BACHELOR PROJECT REPORT

on

PRODUCTION AND CHARACTERIZATION OF
GIANT UNILAMELLAR VESICLES

by

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Section 1: INTRODUCTION

- 1.1 GUVs
- 1.2 GUVs produced from a mixture of lipids
- 1.3 Reasons for research
- 1.4 Project objectives

1.1 GUVs
Giant Unilamellar Vesicles (GUVs) are artificial, closed membranes, composed of amphiphilic phospholipids in an aqueous environment (see fig.1.1a). They are called giant as they can become tens of microns in diameter, and unilamellar since they are comprised of a single bilayer, consisting of two mono-molecular layers of lipids (fig.1.3), which encloses the inner aqueous solution.

Amphiphilic lipids consist of a polar, hydrophilic, headgroup and a non-polar, hydrophobic tail. When introduced into an aqueous environment, the lipids can aggregate spontaneously into micelles for example (see fig.1.2), or into two mirroring one-molecule-thick layers, that are held together weakly by hydrophobic interaction, and thus form patches of bilayer (see fig.1.1b).

One of the methods that can be deployed to let the lipids form GUVs is electroformation, as developed by Angelova and Dimitrov (see also [1]), which will be one of the main objects of study in this project, and will be elaborated on shortly.
1.2 GUVs produced from a mixture of lipids

GUVs composed of only one lipid constituent are always homogeneous. When the temperature of a homogeneous vesicle is brought below the melting temperature of its lipid constituent, the membrane will make a transition to a solid state. Because of this, the temperature of the lipid film must be above its melting point during the electroformation, otherwise the lipid film will yield no vesicles at all.

GUVs with membranes composed of different kinds of lipids can make a transition to a heterogeneous state where they inhibit coexisting phases (see fig. 1.4) [2],[3]. Starting with a membrane inhibiting only one phase, this transition occurs if the temperature is brought below the corresponding mixing/demixing temperature or transition temperature $T_m$. Each present lipid constituent will have a preference for particular phases, in other words the lipids will demix. When the temperature is brought again above $T_m$, the different lipid constituents will mix again, so the membrane becomes homogeneous once more.

Therefore, with lipid mixtures, it is preferred that the temperature is above $T_m$ before the electroformation is initiated, otherwise the lipids will tend to demix during the process, and GUVs within a wide range of lipid compositions will be produced. If the GUV is capable of making a transition to a state with coexisting phases is highly dependant of its lipid composition.

When the demixing-transition is made, one common state of the bilayer is when it consists of two coexisting liquid phases, where the individual lipids diffuse freely within each phase. In this case, island-like domains, also called rafts, of a phase, scattered over the bilayer, appear to be formed embedded into the domain of the other phase. The island-like domains are called rafts because they diffuse through the membrane as a whole, their shape being able to change along the way. The rafts are generally unstable and therefore fuse together quickly to form bigger rafts.

The two phases are the ‘liquid-ordered’ phase and the ‘liquid-disordered’ phase. The liquid-ordered phase is enriched in a saturated lipid, brain sphingomyelin in our case, and cholesterol (see GUV constituents section for details about the constituents). The liquid-disordered phase is enriched in an unsaturated lipid (other than cholesterol), DOPC$^{(*)}$ in our case. The domains are clearly visible because the fluorophores that we use prefer to be in the liquid-disordered phase. The structures of DOPC, brain sphingomyelin and cholesterol are shown in figure 1.5.

$^{(*)}$ DOPC: 1,2-Dioleoyl-sn-Glycero-3-Phosphocholine
Fig. 1.5a: Structure of DOPC [4]

Fig. 1.5b: Structure of brain sphingomyelin

Fig. 1.5c: Structure of cholesterol
1.3 Reasons for research

Vesicles are important objects of study for at least the following reasons. From a physical chemistry viewpoint, the fluid membrane exhibits unique material properties resulting from its molecular architecture. Secondly, the enormous variety of configurations of the membrane is of interest to statistical physicists. And thirdly, membranes occur ubiquitously in biological systems, therefore to be able to produce and study these is of great interest to biophysicists [5].

Instead of producing membranes in the form of vesicles, membranes can also be prepared on a planar support. Although these are certainly of interest, the disadvantage is that the support will interact with such a membrane, thereby changing its behavior. GUV’s however, are free from such interference and have different properties than planar supported membranes, constituting a valuable model system for membranes found in organisms.

One of the aims of this project is to produce GUV’s that are suitable for experiments where the diffusion properties of individual lipid molecules are studied. Biotin lipids that are able to couple to streptavidin conjugated quantum dots were made part of the GUV’s. The quantum dots were excited by a laserbeam, so that the movement of lipid molecules through their bilayer could be tracked with a microscope. These experiments were not part of this project.

1.4 Project objectives

The main objective is to produce Giant Unilamellar Vesicles capable of inhibiting raft-domains and suitable for lipid diffusion experiments, by

- characterizing the general thermal properties of an electroformation chamber
- executing and improving a protocol to produce GUVs
- using various lipid mixtures to produce GUVs with raft-domains and study their properties.
2.1 GUV constituents

Different lipid constituents to produce GUVs were purchased [4]. In order to produce homogeneous GUVs, DMPC (\(\ast\)) or DPPC was used. In order to produce GUVs with raft-domains, a ternary mixture of the lipids DOPC (an unsaturated lipid), brain sphingomyelin (BSM, of which 80% are saturated lipids) and cholesterol (CHOL, unsaturated) was used. To this mixture a 10\(^{-3}\) molar fraction of the fluorophore DiI was added, as well as a 10\(^{-3}\) molar fraction of DPPE-biotin to which the quantum dots couple. DiI does not couple to any lipid molecule, but rather arranges itself between the lipids in domains of the liquid-disordered phase, so that when the GUVs are illuminated with light of the appropriate wavelength, the liquid-disordered phase will emit light, and the rafts are made visible.

\(\ast\) DMPC: 1,2-Dimyristoyl-sn-Glycero-3-Phosphocholine
DPPC: 1,2-Dipalmitoyl-sn-Glycero-3-Phosphocholine
DOPC: 1,2-Dioleoyl-sn-Glycero-3-Phosphocholine
DPPE: 1,2-Dipalmitoyl-sn-Glycero-3-Phosphoethanolamine

2.2 Set-up and general method

The GUVs are produced in a custom build flow chamber (fig. 2.2), as inspired from [6]. The flow-chamber, when closed, consists of two Indium Tin Oxide coated (ITO) glass-slides (7.4 cm x 4.5 cm), separated by a spacer (see fig. 2.3). The ITO coating make each glass-slide electrically conductive on the surface of one side. The spacer is made of the non-conducting material Polydimethylsiloxane (PDMS), which is a widely used silicon-based organic polymer. The ITO-coated sides of the two glasses are faced inward, both touching the PDMS. When the upper glass-slide is not yet placed on top, a circular hole made in the PDMS, allows the soluted lipids to be added onto the lower glass-slide and to be dried there. After the lipids have been thoroughly dried, the flow-chamber is closed and a semi-transparent heating foil is placed upon the upper glass, topped off by two small weights. Then, the two tubes embedded in the PDMS allow a sucrose solution to be injected into the PDMS-hole through one tube, and air is allowed to exit through the other. The sucrose solution is used in the diffusion experiments in compliance with a glucose solution. Their densities and refractive indices differ from each other, which provides a method that makes the GUVs better visible in those experiments. The PDMS-hole or opening, closed from above by the upper glass-slide, is completely filled with sucrose solution, which should reach the upper glass for the electroformation to work in a satisfactory way. Then, through wires connected to the ITO-coated sides of the glasses, a sinusoidal voltage is applied with a computer-controllable function generator, which induces an electric field within the sucrose solution. A thermocouple is attached upon the frame that holds the chamber at the right place on the microscope, and touches the lower ITO-glass from underneath the chamber, to measure indirectly the temperature of the solution [7]. To compensate for the systematic error made this way, a heat conduction model was used to calculate more accurately the temperature of the solution itself.
The electric field applied within the chamber couples to the electric dipole moment of the lipid molecules, making them vibrate with the corresponding frequency. This shakes up the lipids and therefore puts energy in the lipid film, eventually allowing the system to form configurations for which more energy is required, like GUVs. The microscopic dynamics of this process are still unknown. A detailed protocol description will be given in the results section.

The sine function can be chosen to be modulated by a linear function, a so-called ramp. This way the sine function slowly increases in amplitude until a maximum is reached, which might help in producing more homogeneous vesicles, with respect to both spatial symmetry and homogeneity in lipid ratios. Handling this was one of the purposes of a custom Labview program (a V.I., see [8]) that we used. The Labview V.I. was also used as an interface to conveniently control and record the elapsed time during the electroformation. Furthermore, the V.I. could be configured to change the sine wave to a square wave, after the vesicles had reached satisfactory size, for a desired amount of time. The purpose of this square wave was to help the GUVs to detach from the substrate, if they eventually were to be taken out of the chamber.

Some pictures now follow of the set-up components.

![Fig. 2.1: The chamber lies on the microscope frame, at the left there is a power supply, on top of it are the function generator and thermometer.](image-url)
**Fig. 2.2:** Assembling the chamber. Not yet closed, the dried lipids were initially added through the PDMS opening.

**Fig. 2.3a:** one of the two ITO-coated glasses
Fig. 2.3b: PDMS spacers

Fig. 2.3c: a semi-transparent heating foil

Fig. 2.3d: At an early stage, not the heater foil was used, but this small ITO-glass heater, which will be mentioned again in the results section.
Section 3: RESULTS

- 3.1 Characterizing the thermodynamic properties of the set-up
- 3.2 Model for temperature gradient within the chamber
- 3.3 Detailed description of the protocol and its execution; suggestions for improvement
- 3.4 Observations

3.1 Characterizing the thermal properties of the set-up

Because the temperature of the solution must be brought to a certain value, depending on the lipid composition being used, the temperature of the chamber, filled with sucrose, was plotted as a function of the applied voltage, to determine this relationship and the magnitude of the temperature fluctuations. This was carried out at an early stage when the small ITO-glass-heater was used (fig. 2.3d). However, the results can be expected to be applicable also in the case where the heating-foil is used.

Starting with the set-up at room temperature, a small voltage was applied to the heater, and the temperature was measured every second and immediately plotted in a graph, facilitated by the software supporting the thermometer. This way, while the set-up was heated, an exponential function of the form \( A\left(1 - e^{-\frac{t}{\tau}}\right) + C \) could be recognized to take shape in the graph, see fig. 3.1. The thermal properties therefore have the same dynamics as a RC circuit, the value \( 1/B \) taking on the role of the time constant \( \tau = RC \), with \( R \) the thermal resistance and \( C \) the thermal capacitance. \( \tau \) should be of comparable value for all heating processes. For the heating processes which temperature graphs are depicted in fig. 3.1a,c,d, this is the case and averaging the values give \((1/B)_{\text{average}} = 212 \text{ s} \), thus several minutes, which indicates that the chamber heats up sufficiently fast for our purposes. At the point where the temperature had not deviated from a value for over 1 minute, it was assumed that the temperature would not increase anymore, and thus the ending-temperature had been reached, indicating that an equilibrium had been reached. Therefore, the temperature fluctuations in the system appeared to be minor, and their magnitude being \( 0.1^\circ \text{C} \). Thus, the system's temperature could be brought to a desired value accurate to \( 0.1^\circ \text{C} \) and be expected to remain at that value for minutes with minor fluctuation.

After each measurement-session, the applied voltage was increased by 0.5 V and the process was repeated. The acquired final temperatures were plotted, see fig. 3.2, the resulting graph was fit with a quadratic function. The best fit was found to be the function \( T_{\text{final}} = 1.67 \ V^2 + 23.41 \), with \( \Delta T = T_{\text{final}} - T_{\text{room}} = T_{\text{final}} - 23.41 \) taking the role of supplied power in the analog formule \( P = V^2/R \) from electrodynamics.

Several graphs will now follow of temperature measurements with different applied voltages. The time after which the temperature fluctuations become minimal is omitted in most of the graphs. Except in fig 3.1d (4.5V) it is clear that fluctuations were almost absent immediately after the final temperature had been reached, regarding the straight horizontal line at the end.
Fig. 3.1a: \( T \) as function of \( t \), with applied voltage of \( 3.5 \) V, \( T_{\text{final}} = 32.9^\circ \text{C} \), with fit:
\[
T = 10.17 \left( 1 - e^{-1/271 \ t} \right) + 23.02
\]

Fig. 3.1b: \( T \) as function of \( t \), cooling down from \( 32.9^\circ \text{C} \) with no voltage applied. Fit:
\[
T = 11.22 \left( e^{-1/215 \ t} \right) + 22.11
\]
Fig. 3.1c: T as function of t, Voltage = 4 V, T_{final} = 42.4° C. Fit:

\[ T = 18.20 \left(1 - e^{-1/171 \cdot t}\right) + 24.20 \]

Fig. 3.1d: T as function of t, Voltage = 4.5 V, T_{final} = 56.9° C. Fit:

\[ T = 31.90 \left(1 - e^{-1/196 \cdot t}\right) + 25.10 \]
With the small ITO-glass heater, the temperature profile was measured across the heater (but still under the lower ITO-glass) at points indicated by the arrow in fig. 3.3. As can be seen from fig. 3.4, no significant temperature gradient was observed. Also there was no mentionable change in temperature in the perpendicular direction. Therefore the temperature is sufficiently constant throughout the solution in the chamber, so the temperature gradient does not significantly contribute to the wide spread in composition of the vesicles produced in electroformation while the temperature as measured by the thermocouple is at a constant value.

\[ T_{\text{final}} = 1.67 \ V^2 + 23.41 \]

**Fig. 3.2:** \( T_{\text{final}} \) as function of the applied voltage. Fit:

**Fig. 3.3:** The small ITO-glass heater with the arrow indicating the region for the profile measurements
3.2 Model for temperature gradient within the chamber

Because the thermocouple was placed below the chamber, touching the lower ITO-glass, it is of interest to model the temperature gradient within the chamber in the vertical direction, in order to determine the discrepancy between the temperature inside the chamber and the temperature measured. A one-dimensional model based on the heat conduction equation will suffice. Schematically in one dimension, the chamber turned on its side, will look like the following figure.

Fig. 3.4: Temperature profile: temperature as function of displacement (in mm) from the center of the small ITO-glass heater towards its electrodes, measured with applied voltage of 4.5 V, with $T_{\text{final}} = T(0 \text{ mm}) = 57.2 ° \text{C}$. It is clear that the temperature gradient from the center towards the electrodes is minimal.
The heat conduction equation for each section \( \frac{\partial T_i(r,t)}{\partial t} = \kappa \nabla^2 T_i(r,t) \), with thermal diffusivity \( \kappa \), reduces in the one-dimensional and static situation to \( \frac{\partial^2 T_i(x)}{\partial x^2} = 0 \). The general solution is \( T_i(x) = \alpha_i + \beta_i x \). For each of the three sections, from left to right in the figure, the local solutions would be: \( T_1(x) = Ax + B \), \( T_2(x) = Cx + D \), \( T_3(x) = Ex + F \). Because the temperature should be continuous throughout the chamber, \( T_1(x_1) = T_2(x_1) \) and \( T_2(x_2) = T_3(x_3) \). We notice that \( B = T_f \cdot \kappa_1 \), \( F = T_f \cdot \kappa_3 \), and

\[
\begin{align*}
A \cdot x_1 + B &= C \cdot x_1 + D \\
C \cdot x_2 + D &= E \cdot x_2 + F \\
A \cdot L_1 + T_f &= C \cdot L_1 + D \\
C \cdot x_2 + D &= E \cdot x_2 + (\kappa_3 - E \cdot x_3) \\
A \cdot L_1 + T_f &= C \cdot L_1 + D \\
C \cdot x_2 + D &= -L_2 E + T_{ic} \\
\end{align*}
\]

(1)

Additionally, the heat flux should be equal at both sides of the section boundaries, thus

\[
\kappa_4 \frac{\partial T_i(x_i)}{\partial x} = \kappa_2 \frac{\partial T_i(x_i)}{\partial x}, \text{ and } \kappa_2 \frac{\partial T_i(x_i)}{\partial x} = \kappa_3 \frac{\partial T_i(x_i)}{\partial x}, \text{ so } \kappa_1 A = \kappa_2 C = \kappa_3 E \quad (3).
\]

Filling (1) into (2), we get

\[
C x_2 + (A L_1 + T_f - C L_1) = -L_2 E + T_{ic}
\]

\( \Leftrightarrow C L_2 + A L_1 + T_f = -L_2 E + T_{ic} \Leftrightarrow L_2 C + L_1 A + L_3 E = T_d \quad (4), \) with \( \Delta T \equiv T_{ic} - T_f \).

Using (3) with (4), gives us equations for \( A, C \) and \( E \):

\[
\begin{align*}
A &= \Delta T / (L_2 \kappa_{12} + L_1 \kappa_{13}), \\
C &= \Delta T / (L_2 + L_1 \kappa_{21} + L_2 \kappa_{23}), \\
E &= \Delta T / (L_2 \kappa_{32} + L_1 \kappa_{31} + L_1),
\end{align*}
\]

with \( \kappa_{ij} \equiv \kappa_i / \kappa_j \), though only one of these equations is needed because (3) interrelates \( A, C \) and \( E \). Then expressions for these can be plugged into D

\[
\begin{align*}
A \cdot L_1 + T_f - C \cdot L_1 &= , \text{ and } F = T_{ic} - E \cdot x_3 .
\end{align*}
\]

Using the values \( \kappa_{glass} = \kappa_1 = \kappa_3 = 0.0013 \text{ W/(mm*K)}, \kappa_{water} = 0.00058 \text{ W/(mm*K)}, \)

\( L_1 = L_3 = 1.1 \text{ mm}, L_2 = 2.7 \text{ mm, and } x_3 = L_1 + L_2 + L_3 = 4.9 \text{ mm} \), all the coefficients can be determined as a function of \( T_f \) and \( T_{ic} \).

With these values, \( A = 0.121 \cdot \Delta T \)

\[
\begin{align*}
C &= (\kappa_1 / \kappa_2) A = 0.272 \cdot \Delta T , \\
E &= (\kappa_1 / \kappa_3) A = A = 0.121 \cdot \Delta T , \\
D &= -0.167 \cdot \Delta T + T_f , \\
F &= T_{ic} - 0.594 \cdot \Delta T .
\end{align*}
\]
The following graph depicts an example of the temperature gradient in the chamber, as a result of this model, with the values $T_{foil} = 60^\circ C$, $T_{tc} = 37.8^\circ C$, so $\Delta T = -22.2^\circ C$.

This model indicates that a systematic error of several degrees is made when the temperature is measured by the thermocouple located below the lower ITO-glass. The error made with transition temperature measurements comes on top of the error made because of the wide spread in vesicle composition, although it is of the same order, i.e. several degrees.

In a table that will follow, the measured transition temperatures of GUVs of several lipid mixtures are corrected with this model. This way more accurate transition temperature estimates are expected.
3.3 Detailed description of the protocol and its execution; suggestions for improvement

- 3.3.1 Preparations
- 3.3.2 Actual protocol
- 3.3.3 Taking out the vesicles

3.3.1 Preparations

- Making the spacer
- Lipid-solution preparation
- Connecting the wires

- Making the spacer

The PDMS spacer was custom made, using a silicone elastomer kit [9], which comes with a base constituent and a curing agent. These two constituents will chemically react with each other when mixed and the resulting mixture will quickly become solid PDMS. The protocol for making a PDMS spacer now follows. The two constituents are mixed in plastic beaker, with 9 parts base constituent for each part of curing agent, to a sufficiently amount to fill up a petri dish to a height equivalent to the desired thickness of the spacer. The mixture is stirred thoroughly in the beaker, and in order to remove the air bubbles within it is depressurized in a vacuum chamber for half an hour. The petri dish is thoroughly cleaned with water and ethanol. Then, because the silicon tube has to be well surrounded by the silicon from the mixture, the tube must be supported. This can be done by putting double patches of 0.5 mm thick tape at the far ends of the spacer to be, and in the middle where the spacer opening is going to be cut out (see the grey tape patches shown in fig. 3.5). A simple blueprint on paper of the spacer (like the one in fig. 3.5) is placed under the petri dish to be able to see where the tape patches should be placed exactly. The tube is placed and straighted out along the length of the petri dish upon the patches and firmly attached to the underlying tape with more tape. Then the bottom of the petri dish is covered with a tiny amount of vaseline, to help detach the spacer from the glass when it has become solid. Then the mixture can be poured into the petri dish until the spacer is of the desired thickness. The petri dish is then heated to 60°C to speed up the chemical reaction. After 2 hours, the spacer that has become solid can be very gently removed with tweezers, after the edges are cut loose from the petri dish. This must be done very carefully otherwise the spacer will be torn. After this, the spacer is placed upon the blueprint to see where the boundaries of the spacer should lie, and the spacer is cut to its final shape accordingly.
**Lipid-solution preparation**

5 mM chloroform-methanol solutions (volume ratio 9:1) of every lipid constituent to be used in the electroformation were separately prepared. After this, fractions of the initial solutions were added together to make the lipid mixture to use in electroformation. Dil was added to this to make the final mixture.

After making the final mixture, the solutions made were sealed with a layer of argon and stored at -20 °C and can be used for subsequent electroformation sessions for the next few days, during which the original composition of the solution will be reasonably maintained. After a few days this will no longer be the case because of chloroform and methanol evaporation and other decay effects.

**Wire-connections**

*The practical instructions are printed in blue.*

First, the wires must be connected to the ITO-coated side of each glass-slide. Initially we used copper-tape to attach the copper-wire to the glasses and to a third, smaller and thinner ITO-glass which we then used as heating-device. After some experiments it became clear that the tape would slowly detach from the glass, so that the electrical resistance would fluctuate rather fast, which resulted in temperature fluctuations.

- To improve this, a heating-foil (fig. 2.3c) was used instead of the small ITO-glass, and each copper-wire for the primary ITO-glasses was soldered to a copper-strip (fig.2.3a) of the same length of the ITO-glasses and width 1 cm. The copper-strips were then clipped upon the ITO-glasses, and their wires were connected to the functiongenerator.
3.3.2 Actual protocol

- Step 1, cleaning the set-up parts (20 min.)
- Step 2, adding the lipid solution and drying the lipids (40 min.)
- Step 3, injecting the sucrose solution (20 min.)

- **Step 1, cleaning the set-up parts (20 min.)**
  - The conducting sides of the ITO-glasses as well as both sides of the PDMS have to be thoroughly cleaned. The leftover sucrose from previous electroformation sessions can be best removed with a tissue soaked in distilled water. Then, each conducting side is rinsed with distilled water, after that with ethanol and finally with isopropanol. Immediately after the rinsing the remaining isopropanol is evaporated using a nitrogen stream.
  - The additional advantage of isopropanol is that it improves the adhesion of the PDMS to the glasses. This is important because the chamber must be as tightly closed as possible, since avoiding leakage of the sucrose solution out of the chamber is one of the major difficulties during preparation.

- **Step 2, adding the lipid solution and drying the lipids (40 min.)**
  - After the cleaning, the PDMS is placed upon the conducting side of one of the ITO-glasses and pressed upon with a set of tweezers.
  - Then the lower glass with the PDMS on it is placed on a heating plate, and heated up to a sufficient temperature level. After this, a small amount of lipid solution, preferably 20 to 25 µl (no more than 50 µl should be used, otherwise the lipid film will be too thick, see *observations*), is added upon the glass through the opening in the PDMS, using a small pasteur pipet. While doing so, the tip of the pipet can be used to spread out the lipid-solution over the whole of the opening, or to make ‘lines’ of lipid-solution by gently touching the surface of a glass. After this, the chloroform/methanol solution will quickly have evaporated for the most part, leaving only the lipids dried on the substrate. Then the lipids will be thoroughly dried by a nitrogen stream, for an hour. During this, the heating plate keeps the glass on the desired temperature.
  - This use of the pipet tip can be a convenient method because large vesicles are more easily produced when a thin layer of lipids is prepared. When these lines of lipid-solution are prepared you can tell where they have been drawn since after the electroformation the vesicles will have appeared in that area in a row (see fig.3.6).

---

**Fig. 3.6:** Lines of lipid film yield lines of GUVs. The left picture shows the start of the vesicle line from an area crowded with vesicles from where the pipet tip was used to start the line of lipids.
If the lipid-layer in an area is too thick, only small vesicles or none at all will appear there. The thorough drying is necessary to completely evaporate the chloroform and methanol.

- **Step 3, injecting the sucrose solution (20 min.)**
  - After drying, the chamber is now closed by putting on the upper glass, and is transferred to the frame of the microscope. Then, the wires of the glasses are connected to the function-generator. The thermocouple, firmly attached just below the microscope-frame, must touch the lower glass in order to measure the chamber’s temperature. Then, if heating is required, the heating foil, already connected and hot, is placed upon the upper glass, topped off by the weights. Then the chamber’s temperature is once again raised to the desired value. An insulating piece of material can be used between the foil and the weights, to speed up the re-heating of the chamber.

While performing these steps, the chamber will somewhat cool down, but this is not critical, as the lipids will have been completely dried at this point, and the different lipid constituents will not quickly mix if temperature becomes lower than the phase-transition temperature, since most of them will be immobilized.

The heater that we used is semi-transparent so that the light from the condenser can shine through from above, sufficiently illuminating the chamber to examine it using the objective below. The heater doesn’t have to be transparent in our case, because the light from the mercury lamp for fluorescence microscopy shines from below the set-up and also collected there.

- Then a 200 mM sucrose solution, also heated up to the desired temperature using the same heating plate, 1 ml in our case, is very slowly injected into the chamber through one of the tubes, using a syringe, initially filling up the PDMS-opening for roughly only 20 percent of its surface. Then, gently, during less than a second, more pressure is applied to the syringe to make the solution touch the upper ITO-glass. Then there is one part of the opening filled to the top with sucrose, and in the other part there is no sucrose yet. Then, very slowly, pressure is again applied to fill up the opening completely.

If the bottom of the opening is completely flooded before the upper ITO-glass is reached, the solution will prevent the air to escape through the other tube, and a large air bubble will remain in the chamber, which will hinder the electroformation significantly. 1 ml of sucrose solution is sufficient with the dimensions of our spacer. But leakage of the solution during or after injection can be an inconvenient problem. Therefore it is advisable to always prepare twice as much solution as minimally needed, in case the chamber isn’t closed tight enough and more solution is required to sufficiently fill up the chamber.

- After the chamber is filled up, both tube-endings are shut. The Labview V.I. is then configured to let the function generator apply a sinusoidal voltage of 3.3 V, 10 Hz, for the desired number of hours. The time for the ramp and the square wave, as well as its amplitude, are also entered into the V.I.’s interface. Then, when the solution is settled down and there is only little movement occurring, as can be also observed with the microscope, the V.I. is executed.
During the electroformation, the temperature of the solution was held well above the estimate of the mixing/demixing temperature corresponding to the lipid-composition being used, according to results already published [10].

3.3.3 Taking out the vesicles

After the electroformation was ended and many GUVs had reached a minimal desired size, often the GUVs were taken out of the chamber. This was done by simply opening the chamber and pipetting out the solution with the vesicles and putting the solution in an Eppendorf tube. This must be done with the pipet tip cut off, so that the opening becomes 3 mm wide, to decrease the chance that many of the vesicle will be destroyed due to adhesion of the vesicles with a narrow pipet tip. When put in an Eppendorf tube, the vesicle can be conveniently stored in a refrigerator and again pipetted out on a microscope slide for further analysis within 3 or 4 days after storage. The longer the GUVs are stored, the more will deteriorate by losing structure integrity. Deteriorated vesicles showed bits of them flowing freely inside themselves and many strings and tubes sticking inward and outward, making them unusable for further experiments.
3.4 Observations

- 3.4.1 GUV formation
- 3.4.2 Raft-domains formation
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3.4.1 GUV formation

During electroformation, GUVs that often appear to be spherical under the microscope are produced in vast numbers (fig. 3.7). The remarkable flexibility of the membranes allow the GUVs to take on various shapes, some caused by adhesion to other GUVs or the substrate, gravity or a (generally unwanted) current within the solution (fig. 3.8). Furthermore, GUVs uninfluenced by these disturbances still exhibit a variety of shapes, among which shape-transformations can be induced by changing parameters like the temperature or osmotic pressure of the solution.

The vesicles start out as patches of bilayer that are still attached to the substrate and become shaped like a mushroom (fig. 3.9). After that, they may or may not detach from the glass. If a vesicle detaches from the glass, its bilayer will close completely and the vesicle is free to move through the solution.

Fig. 3.7: a typical field of GUVs, illuminated with normal light, the larger ones having a diameter around 60 µm.

Fig. 3.8: GUVs, deformed because of interaction with each other
At each session, the film of dried lipids was clearly seen under the microscope (magnification 40) before and after the sucrose was added. When producing GUV’s of a single lipid, if the temperature was below the melting point when the voltage was applied and then slowly raised, the film remained static until the melting point was reached, after which the lipid film abruptly started to change, and the vesicles started to form. When GUVs were produced from a lipid mixture, the temperature of the chamber was set to be higher than the highest melting point of the constituent lipids before the voltage was applied.

In all cases, vesicles were observed to form in the same way. During the electroformation, the vesicles were clearly seen to vibrate with the frequency of the applied sine wave. Within minutes from the start, small vesicles (radius in the order of 1 μm) started to appear, growing gradually by merging with other vesicles. Within the order of an hour, many vesicles indeed have grown to be giant (in the order of 100 μm), although many have stayed small. Also, vesicles enclosing smaller ones were observed.

Vesicles have the highest probability to grow large (diameter > 50 μm) in areas where the lipid film is of the optimal thickness, i.e. where only a few layers of vesicle-fields are eventually produced. In areas with no vesicles fields at all, most present vesicles will be isolated, so there will be too few vesicles to fuse with, so those vesicles will stay small. If there are more than a few (>3-5) layers in an area, it will be crowded with small vesicles. It is observed that in such areas in most cases the vesicles will not become large, presumably because of interaction with its many neighbours.

At spots where the lipid film is thick, so that more than one layer of vesicles will eventually form during electroformation, a ramp of at least 15 minutes that linearly modulates the sine wave will help to produce vesicles less deformed, but this is no significant effect.

There was no evidence that the optional square wave at the end of the electroformation process was able to help the vesicles to detach from the substrate.
3.4.2 Raft-domains formation

After GUVs from a lipid mixture were allowed to form and grow in the chamber for at least one hour above the required temperature, so that they appeared to be homogeneous during the electroformation, the voltage applied to the glasses was turned off, and the temperature was decreased gradually or abruptly. In both ways, with almost all compositions that eventually would show raft-domains, the rafts appeared in more than 50% of the GUV’s at a certain temperature, and the more the temperature was lowered, the more GUV’s would undergo a phase transition. With almost all of these compositions, eventually 90% to 99% of all GUV’s would show rafts, their transition temperatures lying within a range of a few degrees.

The rafts were small at first (the smallest ones visible were 1 µm big, see fig. 3.10), moved through the bilayer, sometimes quite fast (in the order of 1 µm/s), and changed shape, with their boundaries fluctuating. Also, in some cases within minutes, many merged together to form larger rafts (see fig.3.11). This process would continue until there was only one large raft left (again see fig.3.10, the upper vesicle is 60 µm in diameter and has a single raft of diameter 35 µm).

Heterogeneous states consisting of two liquid domains, as well as at least one of the domains being solid, have been observed.

![Fig. 3.10](image_url): The lower big vesicle shows rafts that appeared a moment before the screenshot was taken. The upper vesicle had already been inhibiting rafts for some time, thus its rafts have fused together to form a single large raft. The lower vesicle has an extension (horizontally in the picture) of 69 µm, its smallest rafts are 1 µm in diameter, its largest rafts extending less than 10 µm. The upper vesicle is 60 µm in diameter and has a single big raft of 35 µm in diameter.
3.4.3 Phase diagram

A phase diagram follows of the lipid mixtures used to produce GUVs, their composition, if there were bright domains on a dark background or vice versa, and their measured transition temperatures $T_m$ if it was determined.

![Phase diagram of used mixtures](image)

<table>
<thead>
<tr>
<th>DOPC (%)</th>
<th>BSM (%)</th>
<th>CHOL (%)</th>
<th>Domains</th>
<th>$T_m$ (° C)</th>
<th>$T_h$ (° C)</th>
<th>$T_c$ (° C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>33</td>
<td>33</td>
<td>33</td>
<td>both kinds</td>
<td>28.7</td>
<td>36.7</td>
<td>29.8</td>
</tr>
<tr>
<td>20</td>
<td>40</td>
<td>40</td>
<td>bright on dark</td>
<td>25.7</td>
<td>28.6</td>
<td>26.1</td>
</tr>
<tr>
<td>15</td>
<td>50</td>
<td>35</td>
<td>bright on dark</td>
<td>24.8</td>
<td>27.8</td>
<td>25.2</td>
</tr>
<tr>
<td>13</td>
<td>62</td>
<td>25</td>
<td>dark on bright</td>
<td>n.d.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.5</td>
<td>67.5</td>
<td>25</td>
<td>bright on dark</td>
<td>31.0</td>
<td>40.7</td>
<td>32.3</td>
</tr>
<tr>
<td>9</td>
<td>66</td>
<td>25</td>
<td>both kinds</td>
<td>34.0</td>
<td>46.9</td>
<td>35.7</td>
</tr>
<tr>
<td>10</td>
<td>70</td>
<td>20</td>
<td>bright on dark</td>
<td>n.d.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>66</td>
<td>20</td>
<td>dark on bright</td>
<td>n.d.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>62</td>
<td>20</td>
<td>dark on bright</td>
<td>33.0</td>
<td>45.2</td>
<td>34.6</td>
</tr>
<tr>
<td>40</td>
<td>40</td>
<td>20</td>
<td>dark on bright</td>
<td>33.9</td>
<td>46.9</td>
<td>35.7</td>
</tr>
</tbody>
</table>

- Mixture with GUVs with bright rafts surrounded by a dark domain.
- Mixture with GUVs with dark rafts surrounded by a bright domain.
- Two mixtures correspond to this point, the (7.5%, 67.5%, 25%) mixture showed GUVs with bright rafts surrounded by a dark domain and the (9%, 66%, 25%) mixture showed both kinds of GUVs.
$T_m$ is the measured transition temperature, $T_h$ is the corresponding temperature of the heater, and $T_c$ is the final corrected transition temperature, as prescribed by the heat conduction model. ‘n.d.’ means not determined.

The diagram suggests that the upper and far right part of the region outlined by the measurements correspond to mixtures yielding vesicles inhibiting bright rafts surrounded by a dark domain, and the lower-left part dark rafts surrounded by a bright domain.

The (33%, 33%, 33%) and (20%, 40%, 40%) mixtures were probably close to the boundary of the region of compositions at which GUVs are capable of inhibiting coexisting phases, because of the low yield in rafts showing vesicles.

The (10%, 70%, 20%) mixture showed small bright rafts that appeared to be static and therefore were probably embedded in a solid domain.

If we compare these results with those already published ([10]), we see some comparisons between this diagram and the ones from the published paper. Especially the common boundary of the areas where the GUVs show bright rafts on dark domains and vice versa seem to be comparable. However it must be considered that the sphingomyelin used in the paper does not contain unsaturated components, while the brain sphingomyelin used in our experiments does, so we expect our results to differ from those described in the paper.

### 3.4.4 Raft bulging

Some GUVs which rafts have fused together so that only a few larger ones remain, show their rafts distinctly bulging out of from its membrane (fig. 3.13). This is referred to as raft bulging, and still is unclear at which circumstances this effect appears. When bulging is present in a GUV, the membrane appears to be stable for the order of an hour, in that its rafts do not fuse together to form a single large raft. This is important, since in the diffusion experiments mentioned in the first chapter, the situation at which GUVs show several smaller rafts instead of only one large raft is required. Bulging is also observed with GUVs inhibiting a single large raft (fig. 3.14).

**Fig. 3.13:** the bright rafts embedded in a dark domain in the left GUV (diameter 55 µm) are clearly bulging out from its membrane, while the rafts in the right GUV are not.
3.4.5 Raft induction by light

During one of the transition temperature measurements, using the (9%,66%,25%) mixture, an unexpected behavior of inhomogeneous vesicles was observed. When an arbitrary field of homogeneous GUVs, illuminated by the mercury lamp, was focussed upon, raft-domains would appear after a few minutes in the vesicles seen by the camara, even while the temperature was held at a constant level. The vesicles just outside the view of the camara remained inactive within the same timescale, as was concluded by moving the camara quickly to neighboring vesicles and back again. Apperantly, the light from the mercury lamp is able to induce the raft-domains in GUVs. Because the lamp is aligned with the camara, the amplitude of the lamp is highest in the middle of the camara's view, and therefore that is where the rafts will be induced first in general.

The procedure consisting of illuminating an arbitrary field of vesicles, measuring the time it would take for the rafts to appear, then comparing with neighboring vesicles that weren't illuminated as heavily, was repeated at least 10 times with the initial mixture. At each session, rafts were successfully induced in almost all vesicles between 2 and 15 minutes. Each vesicle would show its induced rafts after a different amount of time, the smaller ones taking significantly less time than the larger ones. During the inducement sessions, the lightbeam was converged to illuminate a circular field of vesicles within the camara's view (see fig.3.16). After the rafts had appeared in most of the vesicles (see fig.3.17), the beam was diverged again, to compare the heavily illuminated vesicles with their neighbors that were outside the circular area, but still within the camara's view during the inducement. The circular target area was easily distinguished from its surroundings because the converged light had bleached most of the fluorophore present there, leaving a dark spot (fig. 3.18).

The behaviour of the light-induced rafts was completely comparable to the behavior of the rafts that appeared after lowering the temperature below $T_m$. 
**Fig. 3.16:** When none of the vesicles show rafts, light is converged upon an arbitrary vesicle field.

**Fig. 3.17:** A few minutes later, in all vesicles being illuminated by the converged light beam, rafts have appeared.
Oxygen radicals (superoxide), in our case a side product of fluorescence microscopy, are able to react with the cholesterol in the lipid mixtures and is referred to as photo-oxidation [11]. Therefore the transition temperature changes and may go from a value higher than the current temperature to a lower value. In this case the GUVs undergo the phase transition even when the temperature hasn't changed. If a constituent can be added to the GUVs that can chemically adsorb the oxygen radicals before they damage the cholesterol, this effect may be discouraged.

Therefore, in an attempt to significantly shield off this effect 0.5 µl (±)-α-Tocopherol (vitamin E) was added to the (9%, 66%, 25%) mixture prior to electroformation. The oxygen radicals were expected to couple more easily to this vitamin, so that the cholesterol in the membrane would be significantly spared when the raft induction by light was attempted. Then, it should take significantly more time to induce the rafts in the Tocopherol mixture than in a test case without Tocopherol. Naturally, the same mixture without (±)-α-Tocopherol was used in the same experiment as a test case. Both lipid mixtures were dried in separate holes of a new PDMS spacer, placed upon the lower ITO-glass as usual, custom made for this experiment. Both mixtures were subject to the electroformation procedure as usual. After the electroformation was completed, raft-domains were induced by a converged lightbeam in the same way as before. However, no apparent difference was observed in the inducement processes between the (±)-α-Tocopherol mixture and the test case. With both mixtures the rafts were induced within 3 minutes in small vesicles (of

Fig. 3.18: After the light beam is diverged again, the vesicles surrounding the bleached area do not show rafts. Therefore, the light must have induced the rafts.
diameter between 20µm and 40 µm), with 10 different inducement sessions. Therefore, the (±)-α-Tocopherol was ineffective in shielding off the raft inducibility by light. One explanation would be that the (±)-α-Tocopherol, although soluble in fat, was not able to perform its duty effectively because of unfavourable dynamics of the membrane. Also, the (±)-α-Tocopherol may not have been made part of the membranes at all.

3.4.6 Eyeballs

In some instances, GUVs with a single raft that contained a domain by itself were observed. These were called eyeballs (fig. 3.15), for the obvious reason. Again, what circumstances are necessary for this to occur is not clear, but we suspect that again photo-oxidation is responsible for this effect.

Fig.3.15: eyeball GUVs. By this picture, it is hard to be convinced that the ring-like rafts are each part of a GUV, but that is indeed the case.
Section 4

SUMMARY

The raft induction by light section makes clear that the GUV’s composition changes significantly under the influence of light, which must taken in consideration when accurate measurements of GUV properties are to be conducted. GUVs exposed to light may also deteriorate faster, because the membrane may lose some of its structural integrity and small gaps may form, eventually leading to the GUV’s collapse. This effect would come on top of the normal deterioration of GUVs when they are stored for a day at 4°C in the dark, therefore any measurements are preferably done within the 4 or 5 hours after the GUVs were produced, while during that time exposure to light has to be minimalized.

The project objectives have been met. GUVs suitable for diffusion experiments were produced in vast numbers, and their transition temperature was measured in many cases. The thermal properties section shows that with this set-up no automatically operating temperature-control is needed to keep the GUVs at a desired temperature accurate to 0.1°C, which makes the production of GUVs relatively easy. However, in terms of subsequent diffusion experiments, questions remain about in what circumstances special GUV properties ‘come to light’, the effects of photo-oxidation and raft bulging being some of the most important ones.
REFERENCES

[4] the lipids were purchased from Avanti Polar Lipids, inc.; the lipid structures were taken from www.avantilipids.com.
[7] the thermocouple and supporting software were accompanying the HH506RA Multilogger Thermometer, of Omega Engineering Inc.
[8] Labview 7.0 Express, National Instruments.
[10] the estimations were based on "Miscibility phase diagrams of Giant Unilamellar Vesicles containing Sphingomyelin" by S.L.Veatch, S.L.Keller, Physical Review Letters(PRL 94, 148101 (2005)).

Every figure or picture that is not referenced to in the following list, is made or acquired during the project itself, and is not acquired from other studies.

Fig. 1.1b: Vesicle schematic picture from www.azonano.com
Fig. 1.2: Micel picture from www.sakamura-lab.org
Fig. 1.3: Bilayer picture from www.bioteach.ubc.ca
Fig. 1.5: Molecular structure schematics from www.avantilipids.com
Fig. 3.9: Vesicle attached to substrate picture from www.sakamura-lab.org