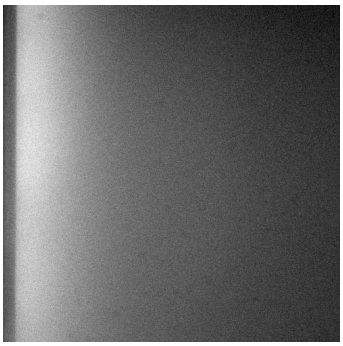
Lipid bilayer formation in a novel SU8/glass flow cell and electrical manipulation of charged fluorescent lipids incorporated in a lipid bilayer.

By

Marcus Jansson



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Thank you!

Introduction

This project is divided in two parts. The first part is to test if it is possible to make a supported lipid bilayer (SPB) in a new type of flow cell. This flow cell is made of negative epoxy resist (SU8) and glass. The final cell channel will have a bottom and top of glass and side walls of SU8. This is possible to achieve by exposing the SU8 through the glass and then develop non cross linked SU8 through holes in the glass. The glass surface will support the formation of a fluid lipid bilayer only if it possible to clean the surface enough after developing away SU8.

The second part is to make a more exotic kind of experiments on these SPBs, experiments involving charged lipids incorporated in the bilayer. The charged lipids are to be moved by applying an electrical field parallel to the bilayer. With patterned diffusive barriers on the glass support the movement of charged fluorescent lipids will be constrained. These barriers are made by scratching the glass surface with a diamond pen. By moving charged lipids towards the wall a system where the force from the electrical field competes with diffusion is created. The resulting steady state can be analyzed by means of fluorescence microscopy. Though a SPB is shown possible to make in the new flow cell, the experiments with applying electrical fields is done in a different flow cell due to practical reasons.

The flow cell

A flow cell is needed when e.g. a biophysical or biochemical reaction taking place in a liquid needs to be controlled. The flow cell in the context of this project concerns the realms of microfluidics and has to be compatible with light experiments with a microscope. When it comes to performing light collecting experiments, the flow cell has to be made of transparent parts, often glass.

Microfluidics is about shrinking down fluidic systems so that a variety of new effects and performances can be utilized, e.g. small sample handling and laminar flows. Here follow some typical techniques in making a microfluidic device.

Flow cell -Mechanical type

A rather robust flow cell can be made by screwing metal or plastic parts together and letting rubber o-rings make it sealed. The technique offers a cell that's reusable because the ease of cleaning the parts and the parts robustness. The glass parts are also interchangeable from the main fluidic device. This gives the cell a long lifetime because a pure glass surface is often used as the active substrate for the chemicals in question. On the other hand with a mechanical cell it is difficult to make channels that are truly in the microfluidic realm and also definable in a satisfactory way. In the biophysics group one type of mechanical flow cell has been used frequently.

Flow cell -PDMS type

PDMS (PolyDiMethylSiloxane) is a soft transparent polymer (fig. 1) which is fluid and hardens when baked in an oven (hardener added).

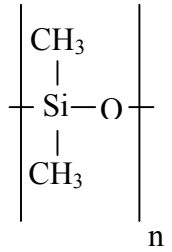

Figure 1: The polymer structure of PDMS.

The typical way of making a flow cell with PDMS is shown in figures 2 and 3, ref 1. A stamp is made by lithography based methods (figure 2). The PDMS is poured on to the stamp and then put in vacuum to get rid of dissolved gases, and after that let to harden in an oven. The PDMS is then pealed of (the stamp is anti stick treated) and after oxygen plasma treatment bonded to glass (figure 3). The chemical structure of PDMS makes it

suitable for bonding to glass or silicon. The oxygen plasma treatment makes the surface covered by SiOH groups which makes a strong bond between PDMS and glass (figure 4).

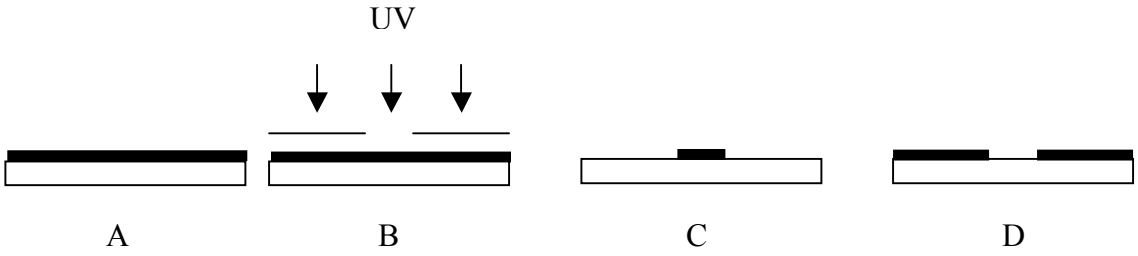

Figure 2A: A photoresist is spun on to a wafer (silicon). B: The photo resist is exposed through a mask with UV. C-D: The photo resist is developed and leaves a part depending on if its negative or positive resist.

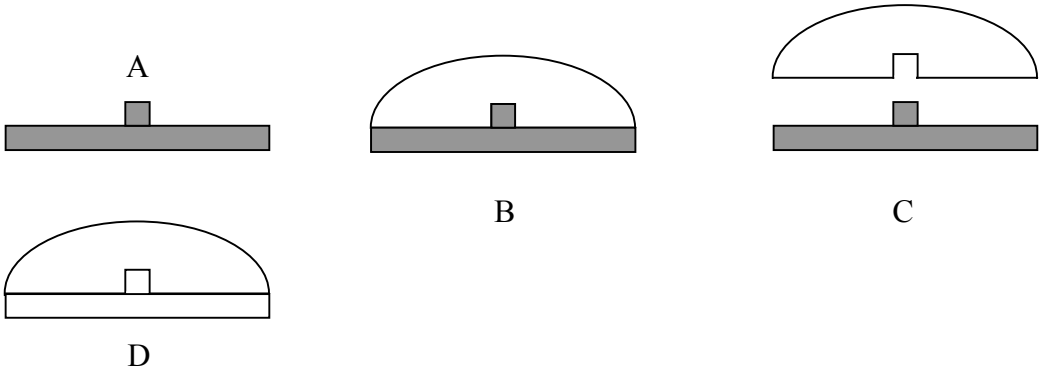

Figure 3A: The mask. B: PDMS is poured on to the mask. C: The mask is peeled of. D: After plasma treatment the PDMS is bonded to a glass substrate.

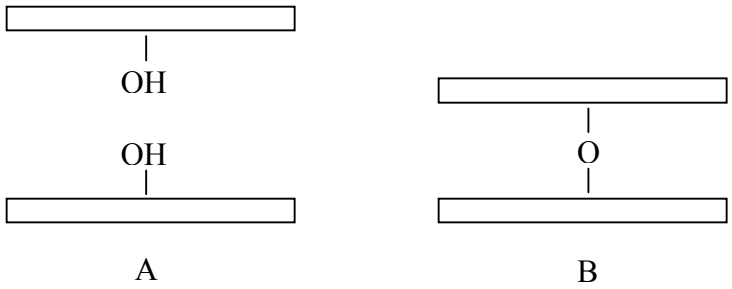

Figure 4A: Two silicon wafers with silanol groups. B: The result when wafers are pressed together.

D A B C

One limitation in making flow channels in PDMS is that the channel can withstand a certain height to width ratio only. This ratio is about 10:1. A higher ratio and the channel collapses. PDMS is hydrophobic but is made hydrophilic during the plasma treatment. This effect is only temporary though and the surface will go back to being hydrophobic which makes it hard to use as a flow cell if its not filled with liquid rather fast. This makes the PDMS cell a little harder to reuse if the channels are small. And because of the elastic nature of PDMS, clogging is a problem when really small channels are concerned. This technique does not offer a glass surface at both the channels top and bottom, but on the other hand PDMS is transparent. The bond between PDMS and glass/silicon is also a covalent bond making the channel robust.

Flow cell-Glass and Silicon type

Glass and Silicon can be bonded to each other directly, refs 2, 3, 4, 5. Two common techniques are fusion bonding and anodic bonding. The main principle is as follows. First one glass is covered with an etch mask of either metal or photo resist. Then a channel is etched in either a wet or dry etch, which gives different results for the shape. A dry etch gives straight walls, in figure 5 a schematic of a wet etch is shown. For thermal fusion another glass is pressed upon the first and melts with it at high temperatures, achieving a close contact with molecular bonds as a result.

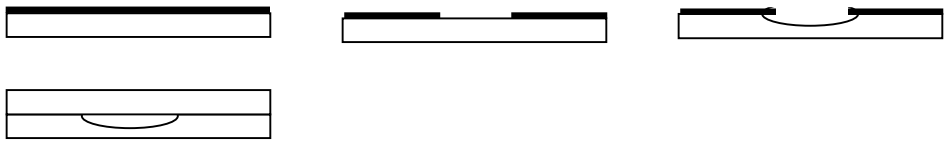

Figure 5A-B: An etch mask is applied and patterned. C: The glass is etched. D: Etch mask is taken away and the wafer is thermally fused with a flat glass.

Anodic bonding is possible if one of the glasses is covered with a thin ion insulating layer, for example amorphous silicon. The glass also has to be rich of sodium oxide. Two electrodes are coupled to the top and bottom wafer and an electric potential is applied (figure 6). The wafers are also heated to mobilize the ions. The temperature needed is below the glass softening temperature. The ions will move towards the electrodes, resulting in a strong force between the wafers which press the wafers close together with a strong bond as the result (figure 7).

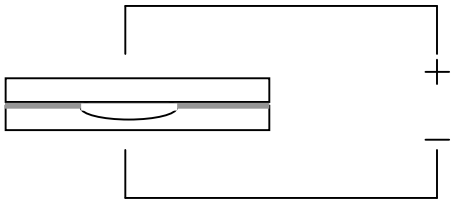

Figure 6: A voltage is applied across the wafers top and bottom. The grey area represents the amorphous silicon.

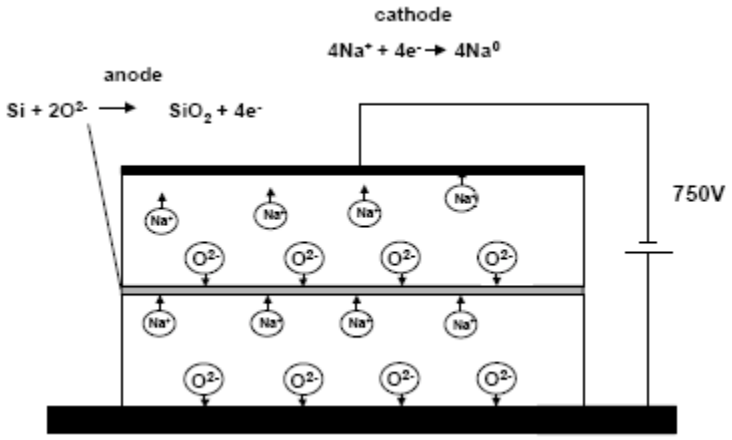

Figure 7: The ions are mobilized and moved by an electric field. Ref 4

Glass and silicon are materials that have well known characteristics and are often used for biological type of experiments. A flow cell of this type can be easily cleaned without taking apart. The use of strong acids is possible because the cell is all made of glass. A treatment with acid is a certain way of resetting the surface. This is not always possible for cells that have parts made by organic compounds.

Flow cell-Adhesive type

Anodic and thermal fusion of glass is taking place at high temperatures. By using adhesives as bonding method the temperature can be significantly lowered and even done in room temperature. SU8 is an epoxy which cross links when exposed to UV light. It is also commonly used as a photo resist because it has good properties for making well defined photo lithography that is meant to stick at surfaces.

There exist a lot of UV curable adhesives on the market with different characteristics. Below follows some ways of using these adhesive techniques in making a flow cell.

A general mass production technique often used is to use a roll to be able to continuously imprint patterns on a surface. Here the roll is used as a way of applying adhesive to an already patterned surface (figure 8), refs 6, 7. The surface could have been patterned with e.g. SU8. A top wafer is then pressed upon the bottom wafer and UV light

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is shun to crosslink the adhesive. The pressing could be done in vacuum to get rid of trapped air.

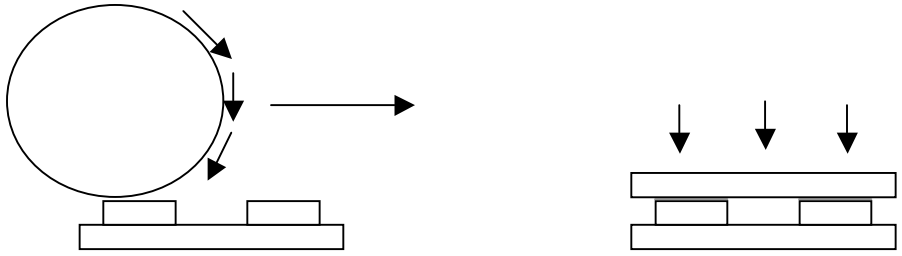

Figure 8A: A thin film with adhesive is rolled over the patterned wafer. B: Another wafer is pressed upon the first.

Another technique relies on low viscosity adhesives which will transport it self by capillary forces between the wafers (figure 9), ref 8. Adhesive that has been pressed out from the edges in to the channel can be rinsed away before cross linking to avoid contamination. A mask can also be used so that adhesive in the channels does not harden and can be rinsed away later. The pattern could be done in SU8 but also etching a pattern could work.

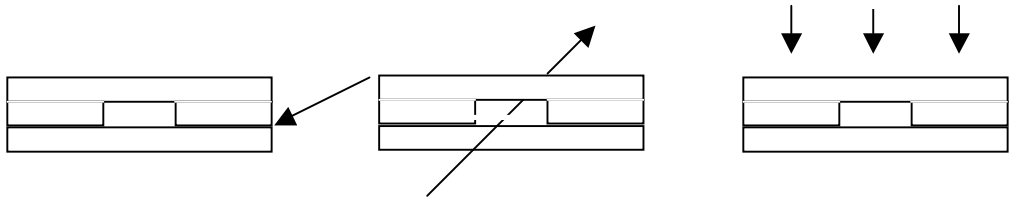

Figure 9A: Low viscosity adhesive is injected between two wafers. B: Adhesive in the channel is flushed away. C. UV light binds the two wafers together.

Another typical mass production technique is the stamp transfer technique, ref 9. A similar technique can also be used for making a flow cell (figure 10). A thin layer of adhesive is spin coated on a wafer. The stamp (also the top or bottom of the cell) is then brought in contact with the adhesive and moved to another wafer and bonded. The adhesive is cross linked with UV light.

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UV

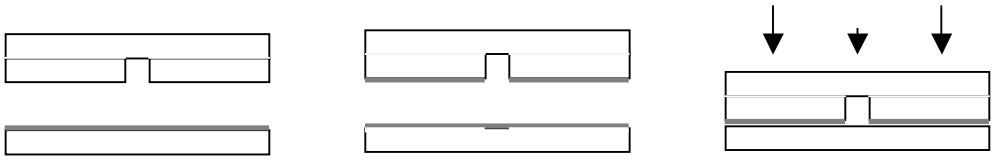

Figure 10A-B: A thin film of adhesive is transferred to the top wafer. C: The wafers are bonded with UV light.

It is relatively easy to work with adhesives because of the room temperature or normal hot plate conditions. One drawback is that depending on the way the flow cell is fabricated the fact that the adhesive is fluid in the initial steps makes it troublesome to work with. Also the long term stability can be questionable. But overall, most adhesive have been tested with a variety of chemical solutions and they have proved to withstand a lot of them. But they can not be used with strong acids because they are an organic type of composition and will dissolve.

Bonding- Some theory and numbers

The bonds in a solid material and between two solids close together are governed by; Van der Waals bonds, Covalent bonds, Metallic bonds and Ionic bonds. The typical strengths for these bonds are listed in list 1, ref 10.

Bonds	Strength kJ/mol
Ionic bonds	590-1050
Covalent bonds	563-710
Metallic bonds	113-347
Van der Waals bonds	
Hydrogen bonds with fluorine	<42
Hydrogen bonds without fluorine	10-26
Other dipole-dipole bonds	4-21
Dipole induce dipole bonds	<2
Dispersion bonds	0.08-42

List 1. The typical bond strength for different types of bonds.

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A microscopically flat surface such as polished silicon has a root mean square surface flatness of 0.3-0.8 nm, resulting in tops and valleys of several nm. Figure 11 shows typical lengths for different bonds and a schematic over a microscopical surface. In order to achieve a tight bond between to such surfaces at least one surface has to be deformed to match the other. The deformation could be plastic or elastic, by diffusion of solid material or wetting by a liquid material. The dominating theory of adhesive bonding is that it is the intermolecular forces that hold surfaces together; the wetting of the surfaces is therefore an important factor in achieving close contact between the molecules.

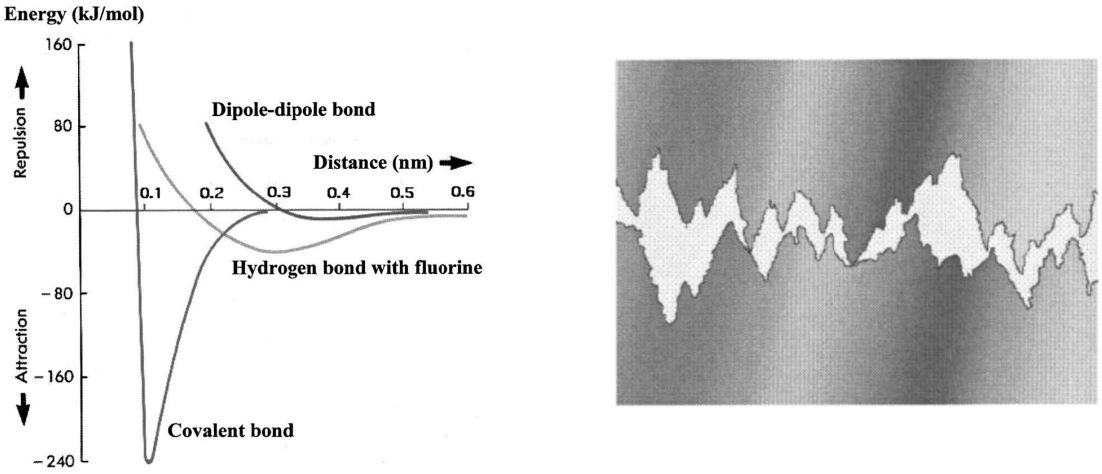

Figure 11A: Bond strength for different bonds and distances. B: A schematic over two surfaces close together. Ref 10.

For wetting to occur the solid has to have a greater surface energy than the liquid (figure 12 and 13). Surface energy is the result of unbalanced cohesive forces at the surface. It is energetically favorable for a surface to attach to another. Or else the world would only exist of surfaces.

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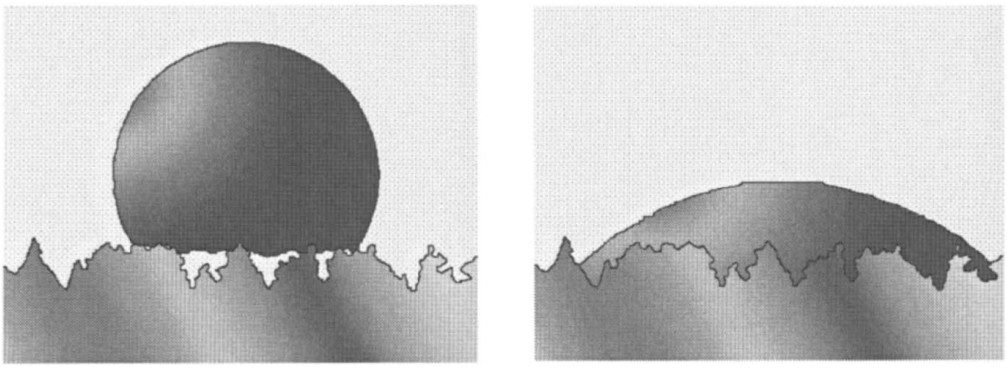

Figure 12A-B: Different wetting properties. Ref 10

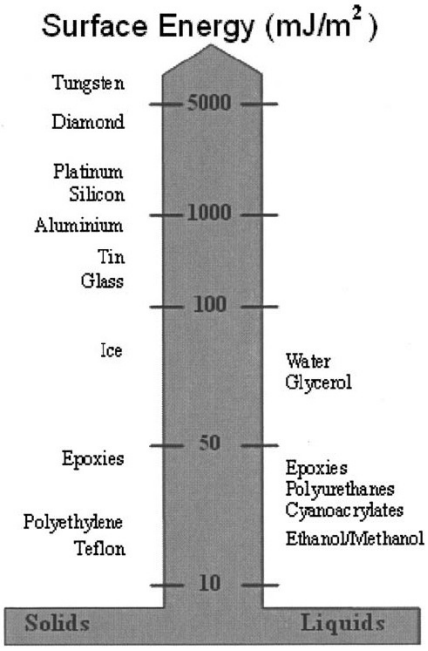

Figure 13: Surface energies for different materials. Ref 10

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Flow cell-The experiment

The experimental flow cell is made of SU8 and glass. The idea is to fill a space between two glass wafers with SU8 and then expose through the glass to define a channel structure. The SU8 that is not exposed is removed through holes in the glass. These holes are the in- and out-let from the channel.

The SU8 is in a solvent which makes it fluid at room temperature. The amount of solvent determines the viscosity. SU8 is in it self a solid under 55° C. The rough way of making a flow cell with this principle is shown in figure 14.

First the glass substrates are cleaned and put on a hot plate. The SU8 is then added to one of the glass wafers and smeared out for more efficient evaporation of solvents. One of the wafers has one or more pairs of sandblasted holes for fluidic connections. When the solvent is evaporated, the second wafer, either the one with holes or without, is pressed upon the first.

To blast holes in a cover glass goes much faster than through a microscope slide. In order to avoid excess SU-8 to be pressed out from holes, a tape strip can be used to cover the holes and later taken away. A spacer could be used to define a thickness. In most cases a small piece from a document plastic sheet was used. It has a thickness of 30 μm to 60 μm.

After the wafers have been pressed together, the flow cell is removed from the hot plate and let to cool. The channel is now ready to be defined with UV light. A small piece of aluminum foil defining a channel between the two holes is applied as a lithography mask. After exposure the flow cell is heated again to achieve cross linking.

The whole cell is then developed and the unexposed SU-8 defining the channel is dissolved through the holes. After development the flow cell is cleaned, dried and put into a UV-ozone chamber for extra cleaning.

To be able to connect tubings to the flow cell, short tubings with a larger diameter are cut and glued with silicon to the in and out let.

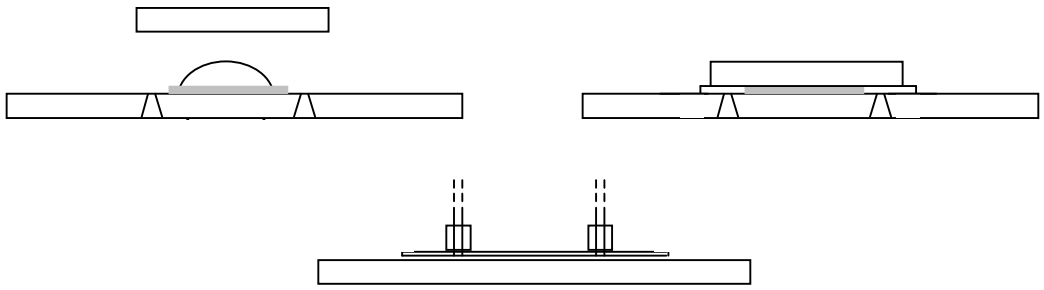


Figure 14A: A drop with SU8 together with spacers is put on to the glass. B: The SU8 is smeared out and soft baked and later a top glass is attached. C: After exposure through the top glass and development, in and out let is glued to the channel.

A B

The ideal cell made in the way described above should have perfectly developed walls and be fast and easy to make. This project is partly to test this. To sum all up, before going in to more details, the walls will not be perfect and the time and ease in making a cell will be hindered by some factors, which are presented below.

Pre bake- evaporating solvent from the SU-8

Before the cells top and bottom is closed all the solvent from the SU-8 has to be evaporated. When a thin layer of SU-8 is spun by a spinner the evaporation goes relatively fast and contributes very little to the total fabrication time. For the method above the evaporation took longer time and was hard to make standardized and optimized because the thickness was rather different from time to time. But there are some problems when a spun SU8 layer is to be used.

First a round glass substrate is best to use because of the symmetry preferable needed when spinning. At the edges of the glass an “edge bead” will occur, that is the SU-8 accumulates. When pressing two wafers together the bead will obviously hinder a perfect bond. The edge bead problem can be walked around by letting a larger round disk be pressed together with a smaller (figure 15). There is another problem though. The SU8 will stick to the next surface in a way which results in small air pockets getting trapped. This could be solved by pressing the wafers together in a vacuum system. A spin/vacuum approach has not been tested.

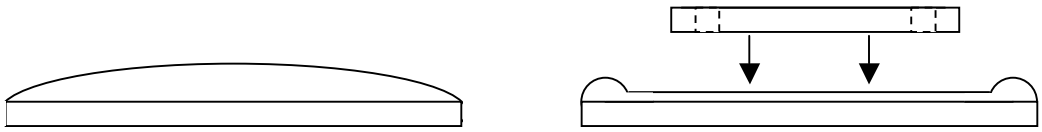


Figure 15A: The SU8 is spun to a defined thickness. B: The top glass is pressed onto the bottom glass under vacuum.

Development- removing non exposed SU8

Removing non exposed SU8 seemed to be the most uncontrollable part of making the cell. The cell is developed in a solvent during sonication. Because of the small channels it takes a long time for the solvent to dissolve and transport the SU8 out from the channel. During this extensive period of time and the powerful agitation from the sonicator the walls of the channels are damaged. This set some design constraints. For example a long channel takes too long to develop, which can be of a problem depending on the use of the cell. A “long” channel is e.g. needed when a prism TIRF setup is used.

Different factors were controlled and varied to try to find the best protocol. Overall the different results between settings were rather small. The fact that the holes are blasted in a way that makes them vary in sizes can be of importance when the relatively large variance in development time between samples is considered. The holes could instead be drilled with a diamond drill to achieve similar conditions.

A few things can be pointed out. If exposing the channel for a longer time this makes a typical effect around the channels to be smaller. It seems like during the development process a small area around the channel expands (Figure 16 and 17). If the exposure is longer the area is a bit smaller. The range tested is 10 to 40 sec. This is not for certain though because there is a large variance in development time.

A more powerful sonication will make the development faster, but instead harmful to the definition of channel walls.

Acetone works well as a developer and is faster than regular SU8 developer. The structures are large in these experiments and the possibility of the acetone to be too aggressive for smaller structures is a risk that is not needed to be addressed here. The walls seem to be the same.

The acetone was warmed to about 50°C, but no evident shorter development time was detected.

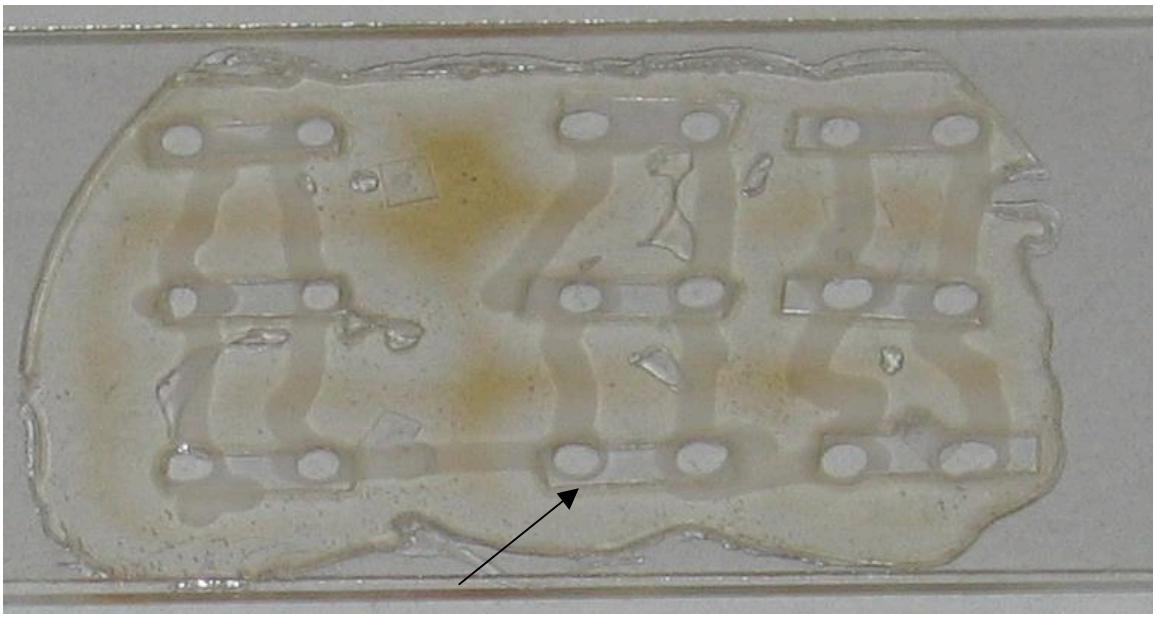


Figure 16: It would in principle be possible to make a lot of channels at the same time. The arrow points at the “swelling” around the walls. Short side 2.5 cm.

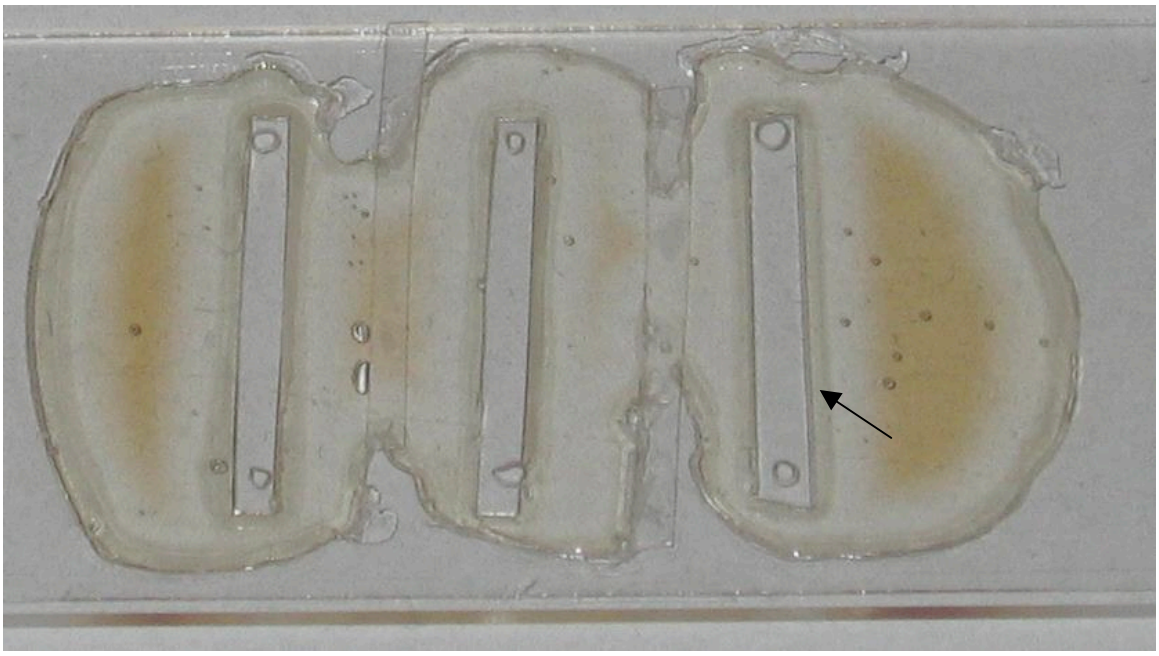


Figure 17: The swelling effect is visible around the edges of the channels (arrow). Also the different sizes of in- and out-lets. Short side 2.5 cm.

Final thoughts

Why is this proposed method to make a flow cell interesting? Primarily because patterning and sealing occurs in one step. The result is also a versatile cell with glass surface at both top and bottom. E.g. the cell is compatible with both prism based TIRF and through-the-objective based TIRF.

One factor is probably very important for good results; the pretreatment of the glass. This issue has not been fully appreciated during these tests. In order to get the SU8 stick as hard as possible a few hours in a hot oven is probably needed. The wafers have been pre treated on a hot plate, but maybe not enough.

One other thing is that the existing SU8 starts to get old. Perhaps it has started to auto crosslink due to light exposure, temperature etc.

How to get liquid in and out

There are different ways of getting fluid through the cell. Techniques we use are syringe pumps and peristaltic pumps. Capillary forces can also be used to drive a fluid.

A syringe pump (figure 18) is a motor chuck that compresses or decompresses normal syringes in a controlled way. A syringe pump is good for high control of flow speeds. One big drawback is that when exchanging fluids either a coupling of many syringe pumps or so called loops are needed. This could be fixed by instead having an empty

syringe drawing fluid from an open syringe through the system. What is then important is that no air is locked in the system. This will introduce slow responses in a microfluidic system. Air is much more easily decompressed than a fluid and fluid moves slowly to balance different air pressures in the device.

Figure 18: A loop system in between the pump and cell to get rid of bubbles during exchange of syringe (exchange of solution)

A peristaltic pump is a pump (figure 19) that works by rolling a cylinder over a soft tube thereby pressing fluid through a system. This technique makes it easy to exchange inlet fluids, because an open reservoir can be used. The reservoir it self can be exchanged, for example eppendorfs for small volume handling. The peristaltic pump cannot produce as high flow speeds as the syringe pump.

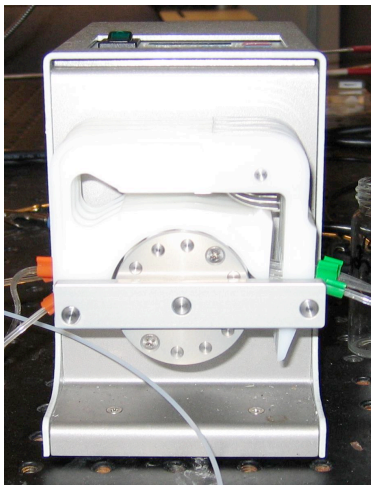


Figure 19: A peristaltic pump

When a more sophisticated technique is not needed, a simple capillary flow can be utilized. For example a tissue at one end of the channel will draw fluid through the system.

Data on the flow speeds using a SU8 cell.

30 s later the spot had at least

The cell managed a 3 mL /min flow speed according to judging from using a syringe pump. A rough estimation of the channels volume gives;

$2cm*2mm*40\mu m=2*10^{-1}dm*2*10^{-2}dm*40*10^{-5}dm=160*10^{-8}dm^3=1.6\mu L$

$3000uL/min=50\mu L/s$ $50/1.6=31$

Which gives that the volume is exchanged ~30 times/s.

Bilayer in the SU8 cell

As mentioned before, it was shown possible to make a bilayer in the SU8 cell. Figure 20 shows recovery after photo bleaching, indicating a fluid bilayer. The quality of the bilayer seemed really good, exept for some dark spots of different sizes. It is hard to know what causes these, but if it is possible to get a perfect bilayer elsewhere on the surface, it should just be a matter of adjusting parameters during development to get rid of the dark spots.

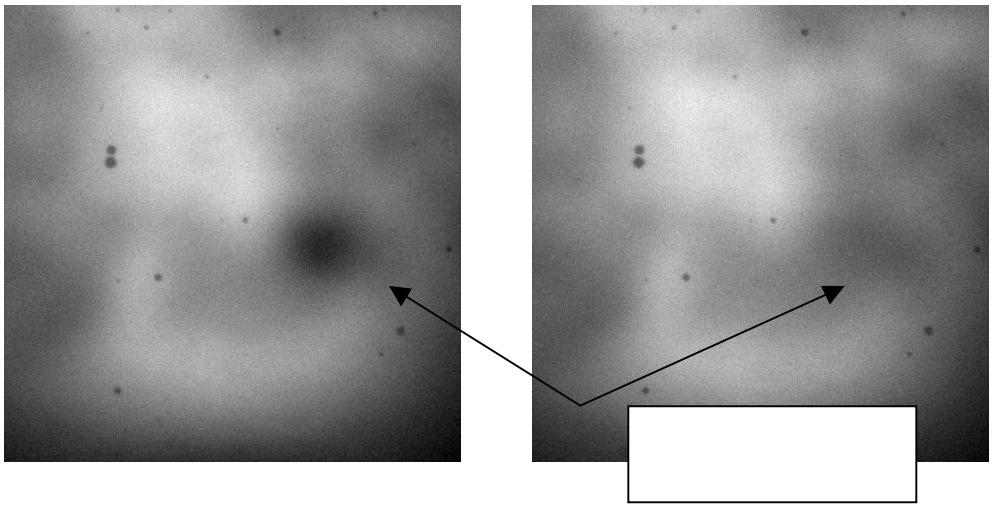


Figure 20: The recovery after photo bleaching is evident. Non homogenous illumination causes the blurry pattern. Small black dots are visible. Images $137*137\ \mu m^2$.

A note about cleaning

The need for cleaning with DI (deionized water) after Isopropanol and developer solvent seems to be of a less importance for bilayer formation. To directly dry the sample with N_2 seems to be enough to get a bilayer, at least if the channel is treated with UV ozone. The question arises though how effective the UV ozone treatment is after the cell has been closed. It is normally used on open surfaces, such as a single wafer, with good exchange of oxygen around it and no glass hindering UV light.

A way of getting rid of the problem that SU8 is still stuck at the surface (probably causing the dark spots) is e.g. to have a layer between the surface and the SU8 which is known to be possible to clean away in a gentle way not affecting the channel definition. A candidate is to evaporate a layer of Al. This layer could perhaps also function as a lithography mask. After development of the SU8 the Al could subsequently be removed (figure 21), resulting in a clean surface.

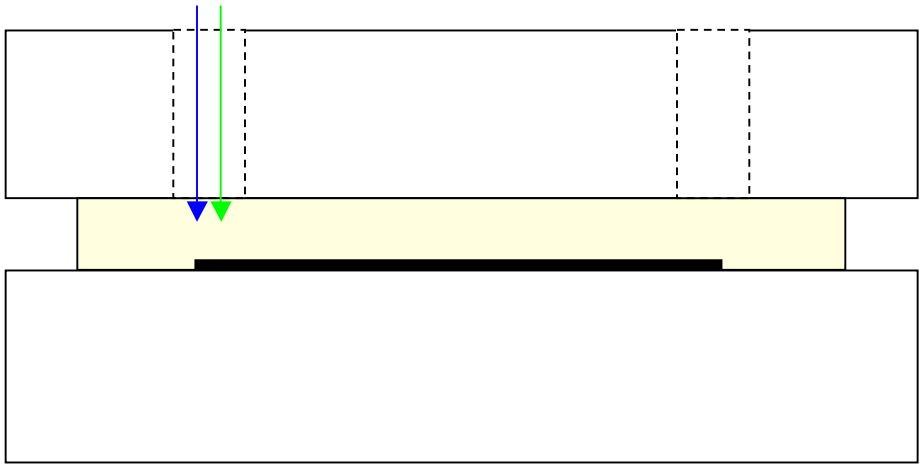


Figure 21: First the SU8 is developed and then the Al.

Electrical experiments

Fabrication

The cell used for the electrophoresis experiments was not the SU8 cell. This could in principle be done but because of practical reasons another cell was constructed. The cell was of an adhesive bonding type and the manufacture of it exploited the capillary effect technique mentioned in part 1. First two cover glasses are put close together, 0.5-1 mm apart. 4 small pieces of plastic sheet are placed according to figure 22 and a third cover glass is placed on top of the other two.

Two drops of UV glue are placed according to figure 22. The capillary forces acting on the UV glue draws it in between the glasses. The capillary forces stops acting when the glue approaches the edge. At least the process goes much more slowly than between two glasses, which makes it possible to wait until the edge has been reached everywhere before the glue gets into the channel. This process is also dependent of the relative sizes between glass and glue thickness. The process takes about 10 min. Next the glasses are put under UV light to crosslink the glue for about 5 min. When the glue crosslink it shrinks and the stress near the spacer makes the glue detach from the glass. Therefore the spacers are placed away from the channel.

A glass slide is prepared by scratching it with a diamond pen in a cross like fashion or in straights line perpendicular to the thought channel. The glass slide is cleaned in detergent and DI. A line is then drawn with a marker pen over where the channel is supposed to be. This is done to get a protecting film from fumes from the UV glue and is later cleaned away. This is just done to prove that is possible; whether the fumes actually contaminate is another question. Some post cleaning impact of the marker pen can be seen in the top left square of figure 25. This square is made of chromium, just as the lower left square. But the top one has been marked with a marker pen. Fluorescent vesicles seem to be attached more easily to the part where the marker pen has been. To the right of the figure there is glass supporting a bilayer. The bilayer behaved the same for places where the marker pen had been or not.

The channel is then defined when the three cover glasses are glued together with the slide in the same way as before. UV light is used to crosslink the whole thing, resulting in a channel with approximate thickness of about 200 – 300 μm. The cell is then cleaned in acetone and isopropanol and dried and put in a UV ozone chamber.

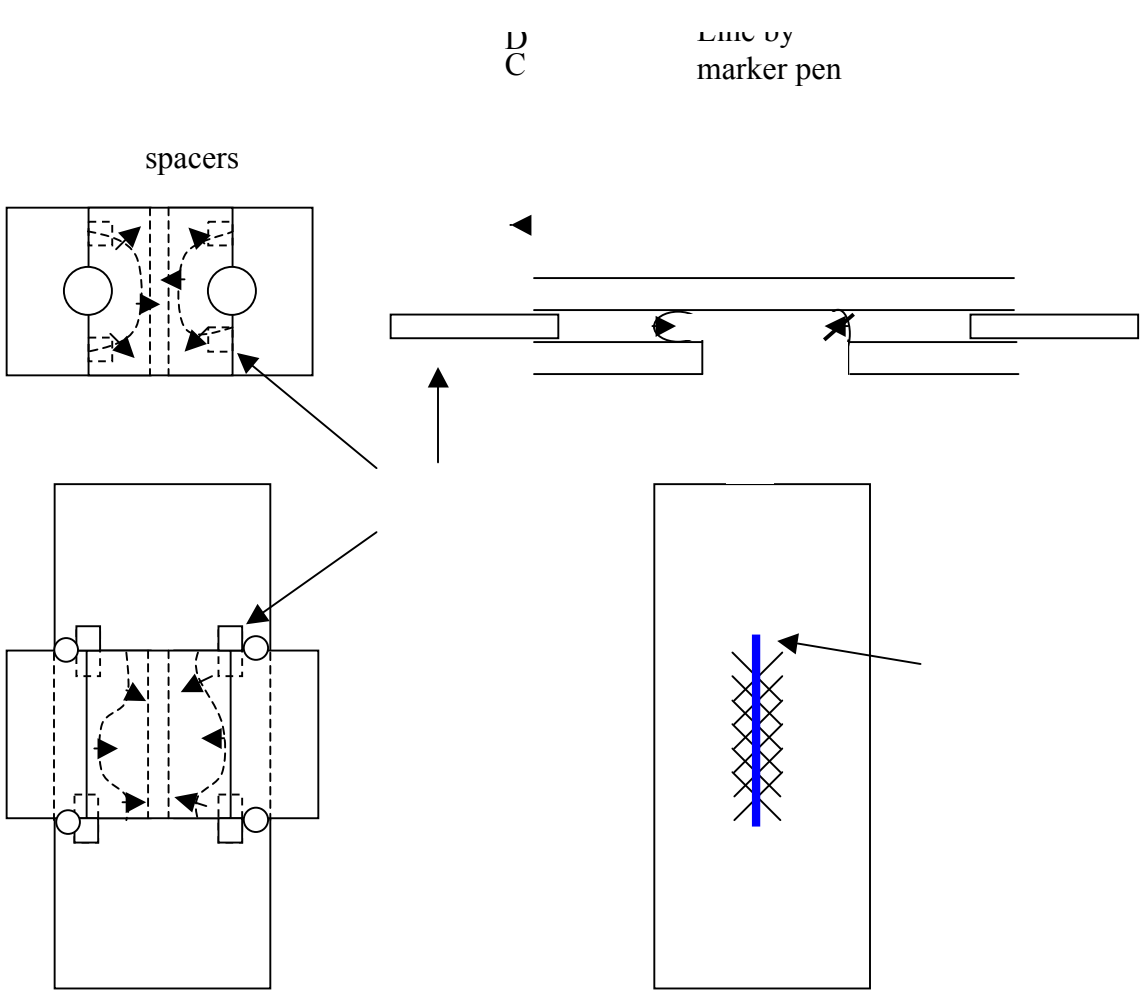


Figure 22A: Three cover glasses are glued together. B: The cover glass piece is glued to a glass slide. C: Capillary forces stop acting when the glue meets the edge. D: Alternatively the glass slides surface is covered by a marker pen before gluing.

After the UV ozone treatment, in and out tubings of rubber are glued with silicon (figure 23). One of the bigger tubing has a smaller tubing glued to its side. This is the actual out let. The big end is used for letting gas out during the electrophoresis experiments. Gas easily forms if there are salts in the solution and if the voltage is high. The whole chuck is then coupled to a peristaltic pump or even a single syringe (no pump) that is handled manually (figure 24). The left tubing is filled with solution which is drawn through the small tubing in the channels other end.

Two platinum wires are then coupled to the device by sticking them into the rubber reservoirs and applying a voltage with a regular power box (figure 23). Another way to couple the electrodes are to stick them through the tubings perpendicular to the channel. In this way the field strength is more quantifiable.

x x

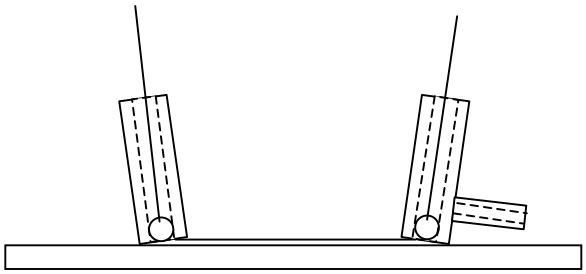


Figure 23: In- and out-let for the electrophoresis cell and alternative ways of attaching electrodes, the cross in the circle means the electrode points in to the picture.

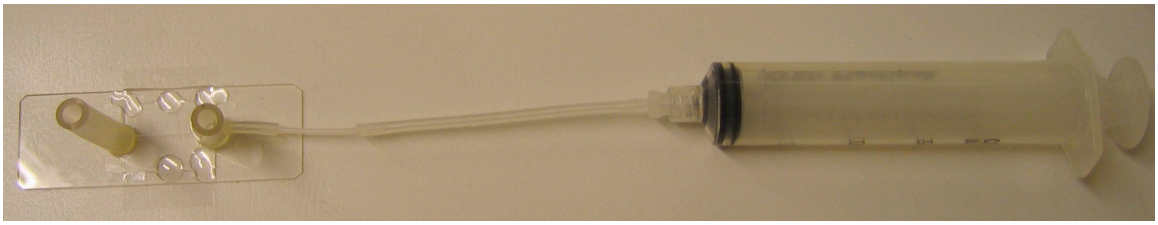


Figure 24: Solution is sucked by hand through the channel.

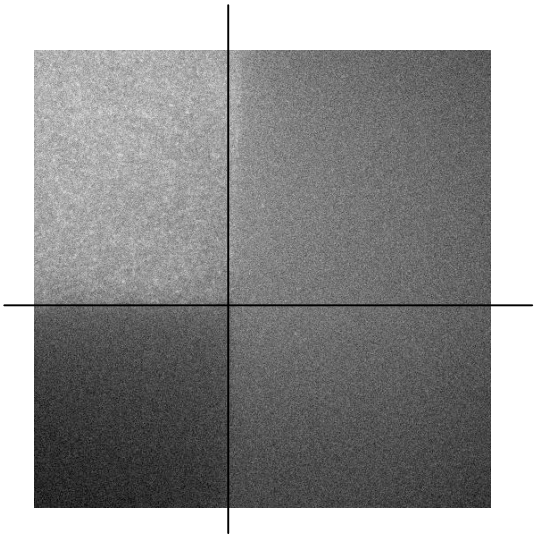


Figure 25: Top left part; Chromium + marker pen = fluorescence, lower left; Chromium no marker pen = no fluorescence. Top right; glass + marker pen = bilayer, lower right; glass no marker pen = bilayer. Image 137µm*137µm

Alternatives to scratches

In order to produce diffusion barriers there are a bunch of different techniques. The technique used in this report was to simply scratch the glass surface with a diamond pen. This technique is not especially reproducible. Instead a deposition of chromium was done using a double layer lift off process (figure 42). First a layer of LOR 3A is deposited on to the substrate and after that a layer of S1813. When later developing the resists an under cut is the result. This makes the edges of the evaporated metal more defined. The rest of the resist is then washed away to expose the glass surface underneath.

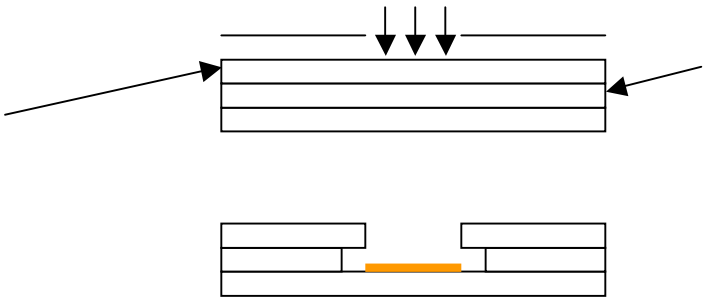


Figure 42: The two layer lift off system

When applying voltage to the system the chrome started to dissolve which made it incompatible with these kinds of experiments (figure 43). Chromium has been used in very similar experiments and is reported to work. This can be because of the different dimensions used for the chromium lines etc, different voltages applied or the amount of time the voltage is applied. The rate with which the chromium dissolved seems to be voltage dependent. For 30 V/cm a 300 Å layer of chromium dissolved in seconds. Bubbles were then evident in the channel, indicating gas products.

Another technique tested was to directly use SU8 as a barrier material. These experiments failed because of difficulties to make the SU8 stick to the glass substrate. An experiment in making barriers in the positive resist S1813 was made (figure 44). The problem here was that the surface was not clean enough to get a functioning bilayer. And also both the texas red and the S1813 is red which can cause a contrast problem. Instead of trying to use other metals than chromium and or adjusting parameters for SU8 and S1813, the approach with scratches was used for this report. Another type of technique often used to produce barriers is of chemical type where molecules are printed with a stamp on to the substrate and works as a barrier. This was hard to make in a closed flow cell as that one used. But can in principle be done using an intricate system of laminar flows.

Figure 43: The places where the chrome has been dissolved is clearly visible.

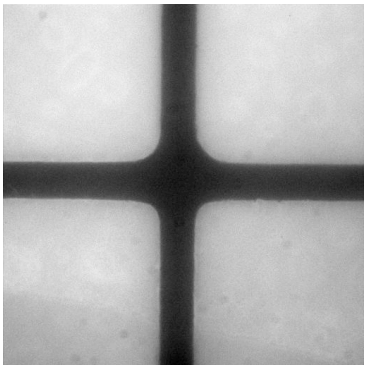


Figure 44: The white parts (fluorescent parts) are S1813.As seen on the picture the glass part that should give a fluorescent background because of a fluorescent bilayer appears black.

The polar lipid

The most interesting property of the polar lipid is its ability to self assembly into larger structures. Different geometries in bulk are the circular (vesicle) and tube like (micelle) structure. When a support is available the structure can follow the support. The structure can be both of a monolayer and bilayer composition. In the following a "bilayer" refers to a supported lipid bilayer structure, SLB. A supported bilayer means that a bilayer is covering a surface.

The origin of the self assembly is hydrophobic/philic interactions between different parts of the lipid and its environment. The tails are made up of hydrophobic hydrocarbon chains and a polar head (hydrophilic). The lipids used in this project are the Egg PC (L-α-Phosphatidylcholine) derived from chicken egg (Fig. 26) and the fluorescent lipid rhodamine DHPE with the fluorescent part attached to the head of the lipid (Fig. 27).

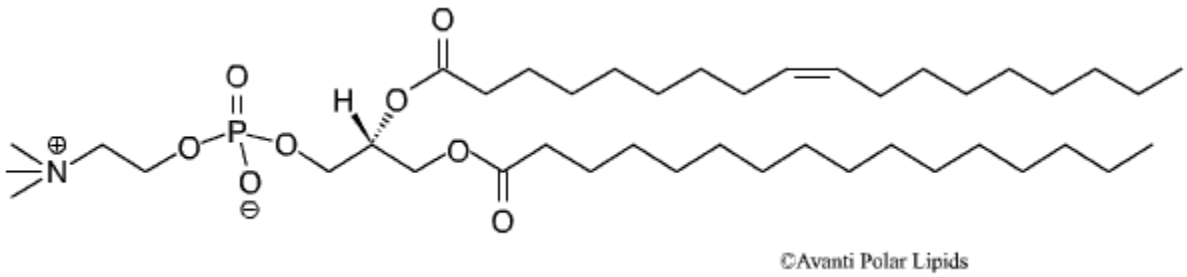


Figure 26: Egg PC, derived from Chicken egg. Ref 11

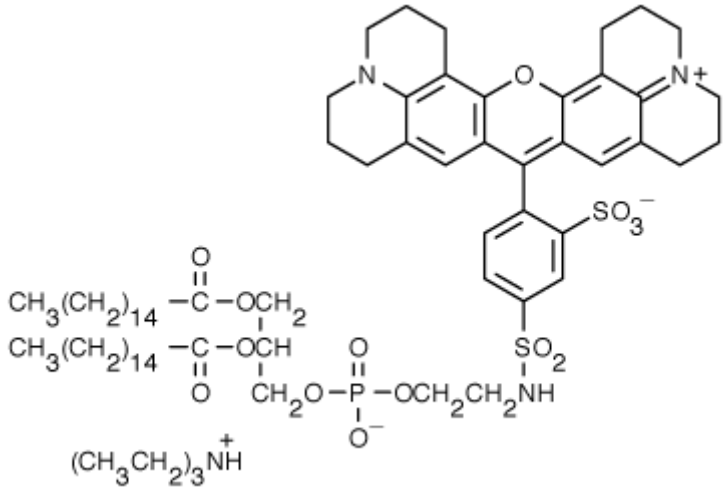


Figure 27: Texas Red DHPE. invitrogen.com

Formation of a bilayer

The technique used for making the SLB is to let small lipid vesicles approach a glass substrate from bulk and spontaneously form a bilayer. The vesicles are fabricated in a simple filtering process where a lipid solution gets pressed through filters with a pore size corresponding to the wanted vesicle diameter. The vesicles are then diluted to and spread over the substrate. What happens during the process of vesicles approaching the substrate depends on parameters such as substrate, lipid and buffer type. Data from two different techniques has enlightened the process of bilayer formation, the quartz crystal microbalance with dissipation monitoring (QCM-D) and the atomic force microscope (AFM).

QCM-D

A QCM consist of an oscillating crystal driven by electrical forces. It will have a resonance frequency which depends on the effective mass coupled to it. It works in analogy to a mechanical pendulum with different mass. The dissipation monitoring measures what is happening with the amplitude of the oscillations if the driving force shuts off. This is a measure of the frictional forces. These techniques working together on the same substrate can give a good understanding of how bilayer formation works.

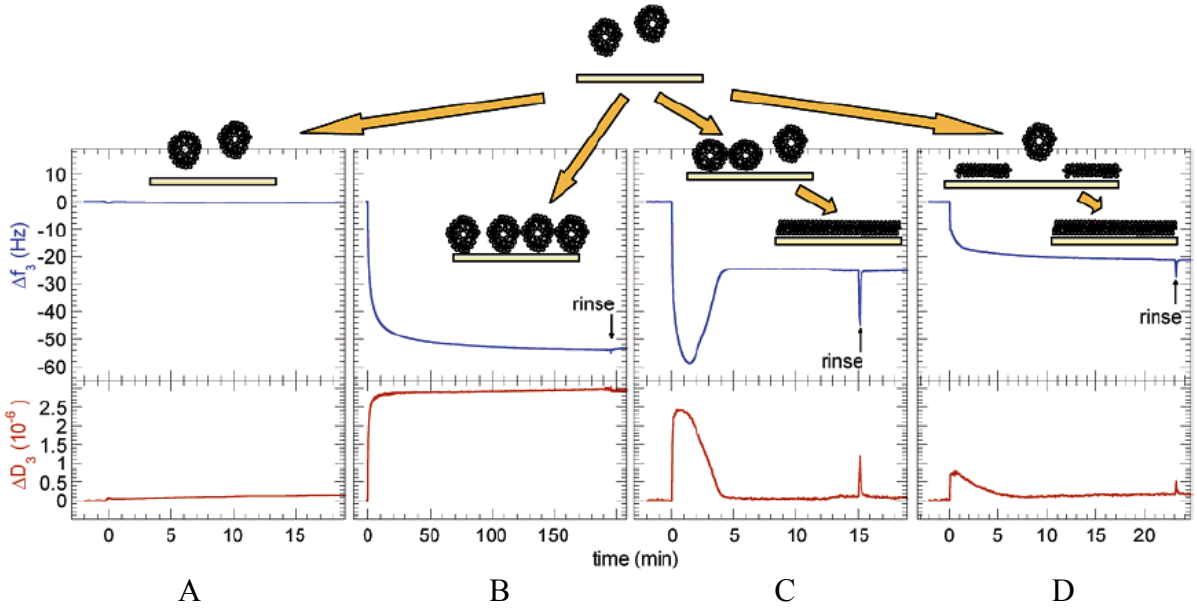


Figure 28A: The frequency and dissipation shift when vesicles are bouncing on the substrate. B: The shift when vesicles are attaching but not fusing. C: Absorbs and fuses. D: Fuses directly. Ref 13

Fig. 28 shows the behavior of the lipids chosen for this project. The shape of the frequency shift is due to release of coupled water when vesicles fuse and the "mirror image" in dissipation is due to the higher frictional coupling from vesicles than a smooth bilayer. The question is what governs the sudden fusion of vesicles. The QCM will in this case only give an ensemble approach on how to understand the formation. For a more detailed picture AFM is a good candidate.

AFM

An AFM consists of a mechanical tip which is interacting with the surface at the same time as its deflection from the surface is monitored. Often a feedback system is implemented which keeps the tip at same physical interaction conditions all time. On a rough surface this means that the tip has to move in the z direction to keep it self at the

"isophysical" point. By controlling the x-y movement in a precise way a topographical map of the surface can be made. There are AMFs which work in a liquid environment which make them very useful in studying bilayers and vesicles.

It seems like at a critical surface coverage the vesicles in case C from fig. 28 starts to fuse. Fig. 29 shows an AFM picture where the coverage of vesicles has been adjusted so interactions between vesicles can be ignored. No fusion has occurred.

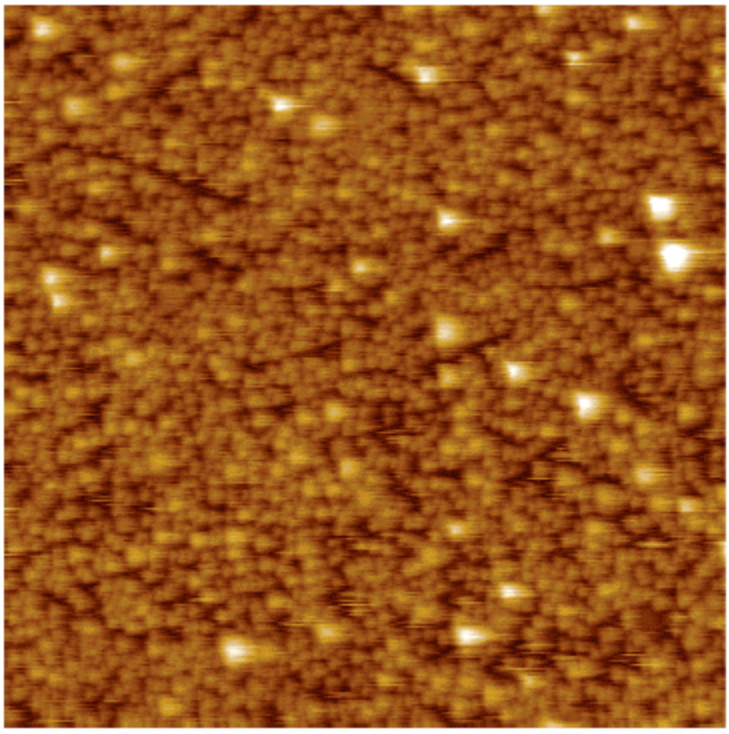


Figure 29: AFM picture of vesicles (made of DOPC/DOPS 4:1). The image size is *50 μm* and the z-scale *50 nm*. Ref 13

The fusion of one vesicle tends to trigger the neighboring vesicles as long as they are sufficiently close together. Fig. 30 shows an AFM scan of relatively tightly packed vesicles. The fusion of one vesicle may in this case be due to stress induced by the AFM tip. But after the first rupture a small cascade of other fusion events occurs. The cascade stops when no "edge effect" can reach more vesicles.

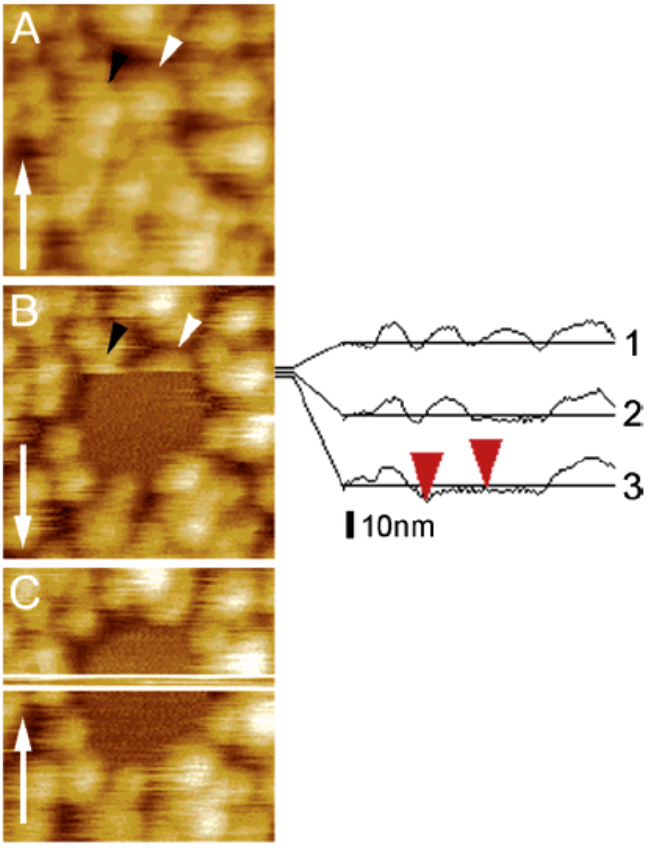


Figure 30A. No rupture. B: The three after each other following horizontal scans show that the vesicles didn't rupture at the same time. C: A small bilayer path is formed but the edge of the patch don't influence more rupture this time. Image size is *250 nm*. Ref 13

To make a perfect bilayer is difficult. Fig. 31 shows AFM pictures of typical bilayers on glass supports and a silicon wafer. The defects take up a small fraction of the whole surface which makes them difficult to quantify using QCM-D techniques. But they are clearly visible in an AFM scan. The defects can be regarded or seen as non fused vesicles or an immobile fraction of fluorescent lipids.

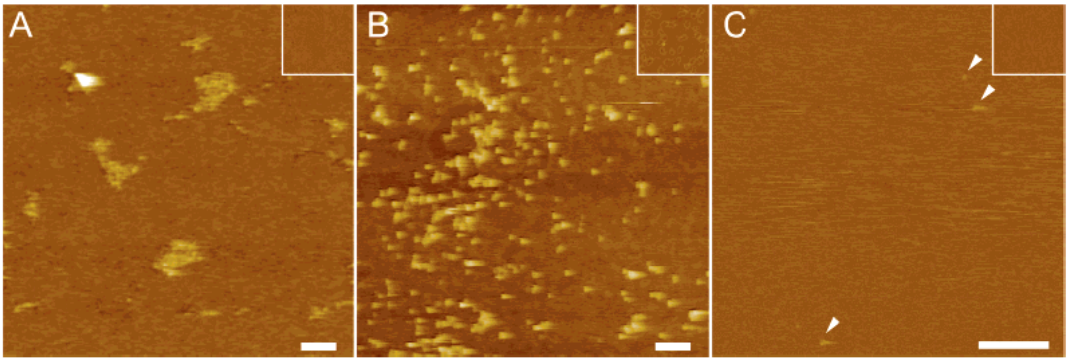


Figure 31A. A typical glass cover slip. 31B: A typical glass slide. 31C: A silicon wafer. The wafer is almost free from defects. Scale bar is *400 nm*. Ref 13

The experiment-theory and results

The experiment is about exploiting the lipid bilayers fluidity. By incorporating charged components in the bilayer these can be manipulated with external electrical fields (figure 32), refs 14, 15. The charged component for this setup is a DHPE lipid labeled with a Texas red fluorescent dye. The lipid part carries a negative charge. This lipid will then be affected by a force and move through the fluid bilayer. The other part of the bilayer is the uncharged lipid Egg PC.

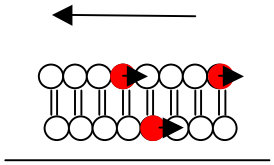


Figure 32. The red head group represent the charged DHPE texas red, and white head group represents the neutral Egg PC. Large arrow, direction of E-field, small arrows forces acting

Figure 33 shows how lipids are moved towards scratched arrow like structures. The field direction has been altered between the two frames. A collection of fluorescent lipids at the right frame can be seen. Suggesting it is possible to concentrate charged components in more than 1 dimension.

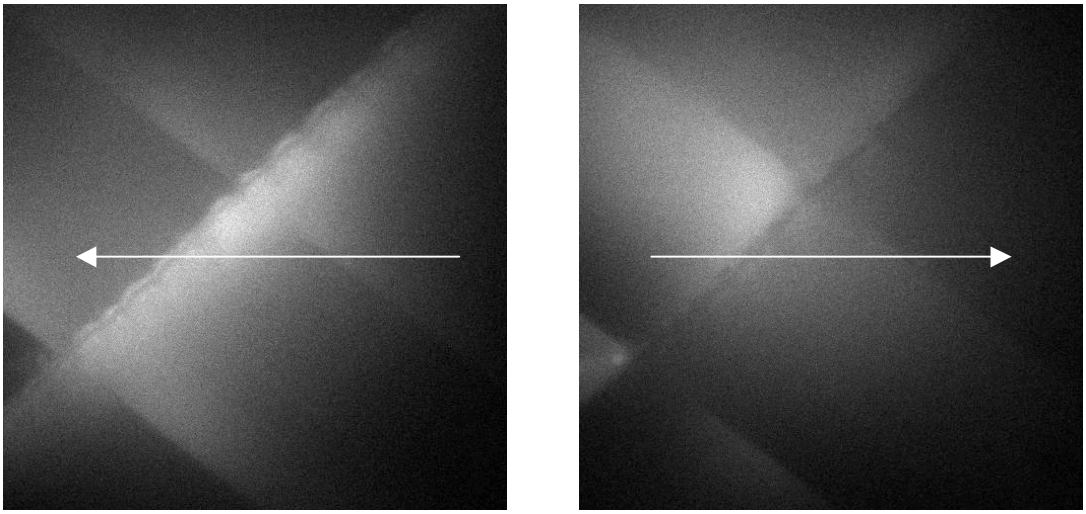


Figure 33: Altered field. Arrows indicates the lipids migration.

With the help of diffusion barriers in the substrate where the lipid can't diffuse, some physical values can be extracted, e.g. the charge of the lipid. A simple model has been proposed for the steady state arising at a barrier when the diffusion, D, and drift, v_d (absolute value in equations), from electrical forces balance. The competition between diffusion and drift;

$$\frac{\partial C}{\partial t} = D\nabla^2 C - v_d \nabla C$$

with conditions;

$$J = -D\nabla C + v_d C = 0$$

and

$$\frac{\partial C}{\partial t} = 0$$, a steady state

Gives the solution

$$C(x) = C_0 e^{-\frac{v_d}{D}x}$$

So by letting the system relax to a steady state, an exponential decay of fluorescent lipids should be seen at the diffusion barrier. The solution assumes that at $x = \infty$ there is no fluorescence which is not the real case due to background fluorescence. But in theory there should be no charged lipids at infinity. By measuring the exponential decay factor (figure 34, 35 and 36)

$$\tau = \frac{v_d}{D}$$

drift velocity or diffusion constant can be estimated depending on which factor is known. There is another approach to get an expression for the exponential decay at the barrier, by simply considering a Boltzmann distribution with the potential arising from the electrical forces.

$$C(x) = C_0 e^{-\frac{Eq}{kT}x}$$

The exponential decay factor is

$$\tau = \frac{Eq}{kT}$$

So by measuring the decay factor, the electrical field and the temperature a value for the charge can be calculated. And instead of measuring the decay factor, a measure of the drift velocity and diffusion constant will give the same result.

$$\tau = \frac{v_d}{D} = \frac{Eq}{kT} \Rightarrow q = \frac{v_d kT}{DE}$$

The drift velocity is possible to measure with the help of the diffusion barrier as well (figure 37, 38 and 39). Let us consider the case if there were fluidity but no diffusion. If charges were driven from a barrier it would be as a step function in intensity moves away from the barrier. With diffusion, this step function will decay, but it is till possible to detect the midpoint and extract the drift velocity. Another approach is to bleach a spot and then turn on the field. The spot will start to drift and by following the middle of the spot the velocity could be extracted. The diffusion constant is measured by looking at how fast a bleached spot is recovered, using a Matlab program written by Peter Jönsson. Ref 16

The data from experiments shown in figures are:

τ = 0.05 μm^{-1}

v_d =0.3 μm/s

D =2.6μm^2/s

T =293 K

E =28V/cm

Inserting the value τ , T and E gives a lipid charge of;

q = 0.46e

where e is the elementary charge.

Instead using the ratio of v_d and D gives a lipid charge of;

q = 1.04e

This value is in really good agreement with the expected. The DHPE texas red should carry a single charge. So why does not the decay constant give the same result? Its value is too small indicating that equilibrium is not reached, there are still lipids that has to "fall down" against the wall. But from figure 36 one can see two profiles with 5 min in between. The profiles looks different, the later one has more lipids close to the wall and less in "bulk". But the profiles still have the same decay factor.

Perhaps an equilibrium with a more correct decay factor could be reached if the experiment where carried along for a longer period of time. And five minutes is a too small time interval to see any effect. Another explanation is that the high concentration of lipids introduces new kinds of forces not considered in the model. The exponential "roll of" (figure 35) at the wall can be due to this kind of effects. On the other hand an explanation for the roll of is probably self quenching of the lipid dyes. And the data fitted to the exponential is chosen so that the role off is not considered, see figure 35. It has also been reported that for high fields for a long period of time irreversible effects near the walls appear which results in that the diffusion is lowered.

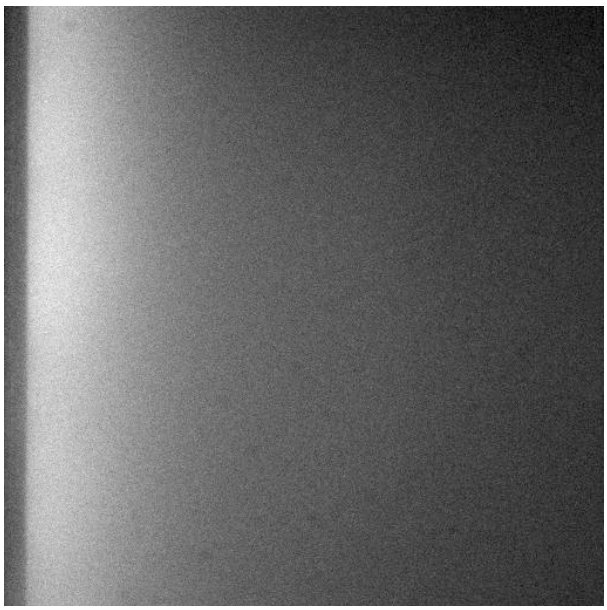


Figure 34: The static profile for 28V/cm. Frame not normalized. The frame for five min later is not shown.

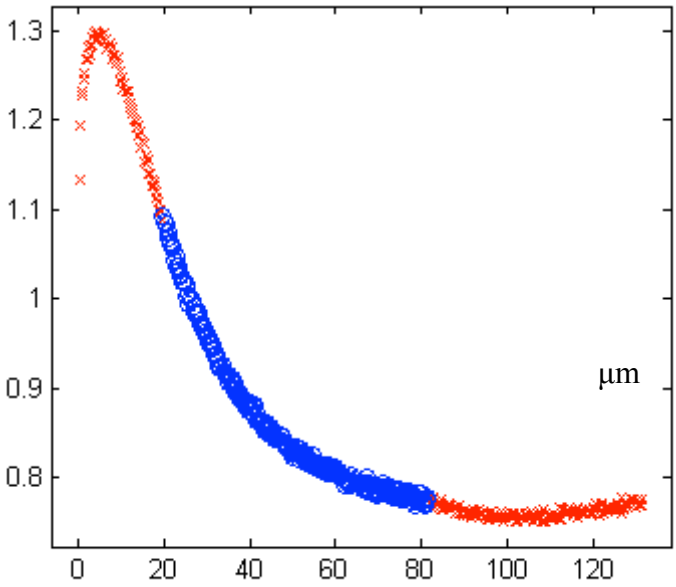
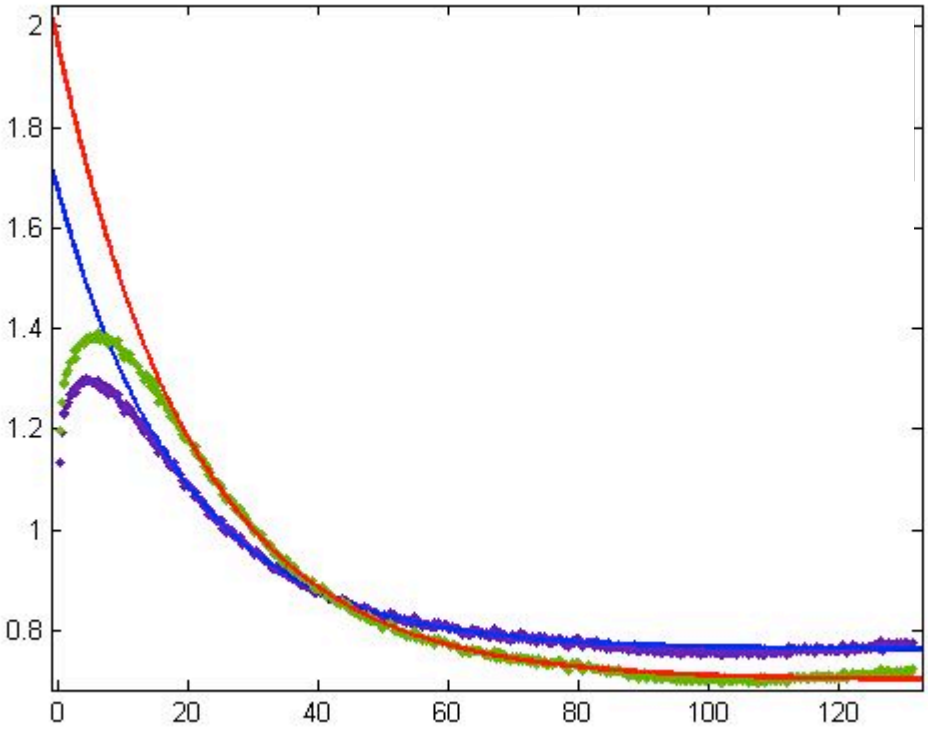


Figure 35: Typical part of the data that is fitted. The “roll of” is clearly visible at the top.



A D B E C

Figure 36: The exponential fit to the data, green/red pair shows the distribution five

r

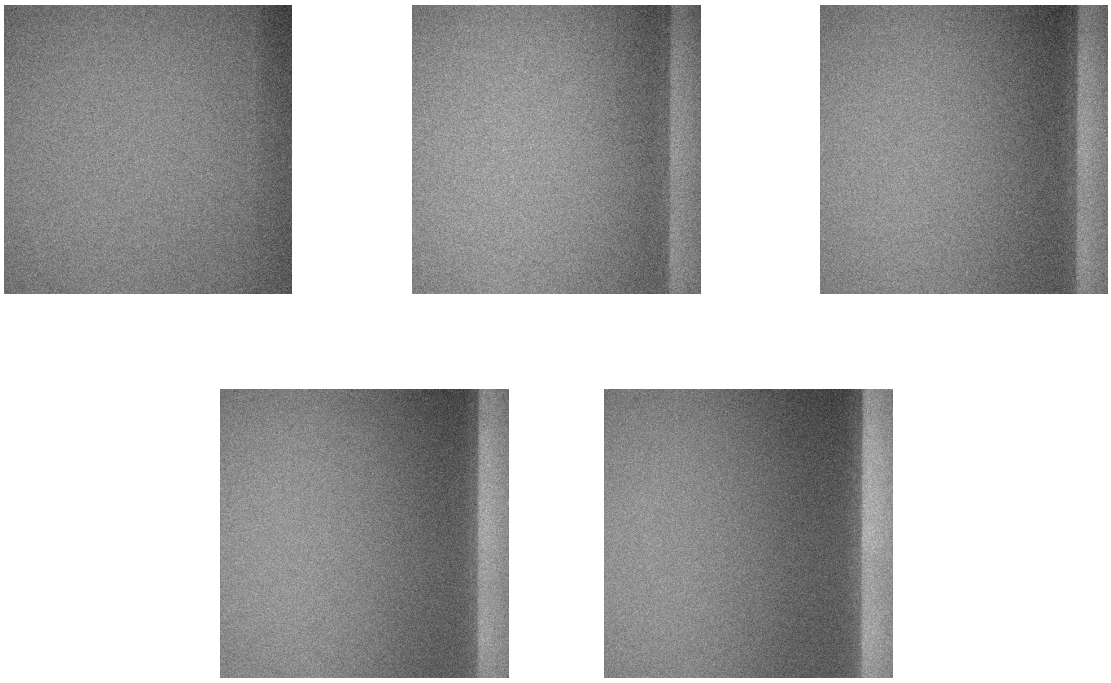


Figure 37A: No field applied. B-E: 28V/cm applied and there is 30 sec between frames. These frames are not normalized for uneven illumination but the tendency is clearly visible.

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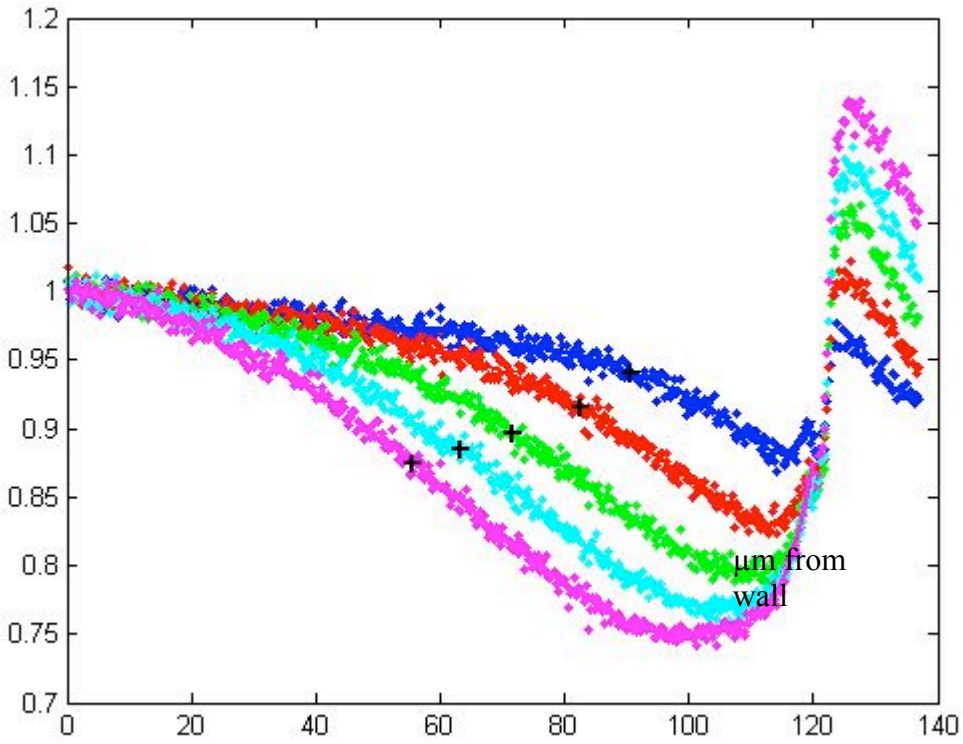

Figure 38A: Plot for the respective vertical average for figure 37B-E: The data is normalized. The crosses are the respective midpoints. Dark blue = 30 sec, red= 60 sec, green 90 sec, light blue = 120 sec, purple = 150 sec.

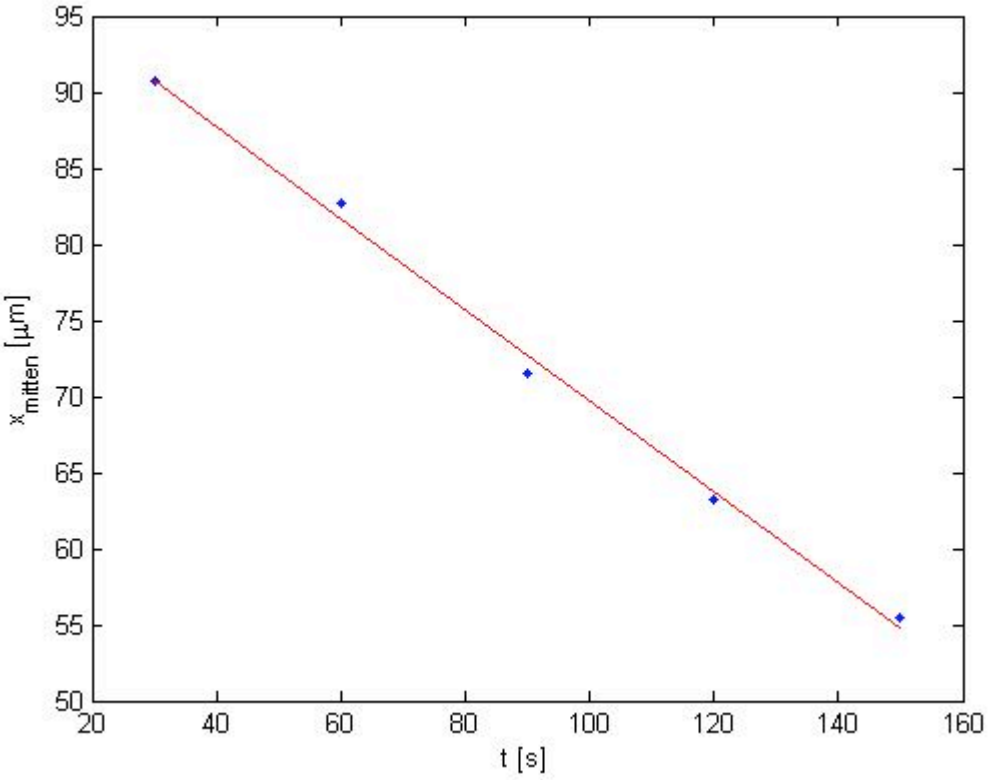

Figure 38: The respective midpoints plotted vs. time. The slope of the curve gives the drift velocity.

There are a few phenomena regarding electrophoresis that should be mentioned. At the surfaces of the walls in the channel a so called electrical double layer is created. This is due to the fact that the surfaces have a net charge. The net charge will attract corresponding ions from the solution resulting in an electrical double layer (figure 39). This layer of ions is attracted to the corresponding electrode, resulting in the whole bulk of fluid being dragged in this direction; even taking with it charged species that would else travel in the other direction (electrophoresis). This effect is called electroosmosis and it could counterbalance the electrophoresis. The velocity profile from electroosmosis is different from the velocity profile from pressure driven flows (figure 40). The electroosmotic flow is also independent of channel radius, which makes it useful in really small channel environment. On the other hand the flow is not easily controlled because the surface charges can be easily altered by contaminations. For this experiment the electroosmosis effect is negliable because there is no extra drag on the lipids incorporated in the bilayer. Further away from the surface electroosmosis do occur though, which probably could be evident from using charged beads in solution. A fluorescent object is seen traveling in the opposite direction relative the movement of the charged lipids (figure 41), this could be from electroosmosis but is impossible to say.

It has been shown that lipid vesicles tethered to a mobile bilayer will react to an applied electrical field in a way that relies on a combination of electroosmosis and

No flow at surface
electrophoresis. This is because the large vesicle (100nm in diameter) will feel the force of the electroosmostic flow.

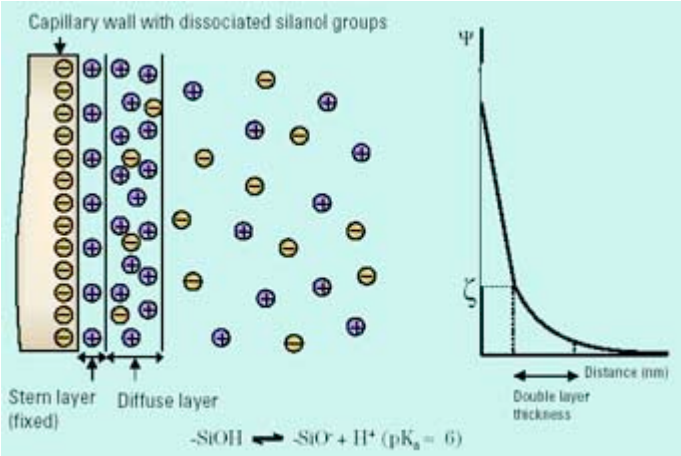

Figure 39: Scematic over the diffuse layer. Ref 14

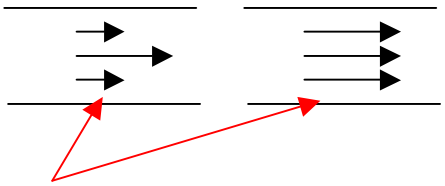

Figure 40: The difference in flow velocity profiles for pressure driven electroosmotic flow. Both profiles have a zero velocity just at the surfaces.

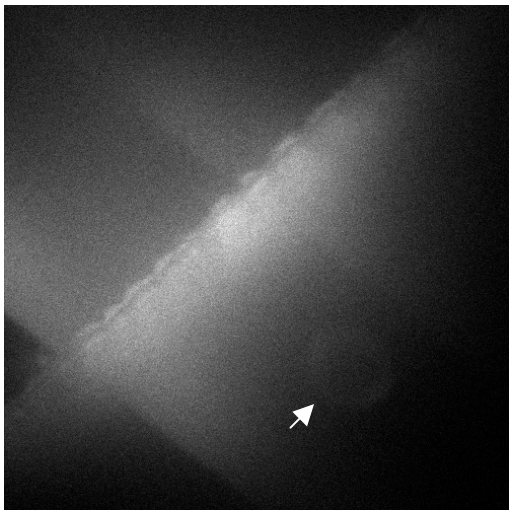

Figure 41: A round fluorescent object travels in opposite direction than the charged lipids.

Conclusion and future work

The SU8/glass flow cell works in principle but there are some issues regarding the ease and speed in making a cell. To spin SU8, drill holes, pre treat the surface sufficiently and assembly in a vacuum system hopefully can give some satisfying results.

The electrophoresis experiments in this report are conducted on an ensemble of charged lipids. With a much lower concentration of fluorescent lipids in the bilayer it is possible to look at single fluorescent molecules. There is a possibility that the observation of single lipids movement because of the electrical field will present data otherwise "averaged out" in the ensemble case.

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The flow cell

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Electrical experiments

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Protocols

Lipids experiment

100 μL of 25mg/mL Egg PC in Methanol
10 μL of 1mg/mL Texas Red DHPE in Chloroform
500 μL Methanol

The lipids are mixed in a round bottle. The bottle is turned and at the same time N_2 is blown in to the bottle so that a thin film of lipids is produced. Later the lipids are held under N_2 flow during 30min. The lipids are then dissolved in:

500 μL HEPES buffer (100 mM NaCl 10mM HEPES pH 7.4)

The lipid solution is then extruded through 100nm filters and then 30nm producing vesicles with a mean diameter around 30nm.

Lipids flowcell

Glass is cleaned in piranha solution for 10 min. The glass is later rinsed in DI and dried, after that they are put in a UV ozone chamber for about 10 min.

Piranha solution 3:1 H_2SO_4: H_2O_2

After that the cell is glued together and is put in Aceton and sonicated and after that Isopropanol and sonicated. The cell is then dried and put in the ozone chamber again for 10 min.

SU8 cell

Glasses are cleaned in piranha and DI and dried on the hot plate (115°C) for an hour. The SU8 is then dropped on the glass slide and smeared out with a stick. The SU8 is let to soft bake for 1h. Small pieces of plastic sheets were placed on to the drop to function as spacers. The cover glass is then pressed upon the glass slide and the whole piece is let to cool. Overflow SU8 that has been pressed through the in let and out let is peeled of. An aluminum peace defining the channel is placed over the in and out let and the piece is exposed to UV light. The piece is then developed in acetone, Isopropanol and DI.