TUDaThesis – Abschlussarbeiten im CD der TU Darmstadt

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IMNS

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Bei einer Thesis des Fachbereichs Architektur entspricht die eingereichte elektronische Fassung dem vorgestellten Modell und den vorgelegten Plänen.

Darmstadt, 27. August 2023

L. Widmayer

Acknowledgment

Ich danke euch

Abstract

Diese Kurzfassung ist kurz.

Contents

1 Introduction

1.1 Motivation

Motivation:Biologische Proben sowohl unterm Licht- als auch Elektronenmikroskob anschauen

1.2 State-of-the-art

1.2.1 Methods for getting rid of ice xD

There are four passive anti-frosing strategies: Inhibition of ice nucleation is archieved by using surface inherent properties and heating to prevent ice crystals to form. Retardation of frosting removes water over time to prevent icing on the surface with water repellent properties such as the lotus effect. Mitigation of frost accumulation prevents already formed ice droplets to further accumulate and forming an ice layer. Last a reduction of ice adhesion so ice needs less force to detach of a surface even until ice droplets are not able to attach to a surface, detaching themself with the force of gravity [8].

1.2.2 PDMS application (in z.b. der Flugindustrie)

1.2.3 Assemblies used at cryogenic temperatures

In advance, two specialized assemblies designed before this master thesis are used throughout the master thesis. These tools created for partially different purposes.

First a bath for sample preparation is used. This Bath is filled with liquid nitrogen for cooling. The second floor is elevated of the bath floor, serving as base plate for other smaller assembly and as work station with dents for other tools and easier use for samples. In normal use, the base plate is submerged in liquid nitrogen. Fixed on the base plate and elevated over the liquid nitrogen, small tanks for other liquids are installed, which can be temperature controlled. Then also elevated a harbor for an harbor-shuttle system is installed for a fast and precise transport of samples.

The Shuttles are also used in cryo light microscopes. The Microscope used for cryotemperatures have an additional box installed, routing Cold nitrogen gas underneath a harbor, where the sample is placed. Heaters are placed around the box and under the harbor to archieve a constant temperature. On top of the Harbor, warm Nitrogen is blown so no ice is forming inside the optical path.

Second, an assembly to lift samples at cryogenic temperatures, also called finger, is used. The finger has two main parts: A metal rod with a slightly pointed tip (Fig. 1.1). Near the tip, the rod is also temperature controlled with a temperature sensor and a heater. On the tip, a glue like HFE can be used to glue onto the sample. The second main part is a 3D printed part, containing the outer shell and routing of the cold gaseous nitrogen. The nitrogen is first flowing inside and around the metal bar for cooling. then, the Gas is flowing through an outer mantle for additional cooling and out on top.

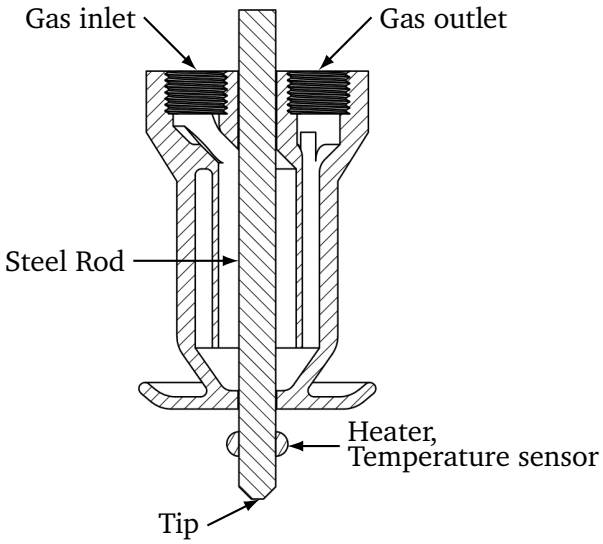


Figure 1.1: Querschnitt finger

The finger is also mounted on three stages and a track. This allows moving in all three axis and moving the finger out of the way with the track. Also the force for lifting is applied through the stages by manually moving the assembly up.

2 Method

Das ist die Methode.

2.1 Task and requirements

In Application, samples (for example cells) are frozen inside a thin ice layer. The sample can be stained with fluorescence and observed with cryo light microscopy. Also a sample can be prepared to study with cryo-transmission electron microscopy (cryo-TEM) . This allows to see samples in an hydrated state. This is only possible in cryo-TEM, as liquid water would evaporate in vaccuum [1].

For sample preparation in cryo light microscopy and cryo-TEM, plunge-freezing is used [1] [4]. This can be done either by hand or with a plunge-freezer, but both methods use the same steps. First, the slide is held by tweezers. then a 2 to 4 ml water drop including the sample is pipetted on the hydrophilic slide. Therefore the droplet spreads over the slide. Then the water droplet is blotted with filter paper, creating a thin film of water which is evaporating quickly. Then the slide is shot in cold liquid under -140°C, for example liquid ethane. A temperature drop of over 100°C freezes the water into a thin ice layer. With this procedure, vitificated ice is formed without a crystal structure.

A vitrificated sample is needed as ice crystals damage the samples and can disturb cryo light microscopy. Vitrificated ice is created by freezing water abruptly into temperatures under -120°C [7]. To create a vitrificated sample, the slide needs a high thermal conductivity to freeze the water quickly. Also the liquid which is used to freeze the sample should not possess the Leidenfrost effect. The vapors which are created with the leidenfrost effect will prevent a rapid temperature drop. As liquid nitrogen has the leidenfrost effect, other liquids like liquid ethane are used.

The motivation for this master thesis is to find a way to use cryo light microscopy and cryo-TEM on the same sample. But currently, no slide is found which has all requirements to be used in plunge-freezing, cryo light microscopy and cryo-TEM. in plunge-freezing, a hydrophile surface is needed to archieve a thin ice layer. Additionally the thermal conductivity of the slide needs to be high for the steep temperature drop needed to create vitificed ice. For light microscopy, a transparent slide is not always required. But a good thermal conductivity is advantageous as less energy is needed to keep the sample cool (WAS FÜR VORRAUSSETZUNGEN GIBT ES DA?). In cryo-TEM, the sample needs to be extremely thin and small. Additionally, only light elements should be used as heavier electrons are disturbing the image in cryo-TEM.

To perform cryo-light microscopy and cryo-TEM, a sample transfer from one slide to another slide is proposed. The slide change must be performed at -140°C to maintain the vitrificated state of the sample. Additionally as the first slide used for plunge freezing is hydrophilic, lifting the sample is not simply possible without designing a new layer. First, I investigated lipids for potential positive characteristics for a sacrificial layer or

detaching it mechanically. then I am trying PDMS and use different mixture ratios and plasma curing to make mechanical removal easier.

2.2 Phospholipids

Phospholipids are the building block of membranes in nature. They own two long hydrophobic chains and a polar head. A membrane is a bilayer of Phospholipids with the hydrophobic parts showing inwards and the hydrophilic head pointing outwards. They are also natural detergents, as they can bind to hydrophobic waste and forming an emulsion, making them removable with polar liquids like water [6].

Phospholipids are generally solvable in Alcohols(???). To apply Phospholipids, the solution is given on the surface. When the solvent dries out, the lipids are bining to the surface creating a layer. this layer can be solved again with the same solvents. If the ice layer is held by lipids, they can be used as a sacrificial layer, being solved at cryogenic temperatures. But to solve this layer, a high solubility at cryogenic temperatures must be given as the surface of the sacrifical layer is only on the edge of the sample.

2.2.1 Parylene

One idea of balancing Hydrophilic and Hydrophobic characteristics is to use Parylene. Parylene is superhydrophobic (SOURCE??? MAYBE NOT), which helps ice not to adhere to the surface. But used alone, an ice layer could not be frozen on top with plung-freesing or by hand as a water drop would not spread on the surface.

For this reason, lipids are used in combination with parylene. The lipids are holding the Ice layer onto the parylene. With a solvent, the lipids can be solved and the parylene will prevent the ice layer to hold on the slide, detaching the layer. Or mechanical pulling on the ice is easier, as parylene is preventing not perfectly covered pieces from adhering on the slide.

2.2.2 Preparation lipids and cover glass

To create the slides with parylene and lipids, cover glasses (5 mm diameter) is first coated with a thin layer of parylene. Then the slides are dipped into lipid solution, covering the whole surface in lipids. then the slides are dried, so lipids can settle on the surface.

Two different lipids are used: DOPC and EGG-PC. DOPC is storaged in powder form. The first step is to solve the DOPC Powder in Ethanol (25 mg / 1 ml). Then, the solution is transferred into several small bottles. EGG-PC is shipped solved in chloroform. Two different ratios were used: 25mg / 1 ml and 10 mg / 1ml. the phioles were broken and then also transferred into several small bottles. small bottles were chosen because if solution is coating the threads of the cap, the bottle cannot be closed airtight anymore, leading to evaporation in the flask. By splitting it into multiple flask and using the solution, only one bottle with a small part of the solution is not airtight.

2.2.3 solubility lipids

Two different solubility experiments are proposed. The first is at room temperature to find solvents which work generally at higher temperatures. With the results, first solution which don't solve the lipids can be left out of the next experiment, as there are only limited baths available. The next one is at cryogenic temperatures to find solvents which also work at cryogenic temperatures.

These tests are conducted to find a fluid to solve a sacrificial layer out of lipids.

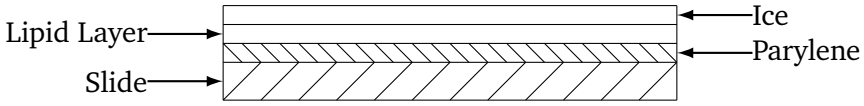


Figure 2.1: Sacrificial layer

at room temperature

First, the potential solvents are picked. For that, the tested liquids needs to be save for humans in such way that no extractor hood is needed. This is needed because the following experiment does not fit under an extractor hood. The tested substances are 4-Methyl Pentene, 3-Methyl Pentene, 1-Pentene, Isopentane, 1-Propanol, Pentane and Ethanol. Each liquid is put in a separate bottle. Then the slides are prepared as previously described.

Then a slide is placed inside each potential solvent. After 15 minutes, the slides are removed and examined under a microscope. then the results are documented in a list. Solvent which managed to solve all lipids off the slide are now tested at cryogenic temperatures.

at cryogenic temperature

As solubility is very temperature dependent, a test is conducted at -140°C. The general process is the same, but the liquids are given in liquid nitrogen cooled baths, which are regulated to the desired temperature.

The Freezing point of tested Solutions are not all below -140°C (Table 2.1). To still test the solubility, they are partially tested at temperatures slightly above their freezing point. Also solution with a high freezing point are mixed with solution with a low freezing point as an attempt to reduce their freezing point.

In the end, the experiment has proven that solving lipids fast and reliable is not possible with tested solvents. As all solvent lipid combinations seem to be endothermic, finding a working solvent lipid combination is very unlikely.

Additionally, some solvents tested are soluble in water. It is unknown whether the solvents could be solved or diffuse inside the ice layer at -140 °C. Therefore the ice layer could be changed in some undesired manner. if a final solvent is found which is soluble in water, this needs to be addressed an tested in future experiments.

solvent	melting point in °C
4-Methyl Pentene	-154
3-Methyl Pentene	-154
1-Pentene	-165
Isopentane	-160
1-Propanol	-126
Pentane	-129
Ethanol	-114

Table 2.1: Melting Point in °C for tested solvents.

2.3 "finger"

The next potential method is mechanically lifting a piece or the whole ice layer off the slide. The challenge is that the sample needs to stay at a vitrified state. The "finger" as previously explained is a device which can lift at a temperature range which guarantees that the sample stays vitrificated. Additionally, a bath previously build was used. Additionally, I constructed a second bath for improves space and flexibility for using samples.

HFE (LANGES WORT) is an oil typically used as an cryoimmersion fluid [3]QUELLE ÜBERPRÜFEN!. Besides that, it has temperature dependent abilities. At freezing temperatures, it does not freeze into a solid at once. It gets more and more viscious before it freezes completely. this temperature dependency is used to first apply the HFE at higher temperatures with low viscosity and pull on the sample at low temperatures.

The process has following steps: First, if not already done, cool down the finger to -140 °C. The sample is prepared and fixed onto a shuttle in the harbor underneath the finger.Then, HFE is applied on the tip. Then, the finger is positioned with the stages over the sample. After checking from different sides an camera, the finger is lowered onto the sample. Then the temperature is reduced and waited until the sample and finger is cooled down. Then the finger is pulled up by turning the stage until it detaches from the sample. After this step, if detaching was successful, the shuttle is changed to an empty shuttle. the Piece is lowered onto it and the piece is unglued by lowering the temperature. then the piece is fixed onto the shuttle.

To collect first insight, samples with parylene and lipids (as described in Section 2.2) are used. by doing those experiments, different variables where determined which could significantly influence of successful detaching. Then the different variables are examined with different experiments to improve the finger. Following variables are determined: the amount of HFE can affect the maximum force and can be falsely applied, the temperature also affects the maximum force, tensile forces and shear forces, the thickness of the ice layer, the layer between slide and ice. In the following, I will examine those in greater detail.

2.3.1 determining needed amount of glue

Using the correct amount of HFE as glue is important for a high repeatability. Too little glue is not able to connect the finger to the sample. too much glue results into a thicker layer, which is the weakest link between finger and sample. additionally, the glue can spread underneath the "window" which is holding down the sample onto the shuttle.

In first experiments, the HFE used as glue was applied with a pincer. TO archieve this, the HFE is given in a cold bath at -140°C. This stops HFE from evaporating. The thickened hfe is now scooped with pincers on the tip of the finger. Though the correct amount is only determinable qualitative.

As an effort to determine the correct amount of HFE a pipette is used. The HFE is pipetted at room temperature, because at lower temperatures the viscosity is already too high. While applying the HFE onto the desired surface, around $4\,\mu l$ is already evaporating. Also, sometimes the hfe does not land on the tip but on the side of the tip, where it is not very useful.

In the first experiment, I dosaged three different amount onto the tip of the finger with the pipette. The amount on the finger did not correspond to the amount of HFE. So other variablilities like the time between loading and unloading and hitting the correct spot on the finger is more relevant than the amount of HFE used.

To still determine the correct glue amount, two pictures representing the lowest and the highest usable HFE amount was picked. then the volume is calculated. this can be used as reference for future work for dosaging the right amount of HFE.

2.3.2 temperature test

Ethoxynonafluorobutane, also called HFE 7200, has a melting point of -138 °C. Below, HFE gets increasingly viscious until it is hard and brittle. This property can be used as a temperature controlled glue. To attach/detach a piece, a temperature at around -138 °C is used so the HFE is nearly liquid. At lower temperatures, HFE hardens and glues the finger onto the piece. First, a temperature of -160 °C is used to glue and attach to the sample. But the temperature regulation is not always able to set the temperature and even lower temperatures are maybe beneficial.

HFE

2.3.3 tensile- vs shear mode

2.3.4 ice thickness

2.4 PDMS

PDMS has generally good properties for ice removal. It is very hydrophobic, so Ice can hardly adhere to the surface.

I used Dowsil Sylgard 184 Silicone elastomer as PDMS [2]. It has two component, Base coat and curing agent. Depending on the Mixture ratio of these two components, the PDMS gets different properties.

In one paper

Additionally,as PDMS is hydrophobic, plasmatreatment is used to make the surface more hydrophilic. Additionally, plasma treatment is changing the structure of the PDMS.

I found that plasma treatment has different effects on the PDMS on different mixture ratios.

Why PDMS was chosen

2.4.1 Preparation of PDMS samples

All PDMS samples with different mixtures are prepared in the same way. The preparation starts with weighting out the needed amount of base coat and curing agent. The mixture is now stirred intensively. Then the mixture is placed in a vacuum bell for 30 minutes to gas out air bubbles. Meanwhile the cover glasses used as slides are cleaned with ethanol or isopropanol. Afterwards, the DPMS mixture is coat-spinned on the cover glass for 5 seconds with 300 rpm and then 120 seconds with 3000 rpm. Then the coated cover glasses are baked in the oven for 30 minutes exept a mixture of 1 base coat and 2 curing agent mixing ratio PDMS. For those 24 hours are needed, as it takes longer to harden and it will result in unwanted effects at plasma curing.

2.4.2 Influence of plasma treatment on PDMS

setup

2.4.3 detaching ice from PDMS

1:2 and 4:1

3 Results

3.1 Lipids

The solubility of lipids at room temperature are tested. For this experiment the cover glasses are coated with lipids. Then a first before picture was taken. Then the cover glass is given into a small container with the potential solvent. In 15 minutes, the cover glass is removed and compared under the microscope with the picture taken before. If streaks created from lipids are still as visible as before, the lipids are categorized as insoluble in this solution. If the streaks partially dissapeared and/or are less visible, the lipids are categorized as partially soluble in this solution. Last if the streaks completely disappear, the lipids are assinged as soluble in the solution (Table 3.1).

potential solvent	solubility EGG-PC	solubility DOPC
4-Methyl Pentene	complete	N/A
3-Methyl Pentene	partial	no
1-Pentene	no	no
Isopentane	no	partial
1-Propanol	complete	complete
Pentane	complete	no
Ethanol	N/A	complete

Table 3.1: list of tested potential solvent and result of solubility tests at room temperature. complete indicates solvents which are able to visibly solve all lipids off a cover glass. Partial solubility indicates solutions which are able to solve lipids, but some stains are left: No solubility indicates no visible changes of tested lipid.

This experiment shows that 3 different solvent exist for each EGG-PC as well as DOPC with high solubility (Table 3.1). Following those results, solvents categorized with "complete" are tested regarding solubility at temperatures of -140°C. As not all solutions are liquid at -140°C (Table 2.1), they are tested at higher temperatures in which they are still liquid, as mentioned in chapter 2.2.3. Also they are tested as mixtures with other solvents with a lower melting point, in hope the resulting mixture has a lower melting point. Additionally all lipids are tested in liquid ethane.

This experiment shows that no tested solvent was able to completely solve lipids at -140°C and a short time frame (Table 3.2). Also a redistribution of lipids was visible in all experiments.

Using solvents to destroy a sacrificial layer, a high solubility is a requirement. In this case, the sacrificial layer would be completely covered by the ice layer but the edges. So the sovents have only a small area to start solving the layer. To solve it completely, a strong solvent is needed to detach the ice layer from the slide. Additionally, as the ice layer needs to stay vitrified, the temperature cannot be raised over -140°C.

Additionally, The solving process of lipids in solutions is probably endothermic. This means that heat is needed to solve lipids, so cold temperature heavily decrease solubility. This effect was observed over the last experiments by all solvents to varying degree. It can be assumed that a majority of solvent lipids mixtures are endothermic which is very disadvantageous for finding a potential solvent lipid candidate. Also strongly exothermic solvents could heat up the ice enough to create ice crystals, which would also not be feasible. So only weakly exothermic solvents are feasible for this task (REFERENZ ZU EXOTHERMIC/ENDOTHERMIC Solvents?).

Solvent	Result
Pentane	Lipids solved 3h at -125°C
4-methyl pentene	insoluble
1:1 volume ratio HFE to 1-Propanol	did not mix, 1-Propanol froze, insufficient solubility
Liquid ethane	insoluble

(a) EGG-PC

Solvent	Result
1:4 volume ratio 1:2 molar ratio Ethanol to Isopentane	insufficient solubility
1:2 volume ratio 1:1 molar ratio 1-Propanol to Isopentane	insoluble
Isopentane	insufficient solubility
1-Propanol	at -130°C insufficient solubility
Liquid ethane	insoluble

(b) DOPC

Table 3.2: in 3.2a for EGG-PC, no sufficient solubility at -140°C was found. In 3.2a, DOPC was tested but also no proper solution was found.

As finding a good solvent lipid combinations seems very unlikely, a new method was tested. In the next section, the finger tool is used to try mechanically detach the ice layer.

3.2 Finger

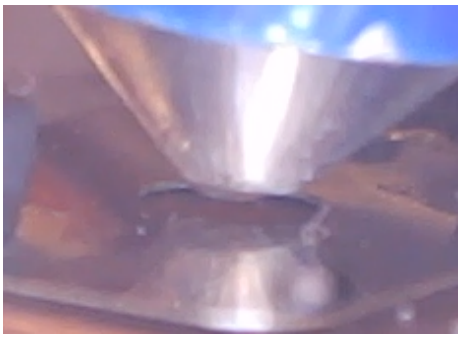
For this section, cover glass coated in Parylene are used as object slide. The slide is then dipped in solution containing lipids for a lipid coating. A ice layer with fluoriscine is frozen with either plunge-freezing or using a pincer and liquid nitrogen. Additionally, the "finger" is used as tool to try lifting off a piece of ice from the frozen layer on top of the lipids. In the next sections, different variables are examined and tested.

3.2.1 Finding right dosage of HFE

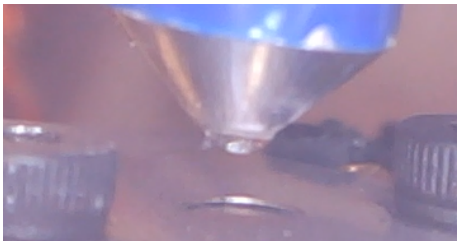
First obvious variable and potential issue source is the amount of HFE used as glue. High dosages of liquid hfe can spread underneath the frame holding the sample, leading to an inefficient force distribution. Also a big glue layer is a weak point between finger and sample, leading to a reduction of maximum force which can be applied. Too little glue will not connect the finger to the sample. Additionally, the dosaging of glue revealed to be a big challenge.

The HFE is dosaged with a pipette. The HFE is "taken up WORD" at room temperature, then the HFE is "released WORD" on the tip of the finger. In between, HFE is evaporating. Around $4\,\mu l$ is evaporating each time. Based on this knowledge, dosaging $4.10\,\mu l$, $4.30\,\mu l$ and $4.50\,\mu l$ is compared and a picture is made.

Results show that pipetting HFE is not reliable. The range spreads of too little to too much HFE even for those dosages. Not only differences in evaporation are playing a role. Correct placement on the tip is a major factor of glue dosaging. Still, a visual estimate for the correct glue dosage can be made by calculating the drop volume out of camera images.



(a) chosen example for lower limit



(b) chosen example upper limit

Figure 3.1: example of upper limit and lower limit for glue dosages. (BETTER PICTURES NEEDED)

To calculate the actual glue dosage, two exemplary Pictures of an Upper and lower limit of glue dosages is picked. Then the Volume is calculated with a formula for the volume of a spherical section. All needed components are calculated out of the estimated contact angle of the glue $\alpha \approx 45$ and the tip diameter of $d = 1.68\,mm$, for the lower range a reduction of d by a factor of $\frac{2}{3}$ is assumed as the drop is not covering the whole tip. The resulting volume range of the glue dosage is $0.11\,\mu l \lessapprox V \lessapprox 0.38\,\mu l$. Also the lower end of this range is desired, but the repetition range in correctly dosaging lower doses is lower.

3.2.2 Temperature over applied force

As the HFE gluing effect is temperature dependent, the temperature needs to be regulated precisely. To narrow in the temperature dependency of HFE, the properties of HFE are observed at different temperatures in a regulated bath. Between -160°C and -170°C, the HFE is increasingly viscious. Under this temperature, HFE Freezes and gets brittle. Over this temperature range, HFE is too fluid to transfer any tensile forces.

In application tests on lipid samples, the temperatures -160°C, -165°C and -170°C are compared. It was observed that decreasing temperatures lead to higher forces transferred to the sample. At the same time, temperatures are not reliably reached under -160°C. As lower temperatures leads to an additional factor for repeatability issues, -160°C is used all other experiments.

Category	Hand-freezed	Plunge-freezed
count executed tries	4	4
unsuccessful	3	3
breaks/movement of ice	1	1
piece lifted with finger	0	0

Table 3.3: Comparison of detachability between hand-freezed and plunge-freezed samples

3.2.3 Tensile mode vs Shear mode

3.2.4 Detaching ice with finger of plunge freezed samples

Next observed possible factor is the thickness of the ice layer. In the following, samples freezed with a plunge-freezer are compared to samples freezed with a pincer in liquid nitrogen. The results are categorized in 4 categories: Not successful pulls don't have visible changes of the flourescent ice layer, Partially successes are visible breaks or clear movement of ice parts on the ice layer, Successful liftoff is a missing piece and a visible piece on the finger, which could be used for future steps. In the results, there is no difference between Hand freezed and plunge-freezed samples regarding detachability. Therefore Ice thickness is not a factor which makes detaching ice easier. As both methods don't show success in detaching ice pieces, it could still be a relevant factor but not a thing which should make a certain solution magically work xD

3.2.5 other observed error sources??

Wrong positioning, forming of ice

3.3 PDMS

Now two mixture ratios of PDMS are compared. For this, samples where coated with 4:1 and 1:2 curing agent to base coat weight ratio. Also for ..., glass without pdms is used. The pulling mashine was used to determine the max force. A plexiglass stamp was used for pulling off the PDMS layer. UV Glue was used to fixate the plexiglass onto the PDMS and is cured with 3 min UV exposure. After pulling, the Area of the separating layers is determined via microscope. Then the max pulling tension is calculated. This was repeated several times. The Result shows, that glass is hardest do pull off. Then 4:1 is harder to separate than 1:2 (Fig. 3.2). In literature, 1:2 weight ratio should have a lower adhesion force on ice too[5]. For those reasons, 1:2 was picked to continue experiments with plasma treatment.

In the next experiment, the effect of plasma curing is investigated. The same setup is used. Samples with a 2:1 weight ratio are additionally plasma treated before quickly clamping on the pulling mashine. Even with low repetition rates, a clear tendency can be observed. With lower and stronger plasma treatment, the durable the PDMS Layer gets (Fig. 3.4). Over the whole range, The needed stress sextubles. Because the repititon rate is low, the exact values should be treated cautiosly. Also the results are not applicable to other mixture ratios, as different behaviour in plasma activation was observed between 2:1 and 4:1 weight ratio. also no glass-like state was observed in 2:1 weight ratio mixture.

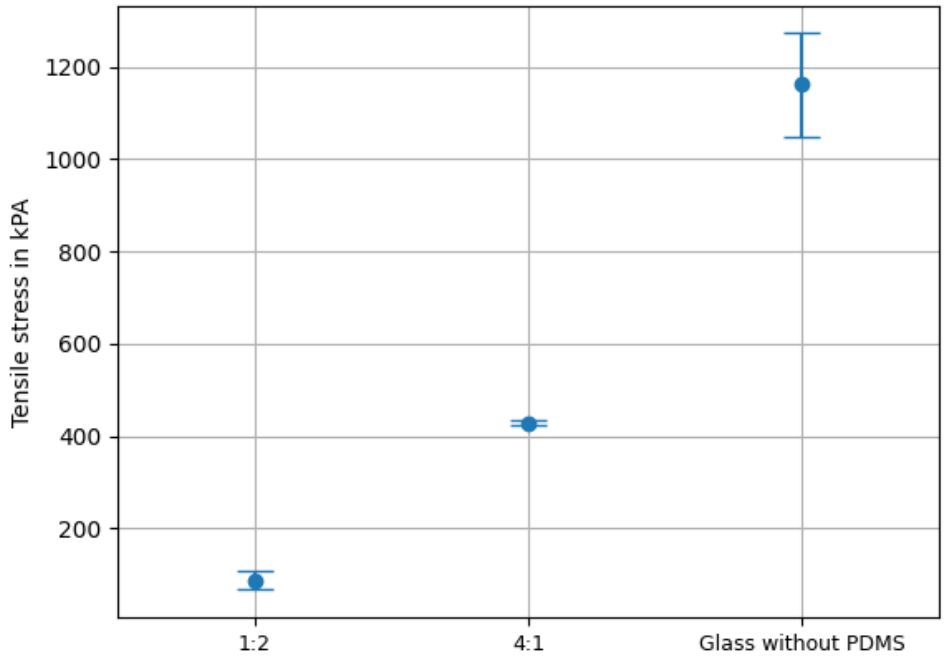


Figure 3.2: Comparison 4:1, 1:2 Base coat to curing Agent and glass without PDMS

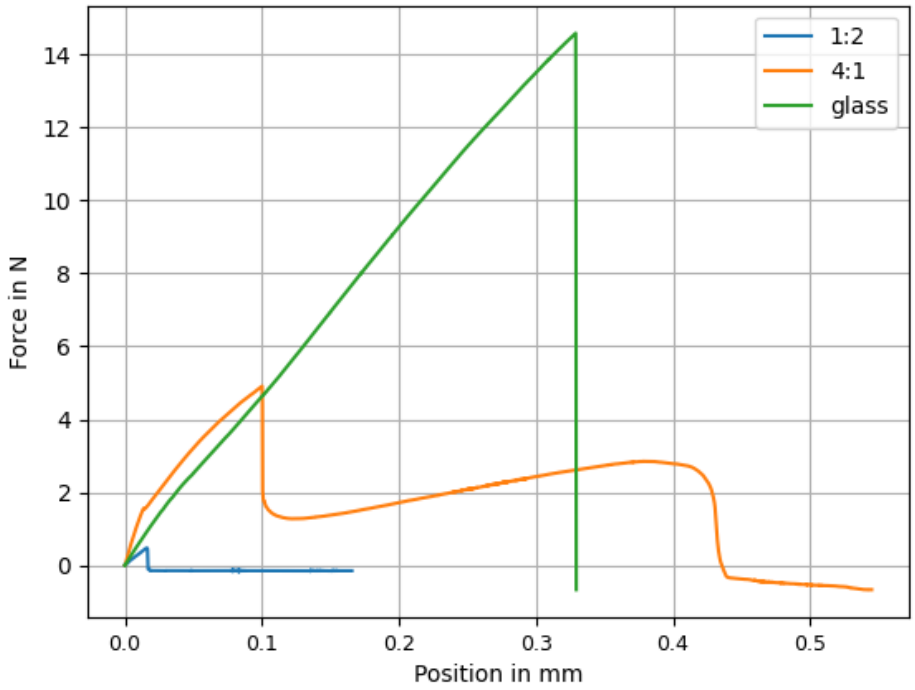


Figure 3.3: force over Time

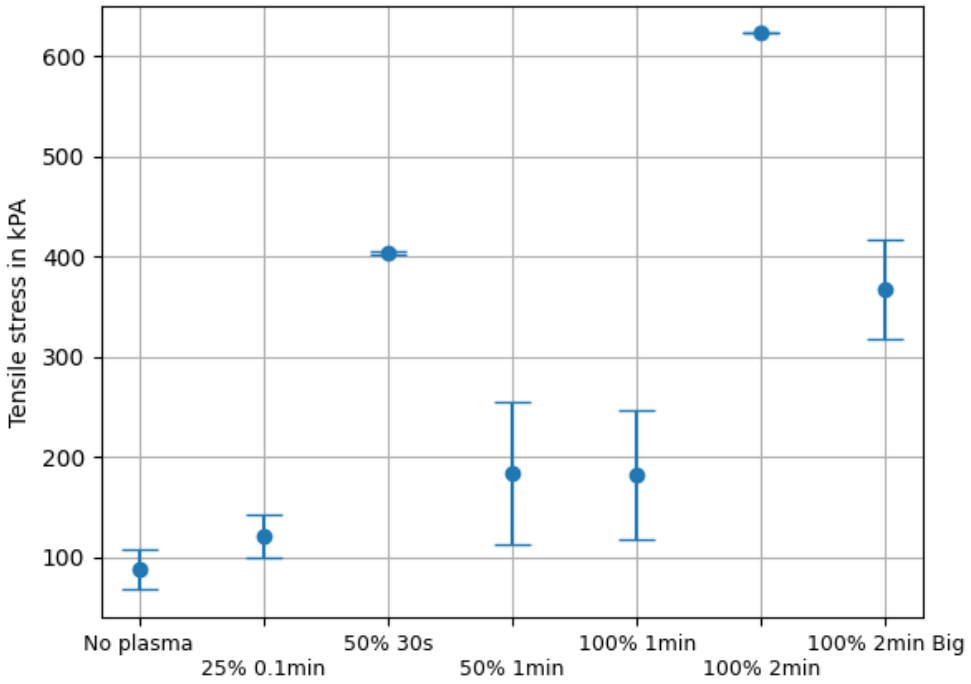


Figure 3.4: PDMS 2:1 Comparison between various Plasma curing strengths and durations.

4 Conclusion

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4.1 Lipide

4.2 Finger

4.3 PDMS

4.4 Ausblick

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