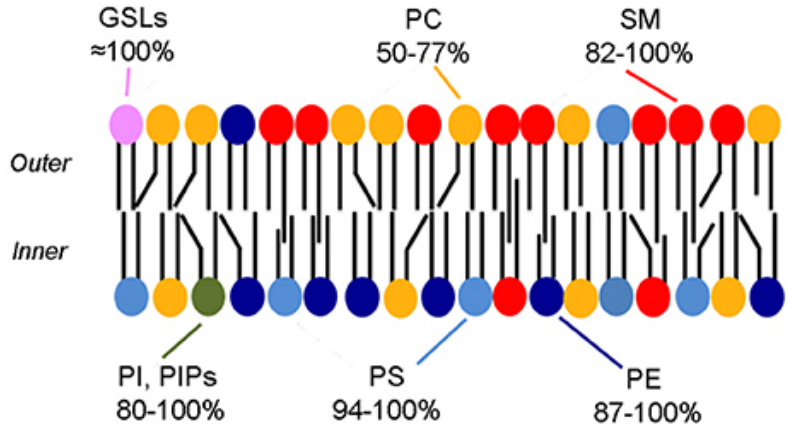

Figure 51. Schematic of the cell membrane asymmetric composition.

Despite the importance of asymmetry for some cellular mechanisms, asymmetric liposomes have been started to be studied in the last years, so there is a still unknown features to be studied. The reason for this is that is not easy to obtain asymmetric liposomes with conventional methods and only recently a method for exchanging lipids between the outer leaflet of different composition liposomes (using a cyclodextrin) that produces asymmetric liposomes has been developed ([47], [56], [57]). The problem with this method is that it is not easy to control the composition of both leaflets.

The microfluidic approach for producing liposomes is a promising tool for studying asymmetrical liposomes with controlled compositions of each bilayer.

Annex B: The road towards symmetrical liposomes production

First experiment

The phases compositions for the first experiment were:

- Outer water phase: 10 wt% PVA in water
- Inner water phase: 8 wt% PEG 6000 and 2 wt% PVA in water. The osmolality of this solution is usually between 100-120 mOsm and is measured each time a new inner water phase solution is made.
- Oil phase: Three different lipid compositions were used:

1. 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC), which is a unsaturated phospholipid was dissolved at a concentration of 14 mg/mL in Chloroform stabilized by ethanol (good solvent) and then Pentane (bad solvent) was added so the Chloroform:Pentane volume ratio is 1:1.8. This way the lipid final concentration in the oil phase is 5 mg/mL a value reported to work in some previous works.

2. 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), which is a saturated phospholipid, and DOPC in a mass ratio of 10:1. Since the molar mass of this two phospholipids is similar the molar ratio of this to phospholipids can be approximated by its mass ratio. To obtain this DPPC and DOPC were dissolved in separated vials at a concentration of 14 mg/mL in Chloroform. Then these two solutions were mixed in a volume ratio of 10:1 and Pentane was added so the Chloroform:Pentane ratio is 1:1.8.

3. Ternary mixture composition so the liposomes formed undergo domain formation (phase separation). In this composition DOPC, DPPC, Cholesterol and fluorescence dyes are mixed in the following proportions that have been previously used in the laboratory.

Molecule	Concentration in Chloroform mg/mL	Mixing volume µL
DOPC	14	340.93
DOPE_Rhodamine (red dye)	0.5	205.03
DPPC	14	233.72
Naphthopyrene (green dye)	0.5	92.96
Cholesterol	14	127.36

After mixing these volumes 1.8 mL of pentane is added so the Chloroform:Pentane ratio is 1:1.8. It is important to note that DOPC, DPPC and Cholesterol are dissolved at the same concentration as done previously, but in

this case the dyes are added so at the end the DOPC+DPPC+Cholesterol concentration is about 3.5 mg/mL below the usual 5 mg/mL value. Besides that, the liposome formation lipid and sterol concentration threshold has been reported in the group to be about 3 mg/mL.

The double emulsions produced are then stored in a phosphate buffer solution (PBS) diluted in water to a final osmolality around 10-20 mOsm smaller than the osmolality of the inner water phase. Three vials containing double emulsions were collected for each lipid composition. No liposomes were obtained from this experiment.

Second experiment

The next change was to reduce the PVA concentration of the outer water solution from 10 to 6 wt%. This new concentration of PVA is used for the rest of the experiments.

The hypothesis to justify the reduction on PVA concentration is that PVA as a surfactant adheres to the double emulsion surface and can induce some surface tensions that lead to the instability and breaking of the double emulsion.

Also the Chloroform:Pentane ratio was reduced for the DOPC and DPPC composition to see if there was any difference in the result. The justification of this step is that reducing the poor organic solvent volume fraction will result in the increase of lipid concentration in the double emulsion when the good solvent is evaporated making it easier for the double emulsions to form liposomes when dewetting. The Chloroform:Pentane ratios tested are listed in the table below:

Lipid composition	Lipid concentration in Chloroform	Chloroform:Pentane
DOPC	14	1:1.8
	12.5	1:1.5
	11	1:1.2
DPPC	14	1:1.8
	11	1:1.2
	9.5	1:0.9
Domain composition	14	1:1.8

No differences in the breaking of double emulsions was observed independently of the vial, recipe or chloroform:pentane ratio. So the chloroform:pentane ratio can be discarded as the main responsible for the double emulsion breaking. Also it is observed that collecting more double emulsions leads to a longer lifetime of double emulsions. This can be because when collecting more double emulsion, the region of the vials where the double emulsions are, saturates faster and the dewetting process is slower so it takes more time for the double emulsions to become unstable.

Third experiment

The next step was to try and change the organic solvents. For this another recipe was used and the poor solvent was substituted by toluene. Also the ratio Chloroform:Toluene is made to have as much toluene as possible while guaranteeing the lipid are completely dissolved. The change of the poor organic solvent is made in order to save time, since the solubility of toluene in water

is an order of magnitude higher than the solubility of pentane so the dewetting process is fastened.

Also we focus on getting liposomes that phase separate, since one can check for sure if the double emulsions have become liposomes by checking the domain formation in confocal microscopes. The next experiment consisted on using the toluene recipe and collect two vials of double emulsions for 5 minutes and 2 vials of double emulsions for 10 minutes.

The toluene recipe used is now described and the program used to calculate the volume is described in Annex C: Program used to calculate mixing volumes for different recipes:

- Molar ratio of DPPC:DOPC:Cholesterol is set to 2:2:1.
- DOPE_Rhodamine (red dye) molar concentration is set to 1%
- Naphthopyrene (green dye) molar concentration is set to 3%
- Final concentration of DPPC+DOPC+Cholesterol is 5 mg/mL

Molecule	Solvent	Concentration mg/mL	Mixing volume µL
DOPC	Toluene	25	394.07
DHPE_Rhodamine	Chloroform	5	82.42
DPPC	Chloroform (insoluble in toluene)	25	367.97
Naphthopyrene	Chloroform	5	58.62
Cholesterol	Toluene	25	96.91

Then to get the final concentration of 5 mg/mL 3294.8 µL of Toluene is added.

The double emulsions collected for longer time (more double emulsions) survived longer reinforcing the conclusions of the previous experiment. No liposomes could be observed using a different solvent recipe, but since this recipe is reported to produce liposomes faster it can be useful to fasten the process.

Fourth experiment

The next changes done were:

- Inner water solution made of 9 wt% PEG 6000 and 1% wt PVA (PVA concentration reduced in inner water phase). This new inner water solution is used for the rest of the experiments.
- Store the double emulsions in vials with a high surface area so the evaporation rate is increased drastically to see if this helps to the formation of liposomes. The vials were made by sticking glass slides together using epoxy adhesive, and one device had a bigger surface area than the other.
- The flow rates are changed so the double emulsions made are as thin as possible so the dewetting process is favoured.
-

The double emulsions of the vial with larger surface area (faster evaporation) broke in less time than the other with less surface area. With this experiments and the result of the previous ones it can be said that evaporation rate can play an important role in the stability of the liposomes

formed but there is another factor that plays a more important role and leads to the instability of the double emulsions, so independently of the evaporation rate, recipe and organic solvents the double emulsions breaks without forming liposomes.

Since the PVA has been reduced in both inner and outer phases and its concentration has been reduced in the collection solution by changing it, the PVA aggregation on the double emulsion creating tension that induces the breaking of the double emulsions can be discarded.

Fifth experiment

The next experiment consisted in increasing the lipid+sterol concentration of the oil phase to 7 mg/mL in the domain liposomes recipe, to see if this increment would solve the problems in liposome production. The recipe used was the one with Chloroform and Pentane as organic solvents in a volume ratio of 1:1.8 and is detailed in the following table:

Molecule	Molar percentage %	Concentration mg/mL	Mixing volume µL
DOPC	39	22.79	371.81
DOPE_Rhodamine	1	5	82.55
DPPC	37	22.79	326.76
Naphthopyrene	3	5	57.52
Cholesterol	20	22.79	161.36

And the 1.8 mL of pentane are added. In this case when collected the double emulsions, some of the collection PBS solution is taken every 2 days with a pipette and substituted with fresh solution. This is done to reduce the PVA dissolved in the collection solution. No liposomes were formed, but from now on the lipid+sterol concentration used is set to 7 mg/mL.

At this point, no improvements in the results have been observed. Analyzing the liposome production three different elements could be the responsible for the breaking of the double emulsions:

1. The solutions used
2. The microfluidic device
3. Storing conditions

Also, at the same time, liposome production in several hours or one they could be achieved using a new organic solvent combination. This new combination of organic solvents is dichloromethane (DCM) as the good solvent and pentane as bad solvent in a volume ratio of 1:0.9. The advantage of this recipe is that DCM has a solubility in water twice as high as the chloroform solubility so the good solvent evaporation is faster and less residue is left when the dewetting process starts. Also as the pentane volume fraction is smaller, we will have less bad solvent in the emulsions increasing the lipid concentration in bad solvent which is beneficial for the dewetting process. When using DCM as good solvent, since it's really volatile, while the device is working a glove full of ice is put near the device so the temperature in the device drops and the evaporation of DCM is avoided. Also, the pressure on the DCM solution when injecting prevents evaporation.

To increase the speed of the dewetting vials with hydrophobic coating on their bottom are used. This way the organic solvent has a higher tendency to evaporate and oil gas bubbles are formed in the bottom.

To discard some elements that could be responsible for the breaking of the double emulsions the device and solutions (DCM recipe) of another member of the laboratory that gets liposomes are used. This way the flow rates, collection vials and storage conditions are as the ones I normally use. Since liposomes where obtained in the hydrophobic treated and normal vials (although the hydrophobic treated vials took less time to dewet) it can be concluded that the problems are in the solution preparation, microfluidic device or in both.

Sixth experiment

Finally, it was noted a difference in the solution preparation that can be critical. The organic solvents for mixings were picked up using pipettes with plastic tips, while other people picked them using glass syringes. To test this new factor DOPC composition double emulsions made with the Chloroform:Pentane 1:1.8 recipe were collected and DOPC composition double emulsions made with the DCM:Pentane 1:0.9 recipe were collected in hydrophobic and normal vials.

This time DOPC liposomes could be observed, leading to the belief that picking the organic solvents with plastic tips could produce that some plastic dissolves in the organic solvent and avoid the formation of liposomes by dewetting

To test this further, phase separation liposomes were tried to made. The DCM:Pentane recipe detailed in the table below was used and double emulsions were collected in hydrophobic vials and normal vials. The solutions were carefully made using glass syringes to manipulate organic solvents.

Molecule	Molar percentage %	Concentration mg/mL	Mixing volume µL
DOPC	39	14.61	425.78
DOPE_Rhodamine	1	5	52.82
DPPC	37	14.61	377.19
Naphthopyrene	3	5	36.81
Cholesterol	20	14.61	107.40

Then 0.9 mL of Pentane is added.

Finally, in this last experiment, phase separated liposomes could be observed using confocal microscopy.

Annex C: Program used to calculate mixing volumes for different recipes

The Matlab program used to calculate the mixing volume for different recipes basically takes as inputs the good solvent bad solvent ratio, final lipid concentration, dye concentration in good solvent, and molar ratios of the different molecules in the recipe. With this inputs it calculates the concentration of the non-dye solution of lipids in good solvent (the dye concentration in good solvent is fixed) and the volumes to mix of the different solutions of molecules in good solvent. Then the appropriate ratio of bad organic solvent must be added.

```
%Good solvent:Bad solvent 1:x
x=1.8;
%Molar masses g/mol
DPPCm=734.039;
DOPCm=786.113;
Cholesterolm=386.65;
DHPE_RHm=1301.715;
Naphthopyrenem=302.38;
%Desired composition %mol/100
DPPCc=0.4425;
DOPCc=0.46255;
Cholesterolc=0.075;
DHPE_RHc=0.0;
Naphthopyrenec=0.02;
%Desired lipid concentration in final solution
DesiredC=5; %mg/mL
%Concentration of lipids in only the good solvent
C=(1+x)*DesiredC; %mg/mL
%Dye concentracion
DC=5; %mg/mL
%Volume of good solvent solution
V=1;
%Calculation of the lipid mass and each non-dye lipid in the good
solvent volume
Lipidmass=C*V;
DPPCrm=DPPCc*DPPCm;
DOPCrm=DOPCc*DOPCm;
Cholesterolrm=Cholesterolc*Cholesterolm;
Totalrm=DPPCrm+DOPCrm+Cholesterolrm;
DPPCmass=Lipidmass*DPPCrm/Totalrm;
DOPCmass=Lipidmass*DOPCrm/Totalrm;
Cholesterolmass=Lipidmass*Cholesterolrm/Totalrm;

%Calculation of the scaling factor of the number of mol in the volume
V of good solvent
%compared to the molar fraction
Redfactor=DPPCmass/(DPPCm*DPPCc*1000);

%Dye volume calculations
DHPE_RHv=DHPE_RHc*Redfactor*DHPE_RHm*1000/DC;
Naphthopyrenev=Naphthopyrenec*Redfactor*Naphthopyrenem*1000/DC;

%Calculation of the concentration of non-dye lipids in good solvent
Restvolume=V-DHPE_RHv-Naphthopyrenev;
```

```
C=(DPPCmass+DOPCmass+Cholesterolmass)/Restvolume
%Calculation of the mixing volumes of the molecules solutions in good
%solvent
DPPCv=1000*DPPCmass/C
DOPCv=1000*DOPCmass/C
Cholesterolv=1000*Cholesterolmass/C
DHPE_RHv=1000*DHPE_RHv
Naphthopyrenev=1000*Naphthopyrenev
```

For the chloroform toluene recipe, since its objective is to maximize the volume fraction of toluene another algorithm was used to calculate the recipe, but since in the development of this work no liposomes have been produced using the toluene recipe, this program is not shown.