Katholieke Universiteit Leuven

Faculty of Bioscience Engineering

Giant unilamellar vesicles production via a double emulsion microfluidic device

Promoter:
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Supervisor:
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Department of Biosystems (BIOSYST)
Division of Mechatronics, Biostatics and Sensors (MeBioS)
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Erasmus dissertation

Sergio Duró Ortiz

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1 Preface

This is the Erasmus Dissertation written in KU Leuven by Sergio Duró Ortiz, corresponding to my Bachelor´s Thesis, since this year I am finishing my degree in Biotechnology, from the University of Zaragoza, Spain.

Two years ago, I was spending a long time thinking about finishing my degree out of my home university, and also out of Spain. One year later, I was selected for doing my Erasmus exchange with the KU Leuven, in which my Bachelor´s Thesis would be included.

When I started looking for a research group to do my final work, I heard from my faculty about Professor Xavier Casadevall, so I searched what his group was focusing on and I saw the opportunity to join their research on artificial cells.

When I first arrived and started the experimentation, nothing had to do with what I had been doing until that moment. These months I have experienced for the first time independent laboratory work and what it entailed, working on my own, not always being supervised by my teachers, and being proactive in terms of the problems that arose during the experiments.

Not all the project has been an easy road to the final result, there have been more problems than expected, with many days without any result but finally, and little by little, those problems were gradually solved until the project´s objective was reached.

I would like to thank Prof. Xavier Casadevall, for giving me the chance to experience first-hand what research work is like. I also want to express my gratitude to my supervisor during the whole project, Jorik Waeterschoot, who has helped me through all the difficulties, as well as being a lab partner. And finally, I would like to mention all my Erasmus friends who have cheered me up in those hard-working days and to my family, for their support throughout this project.

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2 Summary

Synthetic biology represents an emerging field of research that aims at mimicking and understanding living systems and their building blocks from a fundamental bottom-up perspective[(1)]. In numerous studies, lipid vesicles have been identified as ideal candidates for compartments capable of imitating cellular functionalities, due to the structure, size, and biophysical or biochemical properties of the lipid bilayer[(2)].

Over the past decades, microfluidic devices have been developed and widely used for the high-throughput production of droplets and multiple emulsions[(3)].

In this project, a determined microfluidic device will be studied, tested, and analyzed as a way to enable the bottom-up reconstruction of artificial cells. The methodology used will allow to achieve this objective starting from giant unilamellar vesicles (GUVs) and subsequently use biological material to mimic cell-like processes.

PDMS, the material used to build the device, will be the starting point. Due to its properties, it is necessary to adapt the conditions and perform a few treatments before reaching full operativity of the chip, producing GUVs at high throughput without requiring additives.

The first step in the GUV production process is the production of the PDMS microfluidic device fabrication by means of soft-photolithography, PDMS is cured on the master mold, peeled and cut into chips, inlets and outlets are punched and finally they are bound to a cover slide using an air plasma treatment. Next, a channel treatment must be performed before starting any experiment. It consists of selectively flowing certain acid and polymeric solutions through the microchannels, in order to hydrophilize the walls and get a successful production of water-in-oil-in-water double emulsions. Next, different flow rates of the fluids will be tested to finally reach the pursued objective.

After optimizing both the coating process and the flow rates for the correct GUV production, different solutions will be used as inner and outer in addition to the lipid phase.

Finally, another GUV formation method called electroformation will be studied and tested as comparison, operating for the first time an automatic device from ‘Nanion Technologies’ company.

3 List of abbreviations

- BSA: Bovine serum albumine
- DNA: Deoxyribonucleic Acid
- DOPC: 1,2-dioleoyl-sn-glycero-3-phosphocholine
- GUV: Giant unilamellar vesicle
- IA: Inner aqueous
- IPA: Isopropanol
- ITO: Indium-Tin-Oxide
- LO: Lipid oil
- LUV: Large unilamellar vesicle
- O/W: Oil-in-water
- OA: Outer aqueous
- PBS: Phosphate buffered saline
- PDADMAC: poly(diallydimethylammonium chloride)
- PDMS: Poly(dimethylsiloxane)
- PEG: Polyethylene glycol
- PGMEA: Propylene glycol monomethyl ether acetate
- POPC: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
- PSS: poly(sodium 4-styrenesulfonate)
- RF: Radiofrequency
- SUV: Small unilamellar vesicle
- W/O/W: Water-in-oil-in-water
- W/O: Water-in-oil

4 Introduction

4.1 Microfluidics

In this research work, microfluidics will be referred to as the science and technology of systems which studies the process, manipulation and behavior of fluids constrained to a small scale through micro-channels[(4)], featuring dimensions typically on the order of tens to hundreds of micrometers, and small volumes of fluids (down to femtoliters)[(6)] This field involves several disciplines such as physics, biochemistry, nanotechnology, biotechnology, and engineering[(5)].

These microfluidic properties developed along with lab on chip (LOC) devices, microsystems capable of integrating chemical or biological laboratories in a single chip, thanks to the advanced fabrication technologies capable of miniaturizing key fluidic components in order to improve the compactness of the devices and their functionalities.

A key feature of this microscale is the behavior of fluids, which differ from microfluidic behavior in factors as energy dissipation, fluidic resistance, surface tension… Flow within microfluidic devices is almost always laminar, meaning that mixing generally occurs by molecular diffusion, enabling a complete mixing in seconds or minutes. High specificity of chemical and physical properties (pH, temperature, concentration…) can also be ensured resulting in more uniform reaction conditions and higher-grade products in single and multi-step reactions[(7)]. A further advantage connected to this is that volumes of reagents and samples needed can be significantly reduced, producing less waste, and saving costs. Many operations can be executed at the same time thanks to their compact size, shortening the time of most of the experiments. They also offer an excellent parameter control which allows process automation, requiring a low level of expertise and a lot of functionalities (enhanced analytical sensitivity and temperature control, portability, faster reaction time…)[(8.)]

Microfluidic devices can be fabricated from a range of materials using different methods. A microfluidic chip is a pattern of microchannels, molded or engraved, and this network of channels is linked to the macro-environment by several inlets and outlets punched out through the chip[(6)] *(Fig. 1)*. Fluids are injected and evacuated from the chip by these inlets/outlets, and for this purpose several methods are available. The two most used methods are hydrodynamic pumping (application of pressure via syringe pump, hydrostatic forces…) and electroosmotic flow (EOF) pumping (application of voltage difference across charged surfaces inside the microchannels)[(9)].

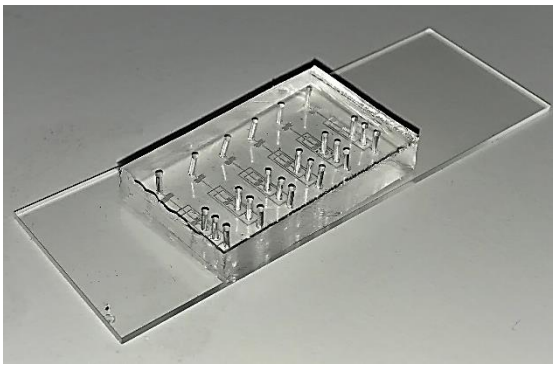

Fig. 1: Microfluidic chips based on PDMS.

The most common chip material employed in the research lab nowadays, and also in this project is the flexible elastomer, poly(dimethylsiloxane) or PDMS.

PDMS ($CH_3[Si(CH_3)_2O]_nSi(CH_3)_3$, n being the number of monomers repetitions) is the most widely used silicon-based organic polymer, because of its versatility and properties[10]. Apart from microfluidics, it is used as a food additive (E9009, shampoos, surfactant, cosmetics, etc.). The choice to make microfluidic chips with this polymer is transparency (it facilitates the observation), low autofluorescence, biocompatibility, deformability, low cost, gas permeability and it is easy to mold. Nevertheless, it adsorbs hydrophobic molecules that can be a problem for some studies in microfluidic devices, and it is sensitive to the exposure to some chemicals. Furthermore, they are also not appropriate for high pressure operations because of the deformability of the microchannels.

For the fabrication of microfluidic devices, PDMS is mixed with a cross-linking agent, poured onto the master mold of interest, and cured to finally obtain an elastomeric replica of the microstructured mold. This elastomer is hydrophobic, this means that polar solvents, such as water, cannot wet it. To overcome this barrier, plasma oxidation treatment can be used to alter the surface chemistry, adding silanol (SiOH) groups to the surface and rendering the PDMS surface hydrophilic for approximately thirty minutes and increasing surface wettability, making it resistant to the adsorption of hydrophobic and negatively charged molecules. In addition, plasma oxidation is used to covalently bond PDMS on an oxidized glass surface (or with another oxidized PDMS) to form bridging Si-O-Si bond at the interface, creating an irreversible seal ideal for microchannel formation and function *(Fig. 2)*. However, most organic solvents can diffuse into the material and cause it to swell and deform[11].

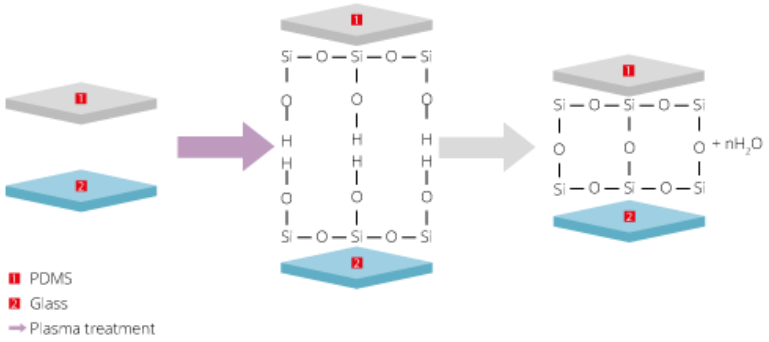

Fig. 2: Schematic representation of PDMS bonding procedure[12]

There are open microfluidics techniques such as open-channel microfluidics, rail-based, paper-based and thread-based, where the fluid is exposed to air or another interface. These techniques make use of capillary forces to move fluids without any need for external energy. Other branch of microfluidic techniques is continuous flow microfluidics, which allow to manipulate the continuous flow of liquid through microchannels thanks to external pressure or mechanical pumps (or combination of capillary forces and electrokinetic mechanisms). Despite there are other techniques in this field like digital microfluidics, where droplets can be placed and manipulated by the application of electric fields (electrowetting), in this project we will focus on droplet-based microfluidics. Droplet-based microfluidics involves the generation of discrete volumes of a solution (dispersed phase) in an immiscible liquid (continuous phase), usually by devices that feature either a T-junction or a flow focusing layout. By modifying the flow rates and composition of the inlet solutions, it is possible to generate water-in-oil (W/O) droplets or oil-in-water (O/W) droplets *(Fig. 3)*, as well as droplets-within-droplets. In these devices, the surface properties of the microchannels are critically important for droplets generation, it should

preferentially be wetted by the continuous phase. As PDMS is inherently hydrophobic, oil-in water droplets can be generated using PDMS microfluidic device after hydrophilic modification of the channel wall. These droplets can be produced at a rate of hundreds or thousands per second in a highly reproducible manner, which allows for quantitative measurements and analysis. Applications include chemical reactions, protein crystallization, material synthesis, single cell analysis, DNA amplification, enzyme kinetics…[7,13]

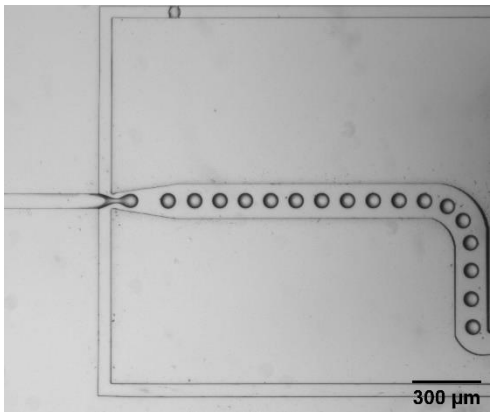

Fig. 3: Generation of O/W droplets in a chip.

4.2 Giant unilamellar vesicles (GUVs)

GUVs are simple model membrane systems of cell-size, which are instrumental to study the function of more complex biological membranes involving heterogeneities in lipid composition, mechanical properties, shape, and chemical properties *(Fig. 4)* . Vesicles are free-standing bilateral models whose size, geometry and composition can be modified with high precision. These include vesicles of sizes ranging from 50-100 nm (small unilamellar vesicles, SUVs) to 1-100 µm (GUVs), but there are also large unilamellar vesicles, LUVs (0.1-1 µm).

The vesicle membrane involves a self-assembly of lipid molecules in water, in which the chemical and mechanical instabilities of phospholipids under high ionic strength conditions (especially multivalent cations), their sensitivity to pH changes, monodispersity, tunability and incorporation of biological material are the main challenges in using protocells for synthetic biology[15,17].

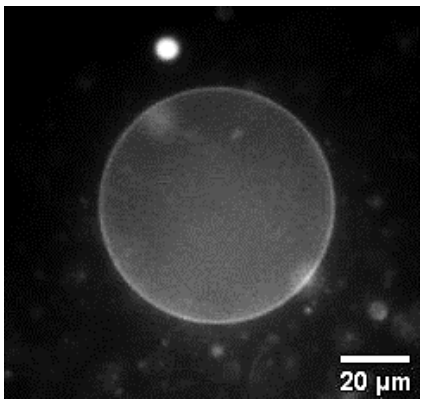

Fig. 4: Fluorescent microscopy image of a Giant unilamellar vesicle, with fluorescent labelled lipid, Liss Rhod PE.

4.3 GUV formation methods

Various methods have been developed to form GUVs, such as gentle hydration, electroformation, droplet transfer method, or by using microfluidics[14].

Currently, electroformation and gentle hydration are the most commonly used strategies for creating GUVs. But, low GUV yields in physiological buffer solutions, being heterogeneous in GUV's size and lipid composition, strong limitations to small amounts of charged lipids and low efficiency biomolecule encapsulation remain the principal drawbacks of these methods towards performing a synthetic cell. Other methods like microfluidic jetting or emulsion transfer overcome these limitations to some extent but compromise the GUV yield. In this project we will focus on electroformation and microfluidics techniques.
Recently, other microfluidic technologies have emerged, in order to improve the high- throughput production of monodisperse GUVs, such as capillary-based microfluidics and dewetting of double emulsion droplets[16,17].

Gentle hydration method, also called spontaneous swelling method, consists of depositing a thin lipid film on a glass substrate, where lipids swell into vesicles by rehydration in aqueous solutions. Despite the recent improvements in electroformation protocols, gentle hydration is still the preferred method when the lipid mixture contains mainly charged lipids when physiological salt conditions are used. On the other hand, this spontaneously swelled GUVs show several defects including different lipid structures attached to the inner or outer membrane, encapsulated vesicles… Other drawbacks are the yield is low and variable, it takes several days to form GUVs, and it only works well for specific conditions and lipid compositions.

Electroformation, it is known to be the best method to produce GUVs because of its high yields of spherical giant vesicles with less variable size, high unilamellarity and fewer defect structures. This method is the same as the spontaneous swelling but with an improvement: application of electric voltage. GUV electroformation should be considered as two-step procedure. Firstly, lipid film is deposited on the electrode (for instance ITO, Indium tin oxide or platinum wire, conductive coating of a glass slide). The next step is the electroformation as such, where electric field affects lipid swelling through direct electrostatic interactions, redistribution of counterions, changes in membrane surface and line tension, and electroosmotic flow effects[14,18]. This requires bilayer separation and bending from the lipid film, and external electrical fields can facilitate both: they can increase the intermembrane attraction and can induce instability of bending, inducing lipid swelling and GUV formation[19] *(Fig. 5)*.

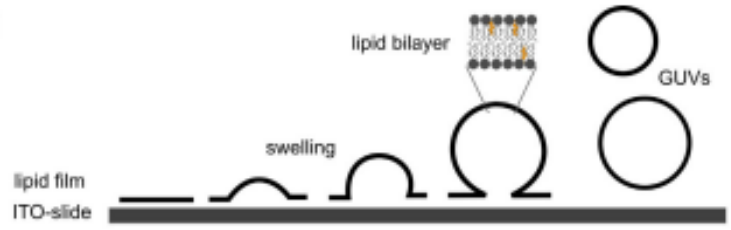

Fig. 5: Schematic representation of GUVs electroformation on ITO-slides[14]

Another method is the so-called droplet transfer, also known as phase-transfer method, water-in-oil method or emulsion-based method. Its principle lies in transferring lipid stabilized water-in-oil droplets through an oil-water interface, where it picks up a second lipid monolayer, resulting in formation of a completed GUV in water with encapsulated contents. One of the main

advantages of this method is that it allows the preparation of asymmetric membranes of GUVs (different lipid composition of the membrane bilayer), and GUVs with different solutions in the inner than in the outer. Towards formation of synthetic cells, this method is a very good approach. This method can further increase reproducibility and control of GUVs production by using microfluidic techniques.

Finally, microfluidic lab-on-a-chip methods are being developed for GUV fabrication, using flow-focusing droplet microfluidics geometries *(Fig. 6)*. Vesicles are produced either by continuous flows using microdevices that focus fluid streams or by pulsed microfluidic jets that deform interfaces and at the end causes the formation of multiple and monodisperse vesicles. Jet of fluid can contain the desired components (e.g., proteins, fluorescent probes…) that are trapped inside the vesicles. This is a great advantage besides the elimination of the washing step of the GUVs to eliminate excess components in the case of electroformation or gentle hydration[14,20].

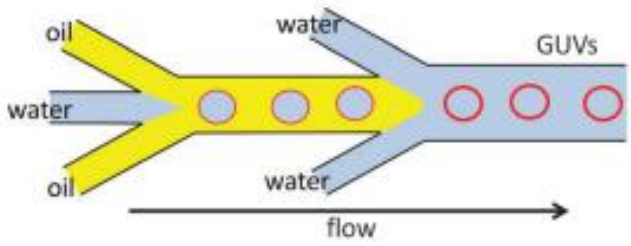

Fig. 6: Schematic representation of microfluidic double emulsion method to create GUVs[17]

Currently, the well-established electroformation and spontaneous swelling to produce giant unilamellar vesicles are limited in the uniform encapsulation of large and charged biomolecules, giving low reliability and reproducibility for this purpose. The emulsion method can be a straightforward and reliable technique to produce basic models for cells, albeit with some drawbacks (lack of size control, low throughput, and dependency on the density of solutions) which, interestingly, can be mitigated by implementing microfluidics to make lipid-based vesicles (microdroplet technology)[3]. Furthermore, droplet-based microfluidics offers superior handling and manipulation of GUVs through better mixing of liquids, mainly due to the laminar flow environment that is typical of microfluidic channels, enabling reactions to be performed in a highly controlled and reproducible manner. In addition, the size and composition of the GUVs can be flexibly designed through adjustment of the flow rate, and the nature of input reagents. Unlike almost all conventional methods including electroformation, this microfluidic technique allows for the continuous production of GUVs in a single workflow. Another advantage of microfluidics is that many reactions within microfluidic environments may be performed under mild conditions, a feature highly beneficial for many bioactive reaction systems[21].

All these mentioned approaches are being used to trying to mimic cells in recent years (artificial cells), but, considering the application of GUVs as compartments in synthetic biology and related fields, and compared with the other methods, the high encapsulation efficiency that goes along with this microfluidic approach can be considered as a major advantage[1]. Its potential to produce configurations of artificial cells in a single platform could form the basis of successful future endeavors in bottom-up synthetic biology specially to produce asymmetric bilayers that are more similar to eukaryotic cells, and it could help progress in the field of artificial cells considerably in the near future[3].

5 Methods

5.1 Microfluidic device fabrication

The PDMS-based microfluidic device fabrication was performed using soft-photolithography. Master molds were prepared by spincoating SU8 2075 on a 3'' silicon wafer using SU8 to a height of 80 µm and curing it through a photomask. SU8 is a commonly used epoxy-based negative photoresist.

SYLGARD®184 was the silicone based elastomeric kit used, it is a two component system with a polymeric base and a curing agent which cross-links with the polymeric matrix (the cleanest PDMS)[6,22].

5.1.1 Master mold protocol

The 3'' silicon wafers were cleaned with acetone followed by drying with N_2. The wafer was then placed on a hot plate at 120ºC for 10 min to dehydrate. Next, it is spin coated with SU-8 2075 photoresist at 500 rpm for 10s with acceleration of 100 rpm/s; second followed by 3000 rpm for 40s with acceleration of 250 rpm/s.

Once coated, the wafer was baked at 65ºC for 3 min and at 95ºC, for 9 min. The soft bake function is to remove the solvent from the resist and make the layer solid. Then, the photomask (cleaned with IPA) was placed in contact with the resist covered wafer and exposed at 250mJ/cm^2 to initiate crosslinking. After exposure the SU-8 2075 needs to be baked again to complete the polymerization, so a post exposure bake was performed: 65ºC during 2 min and 95ºC, 7 min.

Finally, the wafer was placed in a developer solution where unpolymerized SU-8 is dissolved (the final positive replica of the design is obtained). The main developer for SU-8 is PGMEA (propylene glycol monomethyl ether acetate). This process lasts 7 min, and the completed master was then rinsed with the developer and IPA and baked for 10 min at 120ºC[22].

5.1.2 Chip manufacturing protocol

Once the master mold is made, the PDMS microfabrication can be started. The first procedure was the PDMS casting, starting with the addition 22 g of liquid PDMS in a weighing boat, followed by 2,2 g of curing agent (amounts to completely cover a new wafer with a layer of adequate thickness). It is important that Sylgard 184 Silicone Elastomer Kit has to be mixed in a 10 (base):1 (curing agent) ratio by weight for manual mixing[23]. The mixture was stirred thoroughly for couple minutes, put in desiccator until all gas bubbles were removed. Being carefull, the PDMS was poured onto the master mold and it was put in the oven for at least 1 hour at 65ºC.

Afterwards, the cured PDMS was peeled, cut into separate chips and punched with a 1 mm puncher to form inlets and outlets. Tape was used to remove any dust from the PDMS surface.

Finally, the chips need to be bound to a glass slide. For it, a clean glass slide was washed with isopropanol (IPA) and dried. In the plasma cleaner, the chips were placed bottom side up and the glass slide next to them. Next, 30 seconds at high level (RF power: 30 W, Pressure: 0 mbar) after turning on the vacuum pump and the plasma cleaner. After this step, the chips were put on the slides and squeezed gently to ensure contact, then, they were left in the oven for at least 1 hour at 65ºC *(Fig. 7)*.

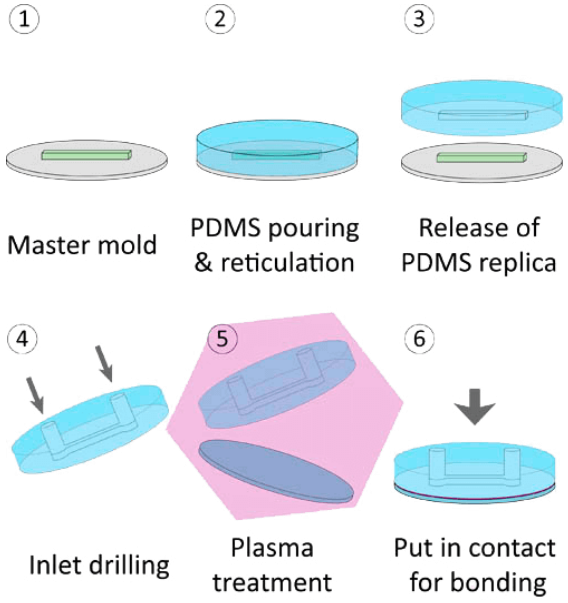

Fig. 7: Schematic representation of the steps followed for the chip manufacturing[11].

5.1.3 Microfluidic device design

The microfluidic design consists of two consecutive cross-junctions allowing for hydrodynamic flow focusing, as depicted in *Fig. 7a*. This involves microdroplet technology to create double emulsions of water-in-oil-in-water (W/O/W) with lipids in the oil phase. An inner aqueous solution (IA) is sheared by an oil phase containing lipids (LO), resulting in the formation of W/O single emulsions whose interface is assembled with a monolayer of lipids, thanks to their amphiphilic nature. This single emulsion is sheared again into droplets by an outer aqueous solution (OA) to form W/O/W double emulsions.

Apart from the standard cross-junction to produce droplets, there are two serpentine-shaped channels to reduce the risk of fluid backflow, add fluid resistance and increase flow stability *(Fig. 8b)*. In addition, the width of the second serpentine module decreases every turn, providing increased fluidic velocities and more shear force for excess oil removal *(Fig. 8c)*.

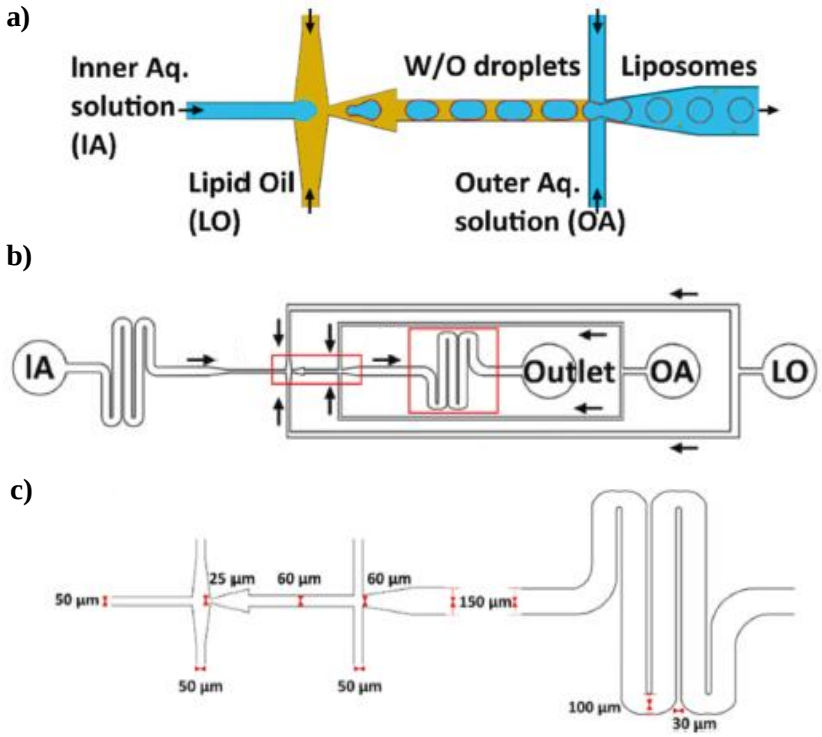

Fig.8: Schematic representation of the chip design. a: Double cross-junction with its respective solution inlets resulting in GUV production. b: Inlets and outlet of the device, including flow directions through the channels marked with black arrows. c: Dimensions of the channels[3].

5.2 Surface coating of the microchannels

After the PDMS-device had been fabricated, a channel treatment was performed before double emulsion production was possible. For the successful production of W/O/W double emulsions based on hydrodynamic flow focusing, the inner walls of the external channel (where the continuous outer aqueous phase is flowing) must be hydrophilic, this is, from OA inlet to outlet (see *Fig 9*). Therefore, PDMS is ideally suited for the generation of W/O droplets, but it prevents the production of O/W droplets with untreated material. Without such a treatment, the wetting of the continuous OA phase on the channel walls would be weak due to the intrinsic hydrophobic character of the PDMS. Consequently, the shear stresses at the second junction would not be large enough to initiate the double emulsion production[1,3,29].

For the channel hydrophilization, a series of chemical reagents were drawn into the microfluidic chip using a syringe of 10 mL and a pipette tip. Since the coating had to be selective, the syringe with a piece of tube was inserted in the OA so that when vacuum force was applied, solutions went from the outlet (where the pipette tip with the solution was placed here) to the OA inlet *(Fig. 9b)*. In every step, chips were observed under the microscope during the coating to check de selectiveness *(Fig. 9a)*.

The next protocol was created combining the coating protocol present in N. Yandrapalli et al. 2021[3], Julien Petit et al. 2016[1] and some additions and modifications I found out were useful for a better chip performance.

First, the channel walls were oxidized by 1:2 mixture of hydrogen peroxide solution (H_2O_2) and hydrochloric acid (HCl) for 2 min. This renders the chip surface negatively charged as such the positively charged polymer poly (diallydimethylammonium chloride) (PDADMAC) will form layers after flushing it through the channels for 4 min. Eventually, a solution of the negative polyelectrolyte poly (sodium 4-styrenesulfonate) (PSS) will do the same after being flushed for the same duration to finalize the channel treatment. After every step, channels were rinsed with ultra-pure water (MiliQ water) for 30 s to remove excess chemical reagents (except 1 min for the last step). After that, no solution flow, only vacuum force was applied (2 min) to remove any liquid inside the channels.

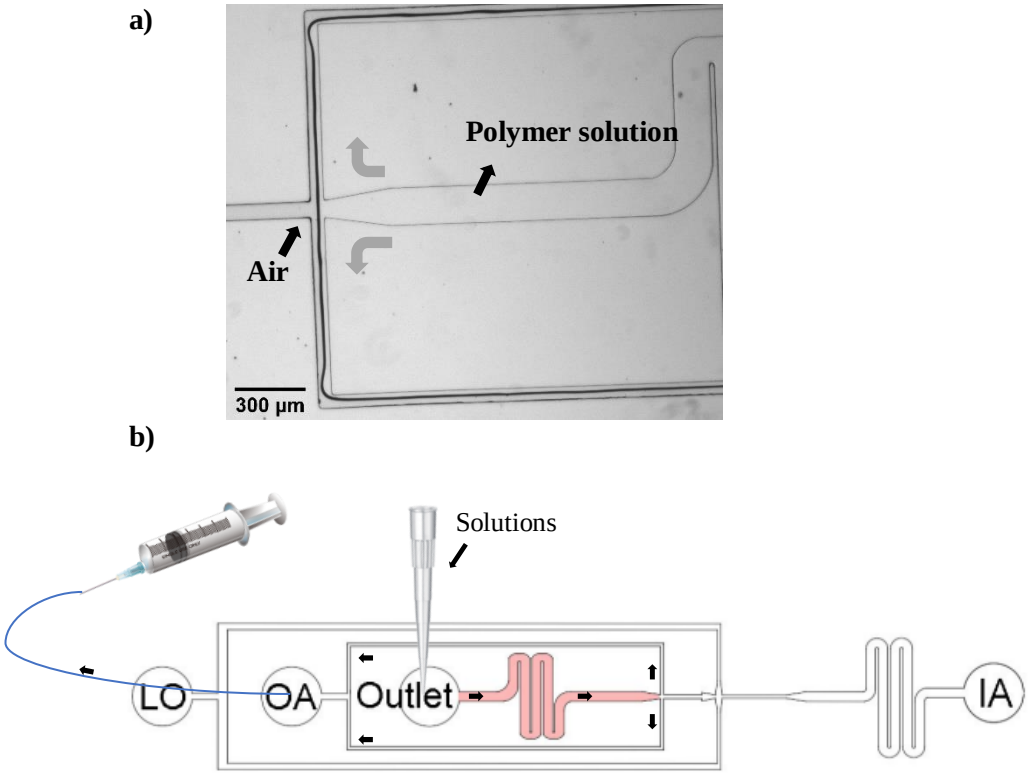

Fig. 9: Selective coating procedure. a: Polymer solution (PDADMAC) being flushed from the outlet to the OA inlet (flow direction marked by grey arrows). The solution does not go through the second cross-junction. b: Illustration of how the coating process is performed. Area of the chip finally coated marked by pink shade[30]. Black arrows indicate the flow direction.

5.2.3 Coating solutions

For the coating process, a 1:2 mixture of HCl (200 µL, 37%) and H_2O_2 (400 µL, 30%) was prepared in a glass vile immediately before flushing it through the chip (1 min before). This way the solution is still reactive, and therefore it can oxidize and negatively charge the surface of the microchannels.

Regarding polymeric solutions, PDADMAC was used at 2 wt.%. 1 g of a 10 wt.% in H_2O PDADMAC solution was added to 4 g of miliQ water, resulting in 5 g of PDADMAC solution at 2 wt.%. For the PSS, a solution of 5 wt.% was prepared dissolving 0.25 g of PSS in miliQ water to get a final solution of 5 g.

60 µL of the acid solution, 50 µL of the polymeric solutions and 150 µL of the miliQ water were used to complete the process.

5.3 Chip operation

To make W/O/W double emulsions, the IA solution containing miliQ water was passed through the first cross-junction to be sheared by an oil phase (LO) producing single emulsions. This single emulsion is in turn sheared at the second junction by miliQ water (OA) creating double-emulsions.

5.3.1 Preparation of solutions

The oil phase (LO) was prepared using 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) with 1-octanol in a concentration of 5 mg/mL total lipid concentration of POPC. 18:1-Liss Rhod PE (fluorescent labelled lipid useful in labelling GUVs[26]) was also added to the solution (0.5 mol%) to enable fluorescent imaging.

Apart from miliQ water in IA and OA, other solutions were prepared to carry out other experiments.

Equimolar solutions of 300 mM sucrose and glucose for IA and OA inlets respectively. 100 mL of each solution were prepared dissolving 10.27 g of sucrose and 5.4 g of α D-glucose in miliQ water. Other pair of solutions were two different buffers: Tris 30 mM; NaCl 300 mM; pH 7,5; Fluorescein 5 μM as IA and the same buffer without fluorescein as OA solution. Finally, other combination was prepared using a *Xenopus* extract from Lendert Gelens research group as IA phase and 275 mM glucose as OA (4.97 g α D-glucose in 100 mL miliQ water).

5.3.2 Operation and flow rates

Chips were operated immediately after coating.

Firstly, three 1 mL syringes were filled with the solutions (two with miliQ water and one with POPC), coupled to neMESYS syringe pumps, and connected to the corresponding inlets via 1 mm diameter plastic tubes (another piece of tube was placed to the outlet of the device). Then, the surface-functionalized chip was wetted by first pumping OA through the channels at a flow rate of 20 μL/min, followed by LO at 2 μL/min and creating O/W droplets *(Fig. 3)*. Finally IA was pumped at 4 μL/min. This strategy prevented the oil from eroding the surface coating (hydrophilicity) of the OA-outlet channel (a layer-by-layer self-assembled polyelectrolyte). Once stabilized, the OA and IA flow rates were increased gradually until 30-40 μL/min and 7-10 μL/min respectively, depending on the double-emulsion formation. LO could also be modified, but always in the range of 2-4 μL/min.

5.3.3 GUV collection

Once the microfluidic device is running and producing GUVs, they were collected in an eppendorf tube. To visualize them, a pipette with a wide orifice tip was used to transfer them to the imaging chamber. Wide orifice tips reduce the shear forces applied on the GUVs, finally resulting in more GUVs surviving the transfer process.

An alternative route to collect the vesicles was to insert the outlet of the plastic tube directly into de chamber or coverslip where they were visualized, or pipetting directly from the outlet of the device without using plastic tube, all with the aim of reducing the damage caused to the GUVs.

5.4 Electroformation

The device used for this purpose was the Vesicle Prep Pro from 'Nanion Technologies', an automated device for preparation of GUVs *(Fig. 10)*. Solvent-free GUVs ranging from 1-30 μm in diameter are formed by electro-swelling (hydration of dry lipid film in an oscillating electric

field). Lipid dissolved in highly volatile solvent (chloroform) is spread on the conductive glass substrate (Indium thin Oxide, ITO, coated glass) of the vesicle chamber, so that after evaporation of the solvent a thin layer of solvent-free lipid is formed on the substrate. With the application of an alternating electrical field the lipid layer releases from the electrode, vesicles are formed and released into the solution.

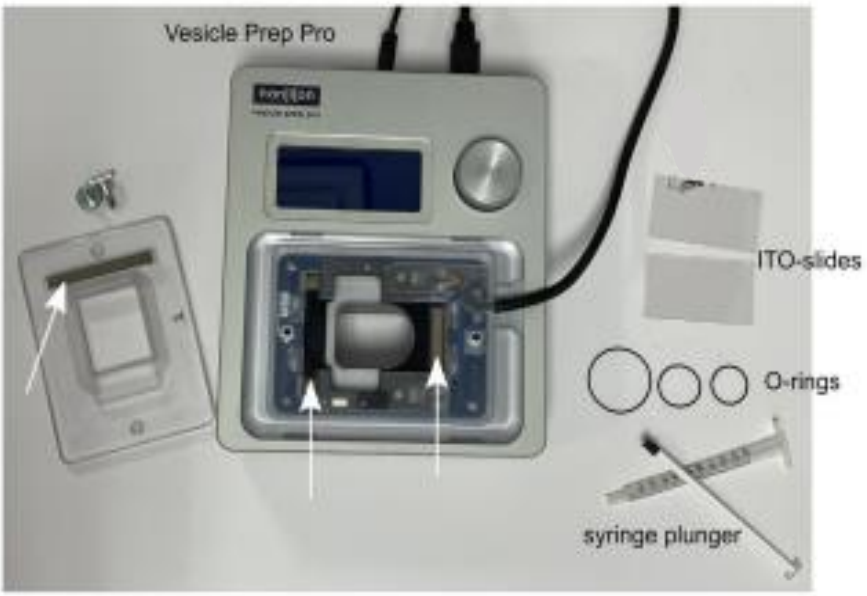

Fig. 10: Vesicle Prep Pro device and its components. White arrows mark the electrodes, which are touching conductive sides of the ITO-slide[14].

5.4.1 Preparation of solutions

5.3.1.1 Lipid solution

A 2 mL lipid solution (1,2-dioleoyl-sn-glycero-3-phosphocoline, DOPC) at 1.5 mg/mL was prepared by taking 0.12 mL of DOPC (25 mg/mL), 1.2 µL of 18:1-Liss-rhod-PE and 1.88 mL of chloroform (highly volatile solvent). Lipid bottles were flushed with N_2 before and after use to prevent oxygen from damaging lipids.

The glass syringes were washed 3 times with IPA, 3 times with H_2O, 3 times again with IPA and lastly, 3 times with chloroform.

5.4.1.2 Inner and outer aqueous phases

Non-ionic solutions must be prepared: 300 mM of sucrose as inner GUV solution and 275 mM of glucose as outer solution. This way, due to the difference of osmolarity, water enters inside the GUVs and it makes them more rigid. In addition, the density of the sucrose solution is higher than the glucose solution, so the vesicles move to the bottom of the sample, making easier to image them.

5.4.2 GUV preparation

First, the conductive sides of the ITO-slides were identified with a multimeter (lipids are deposited on this side of one slide.

Over an area of about 1 to 2 cm of diameter, 20 µL of the lipid solution was deposited slowly on the slide using a glass syringe, being the layer as thin and homogeneous as possible. Then, the glass slide was dried with N_2 and left in the desiccator for at least 1 hour.

Next, the device was assembled. First, the ITO slide was placed in the bottom part of the Vesicle Prep Chamber with the lipid film facing up. Then, a rubber O-ring was carefully coated on one side with silicon grease and put around the dry lipid film. The O-ring creates a chamber around the hydrated lipid solution. Next, 250 µL (for the 16 mm O-ring) of 300 mM sucrose solution was pipetted on top of the lipid film. The second ITO-slide was then placed with the conductive side down on the top of the O-ring resulting in a sandwich construction, making sure it touched the metal strip of the chamber. Finally, the assembly of the device is completed once the screws were placed in the chamber and tightened, so the O-ring got slightly squeezed and formed a tight seal with the slides.

After having the Vesicle Prep Pro Chamber assembled, it was turned on and connected to the Vesicle Prep Pro Station and to the computer so that a protocol can be applied. The applied protocol for DOPC was consulted at GUV formation lipids guideline[(27)], offered by 'Nanion Technologies'. It consisted of 160 min at 36ºC, with a frequency of 5 Hz and an amplitude of 3V.

The vesicles were transferred from the device with a wide orifice pipette without too much delay (since the vesicles are not stable on the ITO slides) and were diluted with the glucose solution (275 mM) or also with the sucrose solution (300 mM).

After removing GUVs solution, ITO slides were washed with distilled water and ethanol until all remain lipids had vanished, then dried with cleaned tissues. O-ring was washed the same way.

5.4.3 Storage of the GUVs

Although after the electroformation the GUVs were visualized directly under the microscope, they can be stored after their production, at 4ºC between 1 to 4 weeks, using sterile solutions and materials for a better conservation[(25)].

5.5 Imaging

All results obtained were observed with an inverted microscope, which are very useful to see biological objects at the bottom of a petri dish or tissue culture flask, but in this case, to see vesicle formation under a layer of PDMS or GUVs inside a chamber/slide. This type of microscopes provide bright-field, phase contrast or fluorescence images.

5.5.1 Bright-field microscopy

Bright-field microscopy is one of the simplest optical microscopy, where illumination light is transmitted through the sample and the contrast is generated by the absorption of light in dense areas of the specimen. Inverted microscopes have both the light source and condenser set up high above the stage and pointing down toward the stage. Moreover, the objectives are located beneath the stage pointing up[(32,33)].

The coating procedure was always done under the microscope, checking that the solutions went through the correct channels without reaching the second cross-junction. What´s more, the exposure time had to be short (0.027 ms) in order to quickly see what was happening and in case it was necessary to apply more vacuum force to the syringe, specially at the moment when the syringe reached its limit and had to return to its initial position. This was a critical step where no pressure is applied to the chip and solutions could go into the wrong channels, so it was important to do this step as quickly as possible.

The GUV production was also observed with bright-field, adjusting the flow rates towards the right formation of single and double emulsions. Finally, the GUVs were collected either in the eppendorf or directly into the chambers/slides.

5.5.2 Inverted fluorescence microscopy

Fluorescence microscopy uses fluorescence to generate an image. The specimen is illuminated with light of a specific wavelength, fluorophores present in the sample absorb the energy of the photons raising to an electronic excited state. When the excited molecule returns to ground state, emits a photon of lower energy, which corresponds to a longer wavelength than the absorbed photon. The emitted light is then separated from the excitation light through wavelength specific filter, transmitting fluoresced light to the detector and creating an image[(34)].

Rhodamine and fluorescein were the two fluorescent labels used, for the LO (and electroformation lipids) and for the IA phases, respectively. The appropriate filters must be used for each experiment during the fluorescent imaging.

For imaging the GUVs, both in bright-field and fluorescence, 'Ibidi' chambered µ-Slide 18 Well Glass Bottom, BSA-coated glass slides and disposable Neubauer counting chamber were used. In cases where the outlet of the chip was not connected directly in the chamber/slides, 40 µL were pipetted from the Eppendorf into the 18 well chamber slide.

5.5.3 BSA-coating

The cover slides utilized for imaging the GUVs need to be coated with Bovine Serum Albumin (BSA) to prevent vesicles from bursting in contact with glass[(28)].

Glass microscopy slides were incubated with a 10 mg/mL BSA in Phosphate buffered saline (PBS) solution overnight. Afterwards, they were washed with PBS and water and left overnight drying at room temperature.

5.6 Different parameters/combinations tested and issues

The main objective of this project was to obtain GUVs via a double emulsion microfluidic device. In the course of the experimentation, many issues appeared, and it was needed to optimize the different steps involved. All the procedures that have given the best results have been described in the previous sections.

5.6.1 Selective coating

The biggest challenge through the research was to optimally obtain the selective coating of the channels *(Fig. 9b)*. Firstly, the coating procedure was performed with syringes of 1 mL using syringe pumps (quickly replaced by hand), applying vacuum from the outlet and placing a pipette tip with the solutions in OA inlet, following the times of the protocol[(3)]. In addition, the rest of the inlets (IA and LO) were blocked using scotch tape or a piece of plastic tube closed at the tip. The purpose of blocking the channels was to prevent the solutions from going to the wrong channels but looking under the microscope, it turned out to be better not to block any of them. The section between the first and the second cross-junction of the chip must be completely hydrophobic so that the lipid phase contacts the walls, surrounding the water and forming W/O droplets. When any of the solution used for the coating process reached the second cross-junction (towards the first cross-junction), this hydrophobicity was modified, and the formation of W/O single emulsions did not take place *(Fig. 11A)*, and thus there was no GUV production. This problem was solved by permuting the positions of the inlet of the solutions (OA) and the syringe (outlet),

improving the control over the flow through the channels. All steps were then performed under the microscope, and a 10 mL syringe replaced the 1 mL one, allowing more vacuum force to be applied.

Another important issue was the concentrations of the different coating solutions and the time they were passing through the chip to finally obtain a functional coating, since the concentrations and times of the protocol we were using was not giving the expected results. An overview of the different combinations tested is attached in *Table 1*.

We discovered that the acid solution must be prepared directly before coating (1 min before flushing it through the channels), in order to enable the oxidization of the surface channels. If the acid solution is not prepared recently, polymer solutions will not form layers, and the lipid phase will adhere to the walls *(Fig. 11B)*. The concentration of hydrochloric acid was also increased from 30% to 37%, but always keeping 1:2 ratio of HCl and H_2O_2. 1:1 relation was also tried, but the channels became dark due to acid erosion *(Fig. 11C)*.

In addition, several problems appeared when the concentrations of the polymer solutions were changed, mainly the emergence of the solid polymers that cover and block the channels *(Fig. 11D)*, so we started to focus more on the time the solutions were inside the chip and not on the concentrations.

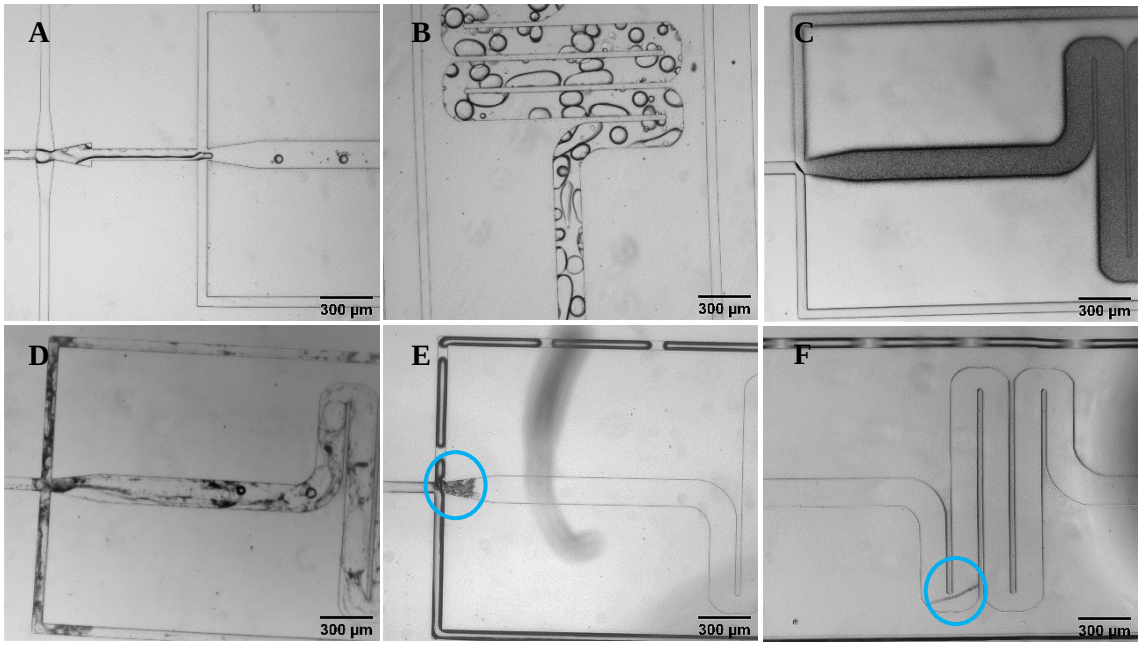

Fig. 11: Different issues during device handling. E and F: Dust particles that came from the outlet during the coating process, requiring discarding the device.

One of the most remarkable improvement was the wait between the coating treatment and the chip operation. Since the coating procedure was very tricky and laborious, it was difficult and time consuming to obtain a faultless process. That is the reason why the pretreatment was done one day and after 24 hours the microfluidic devices were put into operation. However, once coated, most of the time the devices did not work properly, and the coating was not optimally obtained *(Fig. 11B)*. The results achieved changed drastically when the chip operation was performed immediately after the pretreatment of the channels, completely solving this issue even waiting 1 hour in between.

5.6.2 Chip operation

Over the period of the thesis, the operation of the microfluidic chip was improved.

One aspect is the optimization of the flow rates of OA, IA and LO phases during the GUV formation. The syringe pumps used (neMESYS) works with flow rates (μL/min), and it was necessary to find those that were optimal. Initially, all flow rates were less than 5 μL/min, always keeping OA values above the others, followed by IA, and OA was the smallest one (for instance 4, 2 and 1 μL/min respectively). After having tested numerous flow rates combinations, the best results in terms of GUV formation were obtained when the OA flow rate was around 10 times higher than LO and 5 times higher than IA, as mentioned in *5.3.2*.

The last problem was in the GUV collection. In many of the samples no vesicles were observed under the microscope although they were produced inside the chip. This was the reason why sometimes the plastic tube in the outlet was removed and GUVs were pipetted directly from there, in order to reduce vesicle contact with the plastic and thus, more surviving GUVs.

In addition to all the issues mentioned above, dust or dirt sometimes entered the microfluidic device and blocked the channels *(Fig. 11D and 11F)*, making it necessary to throw away the chip and replace it with a new one. Furthermore, many of the chips produced could not be well bonded to the glass slide and detached in the middle of the procedure. These problems occured both in the coating process and in the operation of the chip, being more common in the first one.

6 Results

6.1 Electroformation

GUVs were visualized in a chambered µ-Slide 18 Well Glass Bottom ('Ibidi') except the 1:2 dilution of the GUVs in 275 mM glucose solution, which was placed into BSA-coated glass slides. This dilution was made to not see the accumulation of vesicles at the bottom, due to the difference in densities of the internal and external solutions. This is not the case for the sucrose as outer solution, where GUVs float in it *(Fig. 12)*.

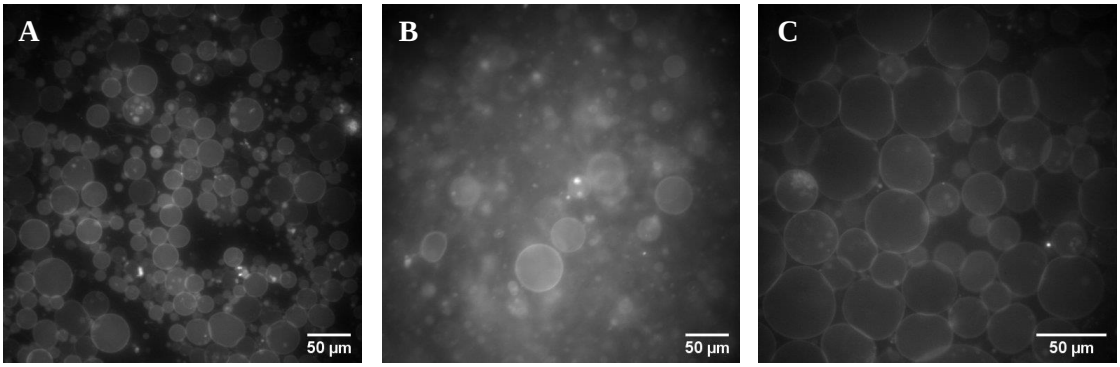

Fig. 12: Fluorescent electroformed GUVs with lipid composition DOPC (1.5 mg/mL) Liss-rhod-PE in chloroform. 275 mM glucose (A) and 300 mM sucrose (B) as outer solutions. C: Dilution 1:2 of the glucose sample.

Although it was the first time the device was tested, the results obtained demonstrated the high yields of unilamellar DOPC GUVs with a somewhat variable size distribution, from 15 to 60 µm.

6.2 GUV production (chip)

The main objective was to produce GUVs using POPC as LO and miliQ water as IA and OA phases. Once this was achieved, we experimented with the encapsulation of different aqueous phases.

If the coating process had been successful, the double emulsions can be continuously produced without collapse or blockages for at least as long as the outer solution in the syringe lasts, being this one that lasted the least due to the high flow rate compared to the others. This time could be between 15 or 20 minutes.

6.2.1 MiliQ water

As explained in detail in'Methods' chapter, the first step in the chip operation is to create O/W droplets by actuating only OA and IA *(Fig. 3)*, and once stabilized, IA phase is initiated to start the formation of double emulsions *(Fig. 13A).* These double emulsions have a very thick lipid layer, so it is necessary to reduce it by gradually increasing both the OA and IA phases flow rates. Then, the desired GUVs begin to appear *(Fig. 13B).* If the OA and IA are further increased to the optimum flow rates, a constant production of GUVs is achieved *(Fig. 13D)* and, on the other hand, single emulsions that were formed between the first and second cross-junction disappear.

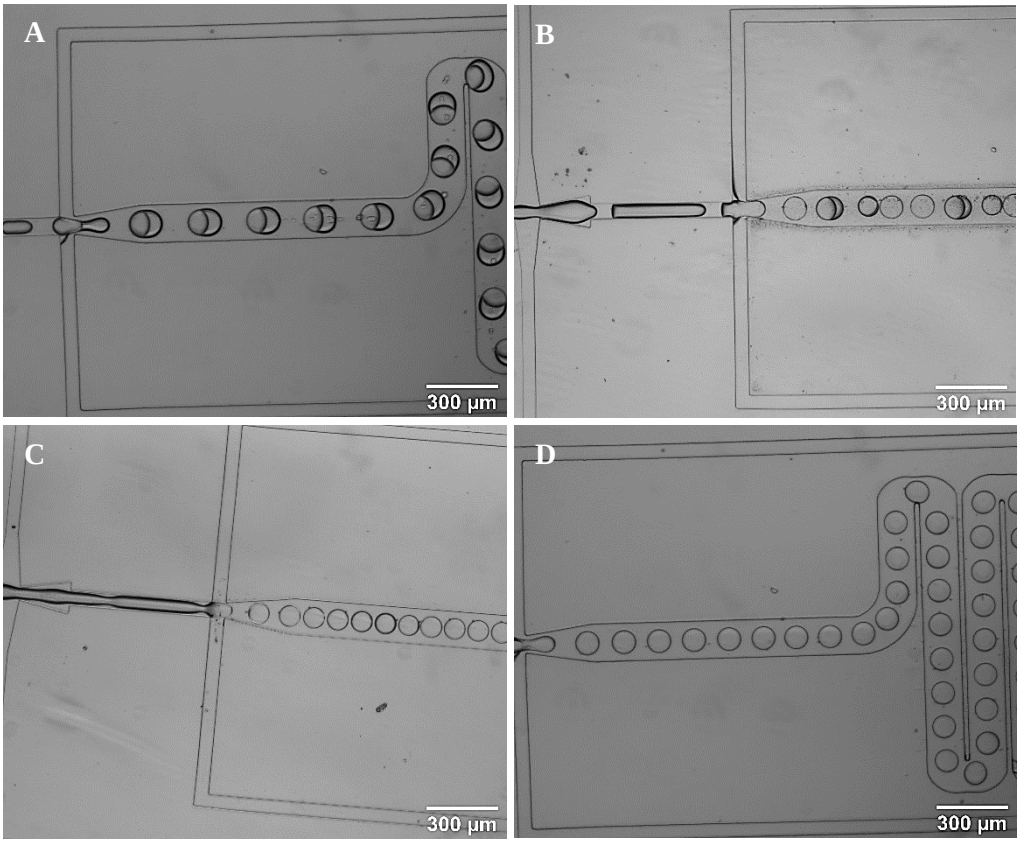

Fig. 13. Bright-field images of the steps followed to reach GUV production.

After all correctly produced GUVs were directed towards the outlet of the chip, they were collected and visualized under the microscope *(Fig. 14)*.

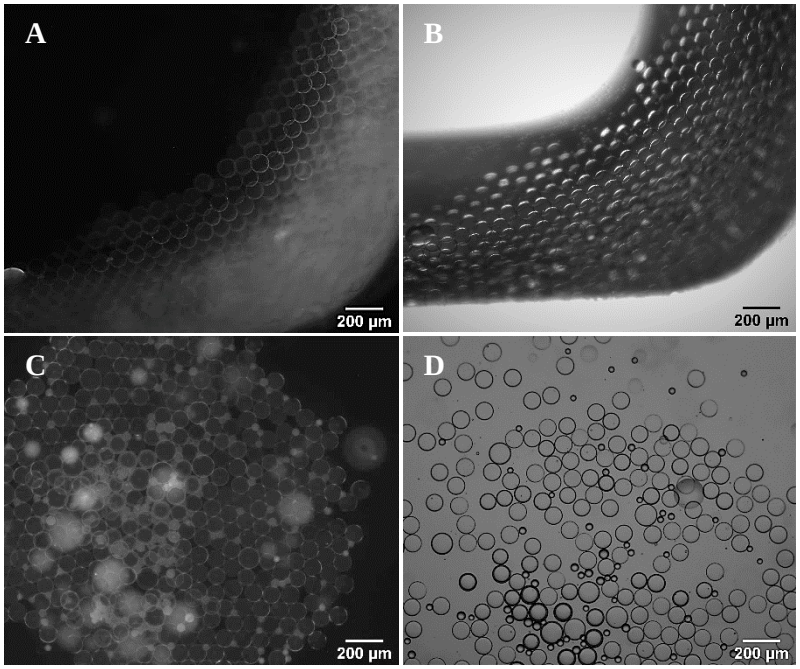

Fig. 14: A-B and C-D show the same region of the sample. A and C was taken under fluorescence microscope and B and D was taken in bright-field. Lipid composition: POPC 5mg/mL and Liss Rhod PE in 1-octanol.

In *Fig. 14A* and *Fig. 14B*, GUVs were collected by directing the plastic tube from the chip outlet into the 'Ibidi' chamber. As can be seen, a large number of GUVs were produced, all of the same size (85 µm). On the other hand, BSA-coated glass slides were also used for imaging *(Fig, 14C* and *Fig. 14D)*. In this case, the vesicles were floating in water along with small oil droplets.

6.2.4 Sucrose and Glucose

In addition to water, two different solutions were used as IA and OA, in this case two equimolar sucrose and glucose solutions of 300 mM, respectively. The objective was to produce vesicles in different conditions and, due to the difference in densities, to direct them to the bottom of the chamber enabling easy visualization.

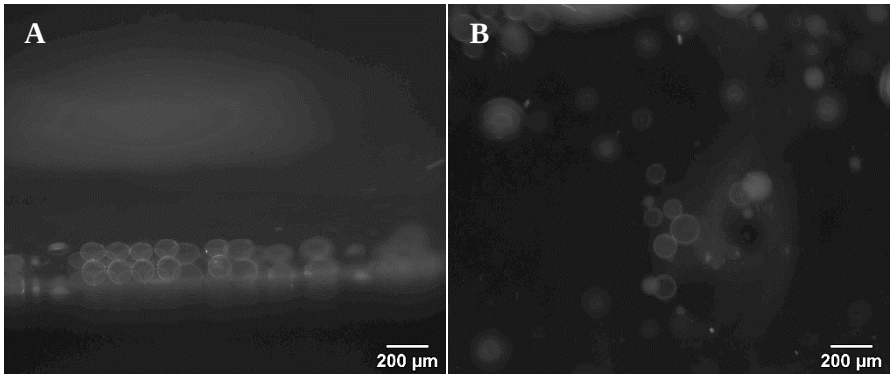

Fig. 15: Pictures of created GUVs under fluorescence microscope. Lipid composition: POPC 5mg/mL and Liss Rhod PE in 1-octanol.

Always following the same steps as with miliQ water to start up the device, the GUVs produced were collected in the 'Ibidi' chamber *(Fig, 15A)* and in BSA-coated glass slide *(Fig, 15B).*

However, after several attempts, the GUVs appeared to be unstable, and they burst very quickly under the microscope despite equimolar solutions. As with water, they went to the edges of the chamber but the small amount of them can be appreciated. On the other hand, it was expected that vesicles would go to the bottom but in *Fig. 15*, they were floating in the glucose solution, making difficult to image them. This was probably because some oil remained on the GUVs, and it makes them float in the solution

6.2.3 Fluorescein buffer

Another of the inner solutions was a buffer containing fluorescein, a fluorophore commonly used in microscopy. The same buffer was added as external solution but without the fluorescent substance. In this case, disposable Neubauer counting chambers were used to visualize the GUVs.

It can be seen that there is no homogeneous size as in the previous cases, the GUVs fuse once they leave the microfluidic device but retain the fluorescent dye *(Fig, 16B).*

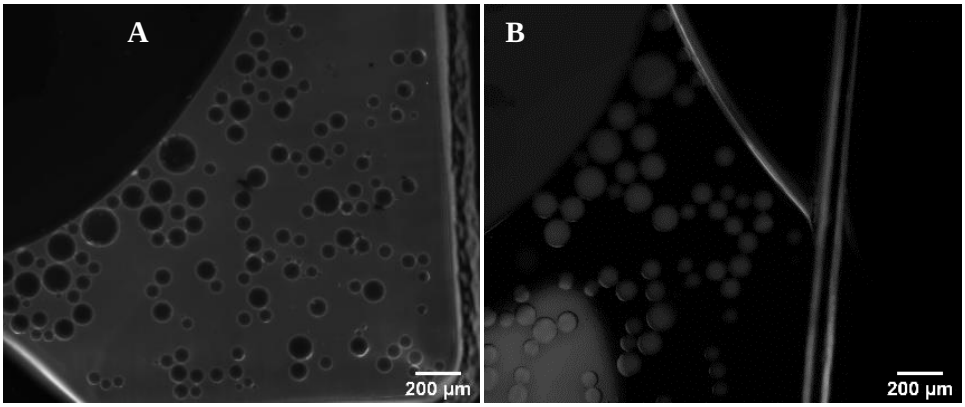

Fig. 16: These three images show the same region of the chamber, but with two different fluorescent filters. Lipid composition: POPC 5mg/mL and Liss Rhod PE in 1-octanol A: GUVs with fluorescent ring of Liss Rhod PE. B: GUVs with fluorescence inside due to fluorescein.

6.2.4 *Xenopus* extract

Since the previous experiments showed that different solutions can be encapsulated, next step is encapsulation of more complex solutions, in this case *Xenopus* extract. After several attempts and repeating exactly the same procedure that had led to successful results in previous experiments, no conclusive result was achieved.

The single emulsions between the first and second cross-junction were not formed, and therefore GUVs were not produced either *(Fig. 17)*. This is probably due to the difference in viscosity between the extract and the 275 mM glucose solution.

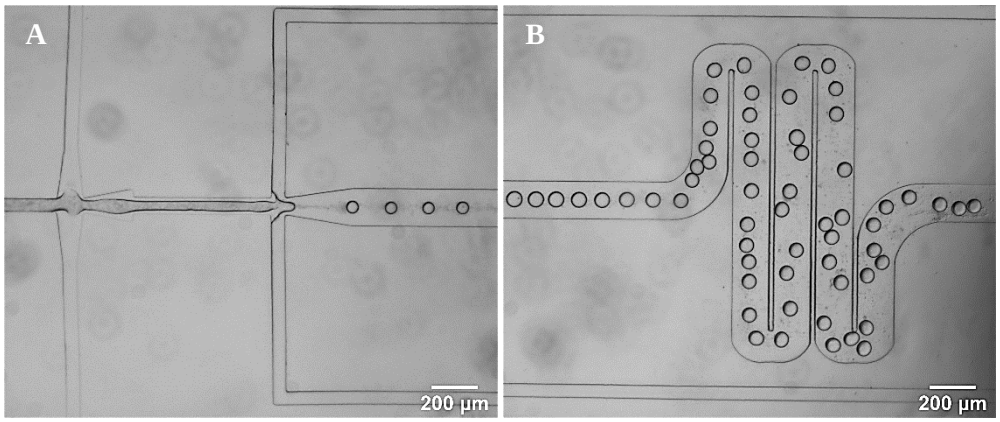

Fig. 17: Images of the cross-cross junctions (A) and the second serpentine module (B) of the microfluidic device. Oil droplets are formed, and the extract enters the outer solution surrounding oil droplets (grey shade is appreciated).

6.3 Other experiments

The previous results were obtained once the coating process had been carried out successfully, but at the same time new combinations were tested, other experiments were performed in parallel, with the aim of achieving the correct selective channel hydrophilization and thus, GUV production.

6.3.1 Polyethylene glycol (PEG) silane coating

One idea we came up with was to completely change the coating method. This consisted of using only one reagent instead of three, called PEG silane, which when it was applied in the same way to the microchannels of the device, a long-term hydrophilization of the surfaces could be achieved[35,36].

This coating process was done as mentioned in 'Methods' section but with the difference that PEG was immediately applied to the PDMS surfaces after the plasma oxidation.

The PEG silane was very difficult to control inside the chip and flowed into the wrong channels, preventing the selective coating *(Fig. 18A)*. Despite that, the PEG silane also crystallized inside the channels and clogged them on some occasions *(Fig. 18B)*.

Even with all these problems, many attempts were made to check whether the channel surfaces (from OA to outlet) had been hydrophilized or not, but it was found that W/O droplets (instead of O/W) appeared after the second cross junction *(Fig. 18C)*, that is, as if the coating had not been done, indicating that the process was not successful.

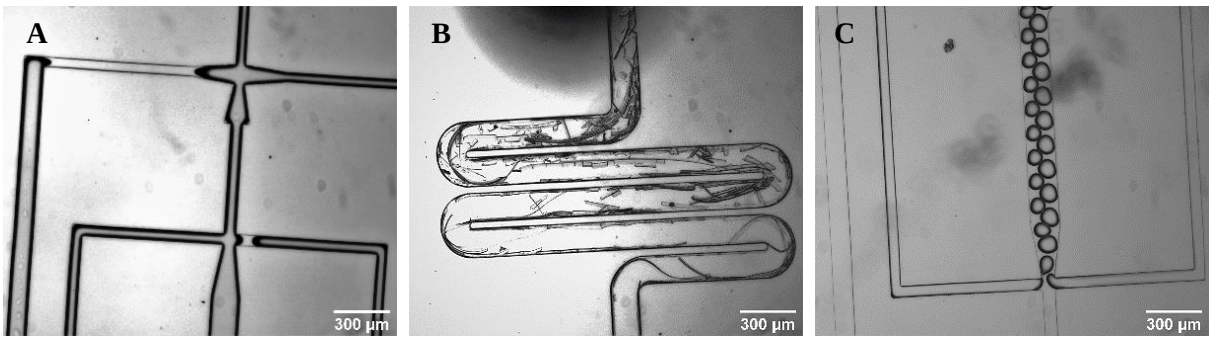

Fig. 18: Images of different chips coated with PEG silane. A. First and second cross-junction of the chip covered by PEG silane. B. First serpentine module totally blocked. C. OA and LO running, forming W/O droplets.

6.3.2 Double layer PDMS chip

Another microfluidic device[37] design was tried due to problems getting the first one to operate. This new design required two layers of PDMS, fabricated in two different wafers in the same way as mentioned in 'Methods' section, but with an additional step of binding the two layers and aligning the channels looking under the microscope.

This idea did not work either, mainly due to the entry of dust particles that clogged the small channels *(Fig. 19A)*, even when using filter syringes. In addition, the coating process was not optimally achieved as the solutions used remained within the channels.

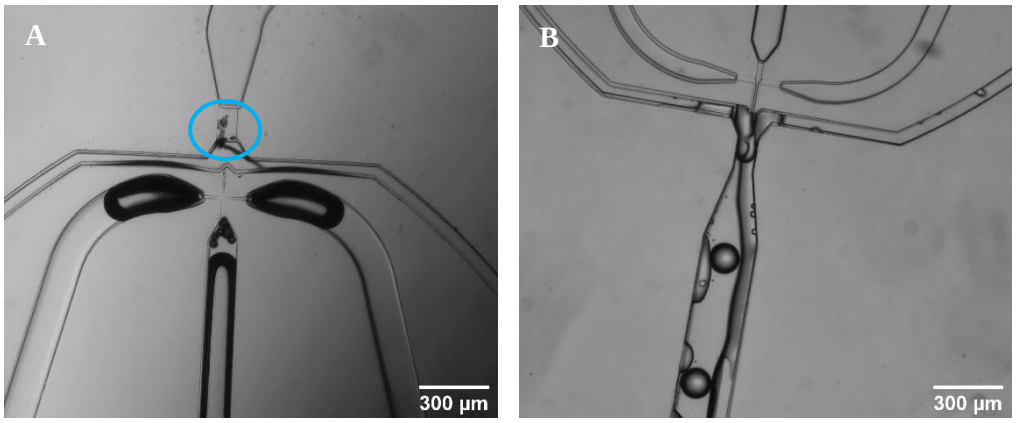

Fig. 19: A: Dust particle (blue circle) in the second cross junction that came from the OA, requiring discarding the chip. B: Running IA, OA and LO phases. Lipid phase stuck on the channel surface preventing the formation of double emulsions.

6.3.3 Chips of 120 µm height

As for the countless attempts to successfully create double emulsions, many of them failed because the thin lipid layer broke on contact with the channel surfaces, and therefore, double emulsions did not reach the outlet of the microfluidic device *(Fig. 20A)*. One of the solutions thought was to increase the height of the channels from 80 to 120 µm, so that the GUVs produced would have more space and would not touch the channel walls.

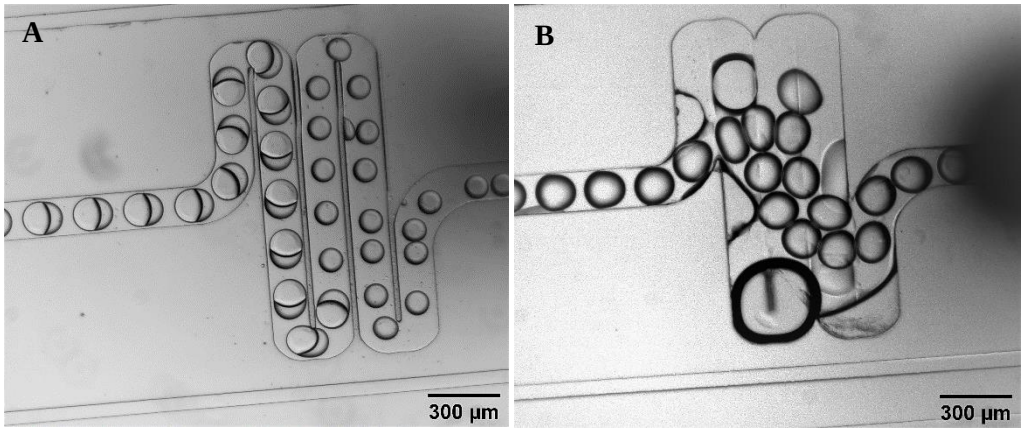

Fig. 120: Images of the second serpentine module of 80 and 120 µm height chips. A: Lipid layer of double emulsions breaks, leading then to O/W droplets. B: O/W droplets passing through the serpentine.

However, all the new 120 µm manufactured chips had the second serpentine module fused like a single channel, and as expected, they did not work *(Fig. 20B)*. In spite of that, it was later discovered that increasing the height of the channels was not necessary, since the optimal flow rates were not being used in the normal height devices (they had not yet been discovered).

7 Conclusions

After a long time searching for solutions to the problems arising from the selective coating of the microfluidic device, which is where most time has been invested in this research work, GUVs were finally produced with a fair degree of reproducibility.

Yet, we were not able to automate the process, so all the steps mentioned have to be done manually, being very laborious and time-consuming. It has been demonstrated that this procedure is essential to achieve a correct operation of the chip, making it necessary to be aware of even the smallest detail.

This work has also been successful in finding the optimal flow rates of the different inlets in order to produce double emulsions. However, the GUV collection process seems not to have been fully optimized, since in many of the samples no vesicles were observed in the chamber even though they were formed inside the chip. Any contact with plastic or PDMS seems to destroy the GUVs.

On the other hand, during the GUV formation via the microfluidic device, a good robustness has been achieved when both the inner and outer solution was the same (water), but when different compositions are used, problems appear in the stability of the vesicles and thus, in their observation. Especially when attempting to encapsulate biological material, where double emulsions did not even get formed in spite of the several combinations of the fluid flow rates that were tested. This is probably due to the different properties (viscosity) and compositions of the two aqueous solutions, and could be optimized by trying to make these two phases as similar as possible.

Another secondary chip design was tested, but it did not lead to any results. The coating process was also necessary, but it could not be carried out properly and in addition, this design featured very narrow channels at the cross-junction, being easily clogged by any small dust particles.

Regarding the electroformation, very satisfactory results were obtained with high yields in an easy and reliable way, even though it was the first time the device was put into operation. Compared to the microfluidic method, electroformation was much easier to carry out because the process is automated, giving more reproducible results than by microfluidics, where it was necessary to manipulate reagents and perform a treatment before the operation. However, the GUVs produced by microfluidics had identical size depending on the fluid flow rates (75-85 µm), unlike electroformation (ranging from 15 to 60 µm).

8 List of tables

8.1 Table 1

Summary of the combinations of concentrations and times tested in the coating procedure, being the last one that have given the best results.

Number	**HCl: H_2O_2 solution**	**PDADMAC**	**PSS**	**MiliQ water**
1	30% HCl and 30% H_2O_2(1:2), 30s	2%, 2min	5%, 2min	30s
2	30% HCl and 30% H_2O_2(1:2), 30s	2.5%, 2min	5%, 2min	30s
3	30% HCl and 30% H_2O_2(1:2), 30s	3%, 2min	10%, 2min	30s
4	30% HCl and 30% H_2O_2(1:2), 30s	4%, 2min	5%, 2min	30s
5	30% HCl and 30% H_2O_2(1:2), 30s	4%, 2min	7.5%, 2min	30s
6	30% HCl and 30% H_2O_2(1:2), 30s	4%, 2min	10%, 2min	30s
7	30% HCl and 30% H_2O_2(1:2), 30s	5%, 2min	5%, 2min	30s
8	30% HCl and 30% H_2O_2(1:2), 30s	2%, 2min	6%, 2min	30s
9	30% HCl and 30% H_2O_2(1:2), 30s	2%, 2min	7.5%, 2min	30s
10	30% HCl and 30% H_2O_2(1:2), 30s	2%, 2min	10%, 2min	30s
11	37% HCl and 30% H_2O_2(1:1), 2min	5%, 2min	2%, 2min	30s
12	37% HCl and 30% H_2O_2(1:1), 30s	5%, 2min	2%, 2min	30s
13	37% HCl and 30% H_2O_2(1:1), 30s	2%, 2min	5%, 2min	30s
14	37% HCl and 30% H_2O_2(1:2), 30s	2%, 2min	5%, 2min	30s
15	37% HCl and 30% H_2O_2(1:2), 30s	2%, 2min	10%, 2min	30s
16	37% HCl and 30% H_2O_2(1:2), 30s	5%, 2min	5%, 2min	30s
17	37% HCl and 30% H_2O_2(1:2), 1min	2%, 2min	5%, 2min	30s
18	37% HCl and 30% H_2O_2(1:2), 2min	2%, 2min	5%, 2min	30s
19	37% HCl and 30% H_2O_2(1:2), 2min	2%, 4min	5%, 4min	30s
20	37% HCl and 30% H_2O_2(1:2), 2min	2%, 4min	5%, 4min	30s and 1 min in last step

9 References

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