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Multifunctional microfluidic tools for surface characterization within single cell analysis

Bachelor of Science Thesis in Chemical Engineering

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Abstract

A challenge with single cell analysis is that it can require isolation and removal of target cell groups. A novel material has been discovered that could possibly resolve this problem. Through modification of thin Teflon AF surface using e-beam lithography, patterned areas can be created facilitating cell adhesion. The adhesion can further be tuned by the solution in which the substrate is immersed in however the origin of this process is still unknown. The aim of this thesis was to, through the use of multifunction microfluidic pipette, see if it is possible to establish an on demand cell detachment procedure using different chelating agents and to monitor change in the electrical properties of Teflon AF using electrical impendence spectroscopy. HEK 293 cells were used in this thesis which was found to have an optimal adherence at around 18-24h, at which 70-90% of the cells adhered to the circular patterns. A problem that was often observed was the adhesion on unexposed Teflon AF areas for which the reason is still unknown. Through experimentation with microfluidic pipette, the use of EDTA, EGTA and BAPTA was shown to have no or irrelevant effect on the cell detachment from the substrate surface. For experiment with electrical impedance spectroscopy a change in the electrical potential was observed stating that the impedance for exposed Teflon AF is higher than unexposed Teflon AF.

The hypothesis that removing Ca^{2+} using chelating agents would detach cells from the patterned Teflon AF surface was found contradicted through a series of experimentation using the microfluidic pipette and through the use of electrical impedance spectroscopy a change in the electrical potential was found between unexposed and exposed Teflon AF.

TABLE OF CONTENT

1. Introduction 1
2. Theory 2
2.1. Cell adhesion 2
2.2. Chelating agent 2
2.2.1. EDTA, EGTA and BAPTA 3
2.3. Teflon AF 4
2.4. E-beam lithography 4
2.5. Fluorescence microscopy 5
2.5.1. Fluorescence 5
2.5.2. Instrumental setup 5
2.6. Electrical impedance spectroscopy 5
2.7 Microfluidic pipette 6
3. Method 8
3.1. Teflon surface 8
3.2. Solution preparation 8
3.3. Cell culturing and cell plating 9
3.4. Cell detachment 10
3.5. Surface cleaning 10
3.6. Electrical impedance spectroscopy 11
4. Results and discussion 12
4.1 Cell adhesion on Teflon AF surfaces 12
4.2. Cell detachment 13
4.3. Electrical impedance spectroscopy 14
5. Conclusion 18
6. Future work 18
6. Acknowledgement 19
References 20
Appendix 22
Appendix 1 Difference in cell adhesion between primary and secondary used Teflon AF surfaces 22
Appendix 2 Electrical impedance spectroscopy, moving across a single pattern. Change in magnitude over time observed in different frequencies. 23
Appendix 3 Electrical impedance spectroscopy, moving across three patterns. Change in magnitude over time observed in different frequencies. 26

1. INTRODUCTION

A recent challenge within the field of biomedical engineering has been to create a surface that not only mimics certain biological features, but also facilitates adhesion and interfacing of biological structures.[1] Many types of surfaces and structures have been studied and tested for the purpose of single cell analysis. The difficulties faced when analyzing single cells are that it can require isolation and removal of target cell groups which has directed attention to the use of polymer based surfaces and especially amorphous fluoroplastic polymers such as Teflon AF that could offer a solution for this problem. A recent study by Ilja, Czolkos et al. successfully fabricated a small-scale patterned Teflon AF surfaces, able to mimic certain desirable biological features.[1] One of the key features of Teflon AF is its super hydrophobicity which prevents cells from adhering to the surface. By using different patterning methods the super hydrophobicity of Teflon AF can be utilized for controlled cell adhesion allowing cells to be anchored to specific areas of the surface. This project uses e-beam lithography as patterning method for Teflon AF which has been found to improve the anchoring of cells to the substrate surface by making it more hydrophilic.

This anchoring can be further modified by changing the solution in which the substrate is immersed, however the origin of this process is still unknown. Studies have shown that the presence of Ca^{2+}, in some cases, aids the cell-cell and cell-matrix adhesion. Not only does it affect the movement, shape and structure of the cell it is also involved in the intermediate signaling between a few known receptors. It has been speculated that the cell adhesion on Teflon AF is facilitated by calcium, hence the hypothesis is that the removal of Ca^{2+} will allow cells to detach from the surface on demand. To facilitate this, metal binding complexes known as chelating agents will be used. The three chelating agents used in this project are EDTA, EGTA and BAPTA due to their specific affinity towards Ca^{2+}.

Patterning of Teflon AF using e-beam lithography is a recently developed method hence there are several properties of this surface which are still unknown. In this project electrical impedance spectroscopy is used to detect any variances in electrical response between unexposed and exposed Teflon AF.

The aim of this project is to, through the use of microfluidic pipette and electrical impedance spectroscopy, monitor variances on the patterned Teflon AF surface. Microfluidic pipette coupled with fluorescence microscopy will be used to optically observe the cell detachment mechanism when exposed to different ionic concentration of chelating agents. The use of electrical impedance spectroscopy (EIS) will allow us to monitor variances between patterned and regular Teflon AF. The goal of this project is not only to understand the tunable adhesion of the substrate surface but also to outline its major means of control.

2. THEORY

This chapter is aimed to give a theoretical understanding on the main concepts of this project as well as introducing methods and materials used.

2.1. CELL ADHESION

Cell adhesion plays an important role in the assembly of individual eukaryotic cells to form a three dimensional structure through precise, organized and distinctive patterns.[2] It allows for communication between cells which is essential for different functionalities such as growth, replication, differentiation, migration, etc.[2-5] Through the use of outer membrane glycoproteins and carbohydrates known as cell adhesion molecules (CAMs), cells are able to interact with each other, structures and extracellular matrix (ECM). These CAMs are classified into 4 major families, immunoglobulin superfamily (IgSF), integrins, cadherins and selectins.[6-8] The importance of CAMs for the maintenance and performance of cells does also to some extent indicate that any type of abnormalities in them would likely be able to cause degenerative, pathological or other types of diseases.[7, 9, 10] Muscular dystrophies is a known degenerative diseases caused by defect in cell-cell adhesion where dystrophin, a binding protein becomes dysfunctional resulting in deterioration of the muscle tissue.[2]

Studies has shown that CAMs are further divided depending in their anchoring ability in the presence of calcium ions, labeling them either calcium dependent or calcium independent. Cadherin, integrin and selectin are categorized as calcium dependent while IgSF is classified as calcium independent.[8, 11] The presence of calcium aids the adhesion in creating an intermediate connection between cell-cell and cell-matrix. It is also known to be involved in signaling between cells through different protein receptors.

The 4 families can also be classified depending on their different anchoring ability to either cells or matrix, classifying them as homotypic and/or heterotypic. The former refers to adhesion between identical CAMs on other cells while the latter refers to the adhesion between CAMs and an external matrix.[7, 8] Cadherins and certain IgSF are both known to mediate homotypic adhesion which allows for interaction and signaling between cells. Integrin on the other hand is mainly involved in the cell-matrix interaction which plays a central role in the spatial localization of cells. It also works as an informational passage having direct impact on the cell survival, migration and expression of different phenotypes.[9, 10] As previously mentioned, the presence of calcium ions also aids the adhesion between cells or ECMs which could mean that the regulation of calcium ion might implicate the cell detachment.

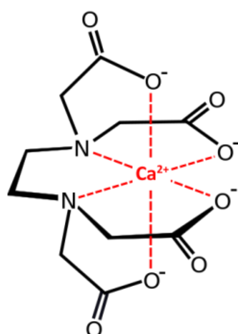


FIGURE 1. EDTA-CALCIUM BONDING

2.2. CHELATING AGENT

Originating from the Greek word "chela" which means the great claw of the lobster, chelating agents are metal binding ligands which binds using two or more electron donating atoms.[12, 13] The bonds formed between the metal ion and each donor atom creates a ring like structure bending the ligand towards the metal ion in a claw type formation (see figure 1). Two requirements needs to be satisfied to form a metal chelate: a suitable functional group and for that

functional group to be able to form a cyclic structure when binding.[14] Although other factors also contribute to the chelation ability these two are the most common.

The stability of the chelator depends on several factors but the more important ones are pK_a of the ligand molecules, substituent, how the donor atom behaves and the metal ion.[14] These factors also determine the affinity of the chelator to the metal ion.

Chelating agents are utilized for metal ion manipulation through reduction of undesirable effect or augmentation of desirable effect. They are used for different applications such as cancer therapy, detoxification, agriculture etc.[12, 13]

2.2.1. EDTA, EGTA AND BAPTA

EDTA, EGTA and BAPTA are three chelators often used in biochemical cell analysis due to their affinity to Ca^{2+} and Mg^{2+}. Their difference in affinity to $Ca^{2+}$is in decreasing order, BAPTA, EGTA and EDTA. While the former two are specified for Ca^{2+} the latter has a more or less similar affinity to Ca^{2+} and Mg^{2+}. Compared to EGTA and EDTA, BAPTA is less affected by pH changes making the uptake and release of Ca^{2+} much quicker. [15] Also the ability to use it at a greater pH range makes it more favorable in some cases. The structures of the different chelators are presented in figure 2, below.

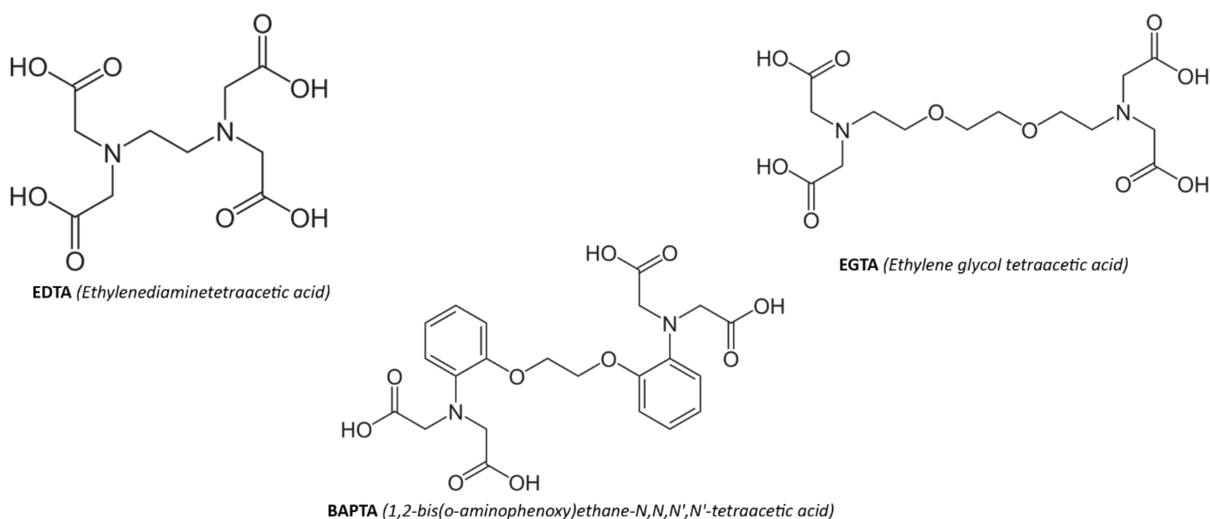


FIGURE 2. CHEMICAL STRUCTURE OF EDTA, EGTA AND BAPTA

2.3. TEFLON AF

Teflon AF is an amorphous fluoroplastic polymer copolymerized of polytetrafluoroethylene (PTFE) and 2,2-bis(triflouromethyl)-4,5-difluoro-1,3-dioxole (shown in figure 3).

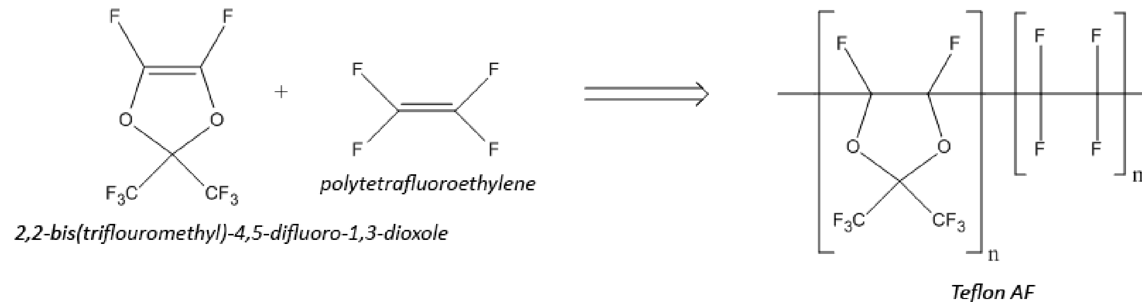


FIGURE 3. TEFLON AF

The material is of interest due to its wide range of properties such as super hydrophobicity, dielectric properties, optical clarity, chemical resistance, mechanical properties and its wide range of thermal stability.[1, 16-19] The super hydrophobicity of Teflon AF makes it suitable for spatial control of cells which allows, on ECM, them to adhere on positions not covered with regular Teflon AF. Anchoring and spatial control of cells to the substrate surface is enhanced using different patterning methods such as photolithography, microcontact printing and microfluidic patterning. E-beam lithography is another type of patterning technique, were the surface is beamed with highly concentrated and precise stream of electrons. Areas that have been exposed to the electron beam becomes more hydrophilic facilitating cell adhesion. The thoughts on how the surface structure appears when it becomes radiated is still unknown.

2.4. E-BEAM LITHOGRAPHY

Modification of surfaces through small-scale patterning can be used to facilitate cell adhesion. One of the most common patterning method is photolithography. This particular method uses light to expose a photosensitive resist creating a template that can be used to obtain a desirable pattern. E-beam lithography in contrast is a new and more costly method but the patterning resolution is higher due to the shorter electron wavelength. It also patterns directly on to the substrate eliminating preprocess chemical treatment which most methods include. The major components in the instrumental setup consist of an electro gun, ion optics and substrate surface. The electron gun is first heated to produce free electrons on the surface were after high voltage is applied, accelerating the electrons towards the substrate. The electron beam passes through the optics which consists of several steps that modulates the beam to have a specific size, current and placement on the surface. The surface is covered with a resist where upon the electrons either induces the deposition of substance to make the pattern rise (addition) or etch away the surfaces to make the pattern sink (subtraction).

2.5. FLUORESCENCE MICROSCOPY

Fluorescence microscopy is an optical microscope that uses the basic concept of fluorescence to generate an image. For the specimen to be observed it can either be auto-fluorescent or it can be stained by fluorescent molecules.

2.5.1. FLUORESCENCE

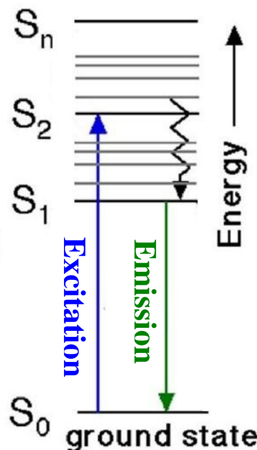


FIGURE 4. JABLONSKI DIAGRAM

The molecular phenomena where a substance emits light after being exposed to another light source is called fluorescence. A Jablonski diagram, figure 4, illustrates the process of fluorescence where a light source is used to excite electrons within a molecule to a higher energy state. Electrons will stay at the exited state for a finite amount of time after which they will start to lose energy through vibration. Finally they will return to ground state through release of energy in the form of fluorescence light (emission). Since some of the energy is lost through vibration, absorbed energy will be higher than emitted. This difference can be observed as a colour change between absorbed and emitted light (if the light used is within the visible spectra). It should also be noted that not all excited molecules return to ground state by fluorescence, other processes might also occur such as collision quenching, fluorescence energy transfer and intersystem crossing.[20]

2.5.2. INSTRUMENTAL SETUP

The setup of a fluorescence spectroscopy is such that the dyed or auto-fluorescent sample is illuminated by a light source with a specific wavelength according to the sample. The light is first directed by a dichromatic mirror which allows photons longer than a certain wavelength through while shorter wavelengths are reflected. Since the light source emits a photon with a short wavelength it is reflected on to the sample. The reflected light goes through the objective and excites the sample; from it a longer wavelength is emitted when the substance molecule relaxes to ground state which passes through the objective and the dichromatic mirror. The emitted light is collected and is then visually observed. Due to the shift in wavelength the light emitted from the main source and the light reflected from the sample usually has different colors.

2.6. ELECTRICAL IMPEDANCE SPECTROSCOPY

Impedance is a term used to analyze the electrical response of a material of interest which would give important information on the physiochemical properties of the system.[21, 22] Electrical impedance spectroscopy has become an area of interest for many biomedical engineers because of its applicability to detect electrical properties of structures, biological specimen and reactions. It is known to be a non-invasive, label-free, real-time method which allows biological entities to be analyzed without any significant disturbance.[21-24] The impedance technology can also be applied to smaller devices making it compatible for research on miniature level.[22] The system where impedance spectroscopy is used for measuring the electrical potential difference of a cell can be compared with a basic model of an

electrical circuit shown in figure 5. The membrane of the cell works as both a capacitor and a resistor. The fluid outside and the cytoplasm inside the cell works as resistors. Two different electrodes are used, the first one stimulates the cell by sending electrical signals while the second collects and records the change in impedance. However it should be noted that understanding the electrical properties of cells is much more complex.

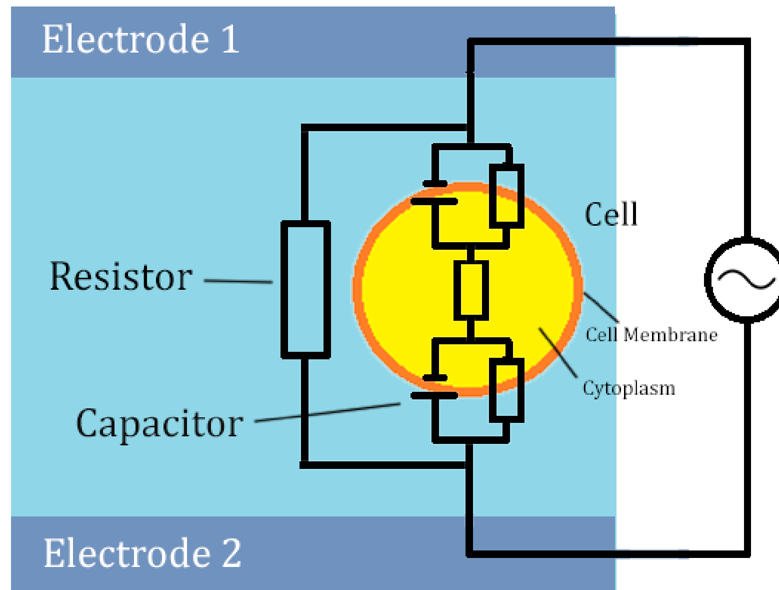


FIGURE 5. SCHEMATIC SHOWING CELL BARRIER ELECTRIC EQUIVALENT CIRUIT MODEL

2.7 Microfluidic pipette

Microfluidic pipette is a recently developed device for the use of fluidically probing and manipulating single cells.[25] With this device it is possible to deliver liquid into an open volume while at the same time recirculating the liquid back into the device. This recirculation is achieved by accurately placed channels under negative and positive pressure, creating a hydrodynamically confined flow volume.[26] The shape of the pipette has been modified into its sharp elongated shape to facilitate the application with other devices.[26] Using the knowledge of recirculation and the shape of the pipette, the device can be positioned to analyze and stimulate single cells without affecting the surrounding liquid.[25, 26] The basic structure of the pipette tip is illustrated in figure 6.

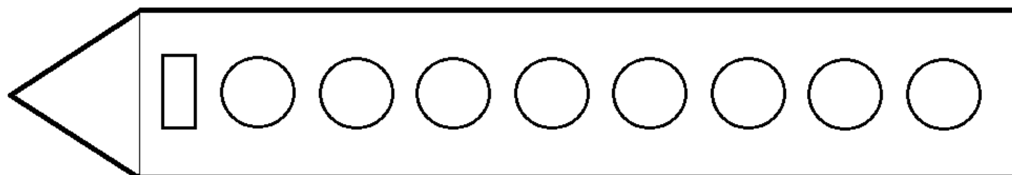


FIGURE 6. SIMPLE STRUCTURE OF THE MICROFLUIDIC TIP WHICH CAN HOLD UP TO 4 DIFFERENT SLUTIONS AT THE SAME TIME.

The tip is fabricated using a soft lithographic moulding technique. The master is produced using a silicon wafer and SU8, a photo patternable polymer. Once developed this master can be readily used to replicate many times. It has 8 on-chip wells, the 4 at the front are used for solutions and the 4 at the end are used for recirculated waste.[25, 26] The pipette is manually inserted into the holder which isolates and connects each individual well to a pressure and valve actuation controller.[26] The positive and negative pressure of the device can actively be controlled through a software. Using the software, pressure and recirculation of the device can then be manipulated to control the effective area the solution exposes. The use of fluorescence microscopy and a fluorescing agent allows the effective area to be observed as shown in figure 7. Further information on the fabrication method and instrumental setup of the microfluidic tip and holder, can be found previously in literature.[25, 26]

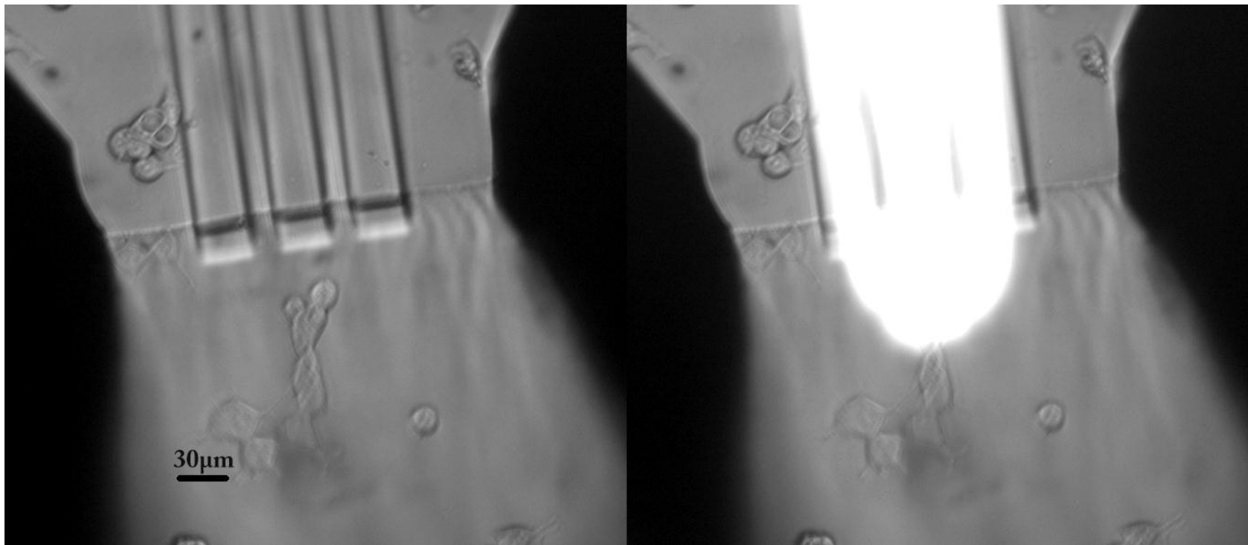


FIGURE 7. EFFECTIVE EXPOSURE AREA VISUALIZED USING FLUORESCING AGENT (FLUORESCEIN)

3. METHOD

3.1. TEFLON SURFACE

The surface was prepared by first coating a layer of indium tin oxide (ITO) onto a glass bottom and then layer of Teflon AF (by DuPont®) on top. E-beam lithography was then used to pattern the surface. The varied types of structures with and without alignments are shown below in figure 8.

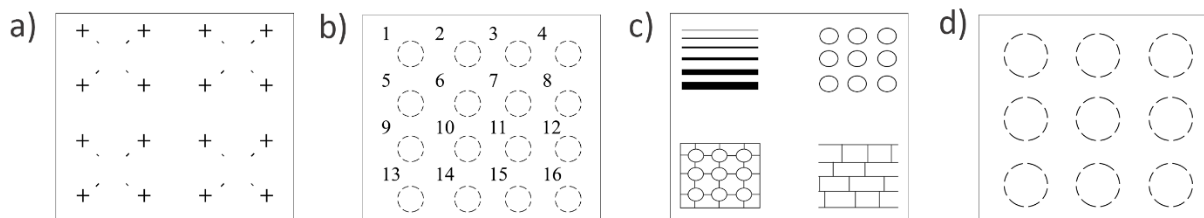


FIGURE 8. DIFFERENT PATTERNED TEFLON AF STRUCTURES WITH AND WITHOUT GOLD ALIGNMNETS.

Structures a), b) and c) have visible gold alignment to mark the patterns position while structure d) has no visible alignments. Also, the structures on a), b) and d) have similar circular patterns while c) has a mixture of 4 different patterns. Silicon oxide as an additional coating layer on the glass was also experimented on, to observe any difference in cell adhesion. A lower e-beam intensity was also tested for printing on the glass.

3.2. SOLUTION PREPARATION

For the experiment 4 different stock solutions, calcium free extracellular buffer and 3 chelators, were prepared as described below in table 1 and 2.

Table 1. *Calcium free Extracellular Buffer (10x, 500ml, pH 7,4)*

Compound	MW (g/mol)	Concentration (mM)	Weight (g)
NaCl	58,44	1400	40,9
KCl	74,56	50	1,9
$MgCl_2$	203,00	10	1,0
HEPES	238,30	100	11,9
D-Glucose	180,00	100	9,0
Total ion concentration		1660	

A beaker was filled with 300mL of Milli-Q water in which all the weighed components were added. The solution was vigorously stirred until all components were dissolved, after which the pH was adjusted to 7,4 using Jenway 3510 pH meter and 2mM NaOH. When pH adjustment was made remaining Milli-Q was added to the 500mL mark.

Table 2. Chelators 2x, 100mL (except for BAPTA, tetrapotassium salt)

Compound	MW (g/mol)	Weight (g)	
		5mM	10mM
EDTA	292,25	0,289	0,585
EGTA	380,35	0,380	0,760
BAPTA, tetrasodium salt	564,37	0,564	
BAPTA, tetrapotassium salt	628,80		0,226

All the chelators except for BAPTA tetrapotassium salt were weighed and put into separate volumetric flasks with 60mL Milli-Q water. Both the EGTA and EDTA solutions were adjusted to pH 7,4, since they are highly insoluble at pH below 6. Neither of the BAPTA salts needed any pH adjustments. After the solutions were mixed, they were adjusted to 100mL with Milli-Q water. A smaller amount, 20mL, of BAPTA tetrapotassium salt was prepared instead.

For the cell detachment experiment with microfluidic pipette, the following solutions were prepared from the stock solutions (Table 3).

***Table3.** Preparation of 10mL ECB and different concentration of chelators*

1x ECB + 1x,5mM EDTA
1x ECB + 1x,5mM EGTA
1x ECB + 1x,5mM BAPTA tetrasodium salt
1x ECB + 1x,10mM EDTA
1x ECB + 1x,10mM EGTA
1x ECB + 1x,10mM BAPTA tetrapotassium salt

1mL of ECB was mixed together with 5mL of 5mM EDTA in a plastic centrifuge tube where after 4mL Milli-Q water was added and further mixed. The same procedure was performed with all the other chelators.

3.3. Cell culturing and cell plating

HEK 293 cells were prepared and plated for the experiment. The growth flask was removed from the incubator and the old medium was exchanged with 1mL of accutase. After 2min, 2mL of new cell growth medium containing 10% HI-FBS (heat inactivated fetal bovine serum), MEM (modified eagle media) and anti-anti (antibiotic-antimycotic) was added. The cells on the surface were forcefully detached using a pipette, releasing the cells into the solution. This slurry was transferred into a centrifuge tube. Two separate cell culturing flasks were prepared with 5mL cell growth medium in each. 100µL and 250µL respectively of the cell slurry was transferred to each separate flask and then placed into the incubator.

The remaining cell slurry was used to plate the surface. 3mL of cell medium was first added to the surface. Once added, 150µL of cells was transferred to it. The surface was then put into the incubator overnight.

3.4. Cell detachment

After approximately 18-24h of incubation, cells were removed from the incubator. The cell growth medium was removed and cells were rinsed 2 times with 1x ECB. The petri dish was then filled with 4mL of the same buffer and placed under the microscope. The microfluidic tip was then loaded with 30µL of 1x ECB, 1x ECB+10mM EDTA, 1xECB+10mM EGTA and 1mM fluorescein. It was placed into the holder and purged for use. After purging and positioning the pipette into the petri dish, the pressure and recirculation values were set to as presented in table 4.

Table 4. *Pressure and recirculation values for microfluidic pipette*

P1(Off)	**P2(On)**
21mbar	170-190mbar
V1(Switch)	**V2(Recirculation)**
-110mbar	-70-90mbar

Visualization of the cell detachment experiment was performed with a Leica DM IRE2 confocal microscope equipped with a 10x Leica objective. The microfluidic pipette was positioned to target a single or a group of cells. Using Thorlabs CMOS camera and uc480 Viewer software, images were taken in the following order:

1. Unexposed
2. Exposed to fluorescein (to observe the exposure area)
3. 1min exposure to 1x ECB
4. 30sek exposure to 1x ECB+1x, 10mM EDTA
5. 1min exposure to 1x ECB+1x, 10mM EDTA
6. 2min exposure to 1x ECB+1x, 10mM EDTA
7. 5min exposure to 1x ECB+1x, 10mM EDTA

The same procedure was performed on different single or group of cells switching between EDTA, BAPTA and EGTA and their concentrations. Cells were exposed for a longer time if any prominent changes were observed (10+ minutes).

3.5. Surface cleaning

After each experiment, all the cells on the surface were first removed by switching the bath solution to 1x ECB+1x, 10mM EGTA. Using fast flow from a 1mL pipette remaining cells were removed from the surface. After removing this solution the surface was dipped into acetone (99,5%), washed with Milli-Q water, then dipped into ethanol (99,7%), washed again with Milli-Q water and then dried with nitrogen gas. All surfaces were cleaned using this protocol before cells were cultured on them.

3.6. Electrical impedance spectroscopy

A small Teflon AF covered area was removed from the surface to access the conductive ITO layer below (see figure 9). The surface was placed under the microscope and positioned so that the ITO area could be connected to the electrode. Afterwards a drop of 1x PBS (phosphate buffer saline) solution or MilliQ was placed on the site of experiment where the front end amplifier (counter electrode) was also positioned. The distance from the surface to the counter electrode was measured and recorded. For the experiment, the counter electrode was moved as followed:

- Across a single pattern
- Across three patterns

The impedance was measured at multiple simultaneous frequencies for of each experiment. All of the tested surfaces had been cleaned using the protocol described previously.

FIGURE 9. THE DIFFERENT LAYERS OF THE SURFACE.

4. Results and discussion

This chapter will present and discuss data on the cell adhesion, cell detachment and electrical properties of Teflon AF in the form of figures attained from the experiment.

4.1 Cell adhesion on Teflon AF surfaces

The adhesion on structured Teflon AF surfaces varied comparing the confluence and spatial control of HEK 293 cell line. Microscopic observation of the cells after incubation showed that most of them presented filopodia, indicating that they have adhered and are healthy on the surface (figure 10). However on most of the structures, the cells adhered not only to patterned regions but too unexposed Teflon AF as well (marked areas in figure 10) which raises suspicion since regular Teflon due to its super hydrophobicity do not readily promote attachment.

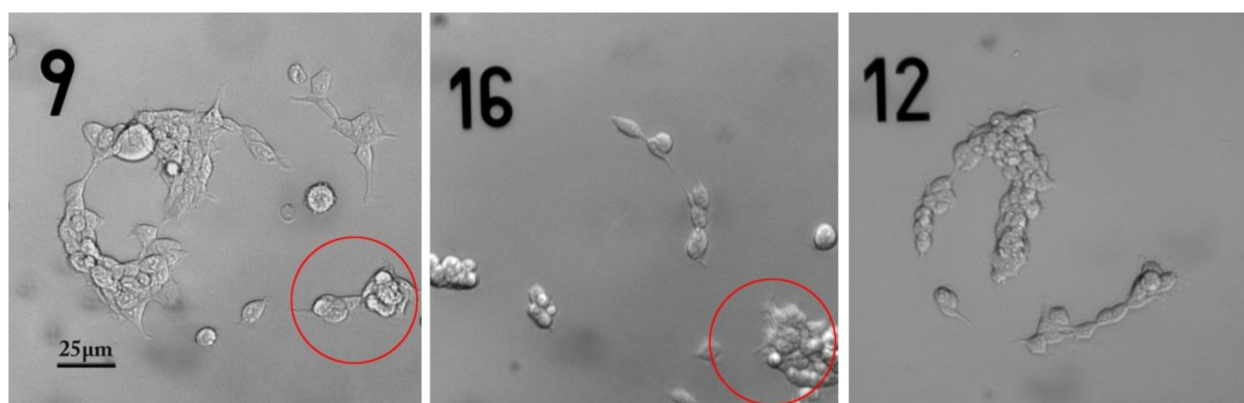


FIGURE 10. CELL ADHESION IN A RING-LIKE STRUCTURE ON NUMBERED TEFLON AF SURFACE, PRIMARY USE. THE MARKED AREAS SHOW ADHESION OF CELLS TO UNEXPOSED TEFLON AF.

Depending on the cell line, the rate at which cells localize onto the exposed pattern is known to differ, HEK 293 shows slower adhesion compared to other cell lines. The incubation time for optimal adhesion, where the HEK 293 attached typically to the circular patterns was found to be between 18-24h. The confluence on patterned regions after this time was around 70-90%. Adhesion on non-exposed Teflon AF was more frequently observed as a result of a longer incubation time. The adhesion on the patterned regions also depended highly on the number of times it had been used. The ring like structure were mostly observed in primary use surfaces with optimal adhesion on exposed Teflon AF while reused surfaces either showed few or no such structures (difference between primary and secondary used surfaces is shown in appendix 1).

The problem regarding adhesion on unexposed Teflon AF was, as previously mentioned, more often observed on surfaces that had been cleaned and reused. The chemicals used for cleaning the surfaces were acetone and ethanol. Both of them are known to remove the majority of biological components from the surface but there is still a possibility that bio fouling remains which would allow cells to adhere to non-exposed areas. Since the Teflon AF layer covering the surface only has a thickness of 50-70nm another possibility is that the cleaning might have inadvertently damaged this thin film and even allowing it to lift off from certain areas. This would expose the base layer of the surface on which cells

would favorably adhere compared to Teflon AF thus explaining the irregular adhesion on unwanted areas. Two other aspects were tested, the use of silicon oxide as an additional coating layer and lowering the e-beam intensity for surface patterning. The former showed no difference in cell anchoring ability neither was any improvement on the cell detachment observed when exposing cells to 10mM EDTA, EGTA or BAPTA. The use of a low intensity e-beam however showed lowered cell attachment which could indicate weaker anchoring ability, but further study on this needs to be performed to rectify this assumption. No improvement on the cell detachment was observed when lower intensity was used.

4.2. Cell detachment

Single or group of cells adhered to the patterned regions of the substrate surface were analyzed using different chelating agents. Direct observation showed some but no relevant effect on cell detachment when applying different chelating agents or varying concentration. A few notable observation needs to be fulfilled when observing cell detachment, namely, the release of the filopodia creating a more spherically shaped cell, vivid cell edge and finally detachment of the cell from the surface. The few cell that did show some effect did so by either spherical transformation or more defined edges however no cells released the surfaces.

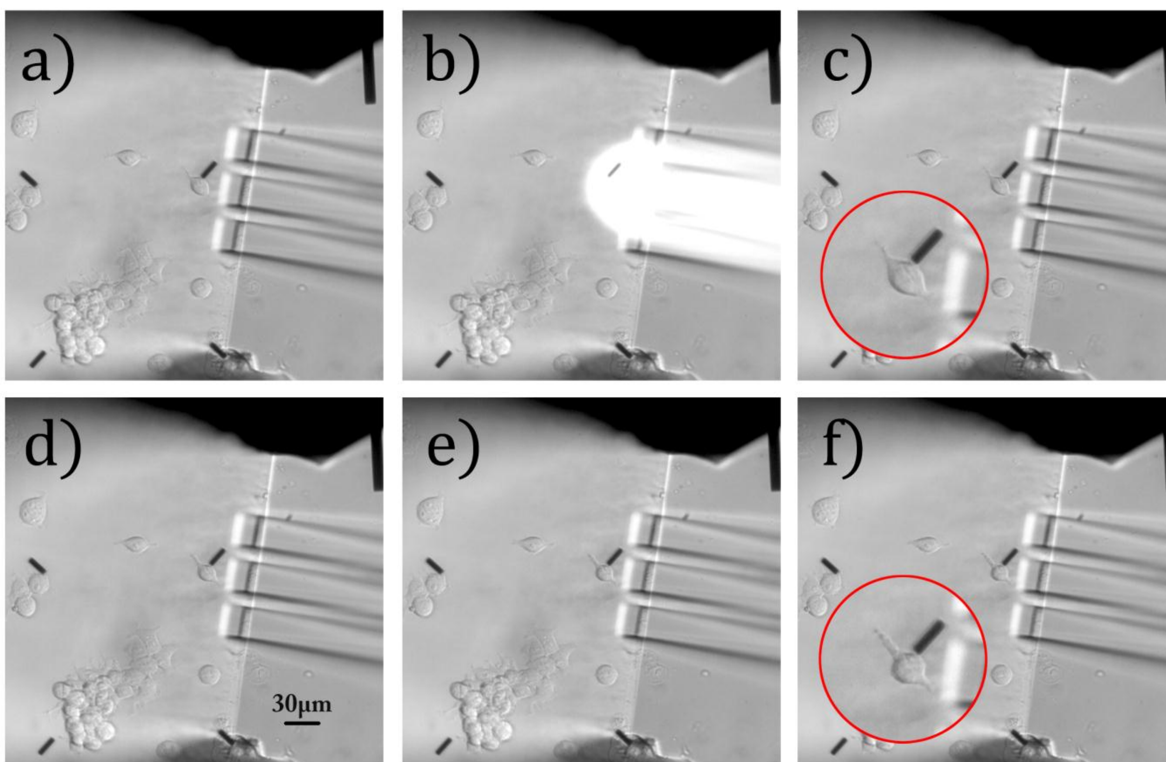
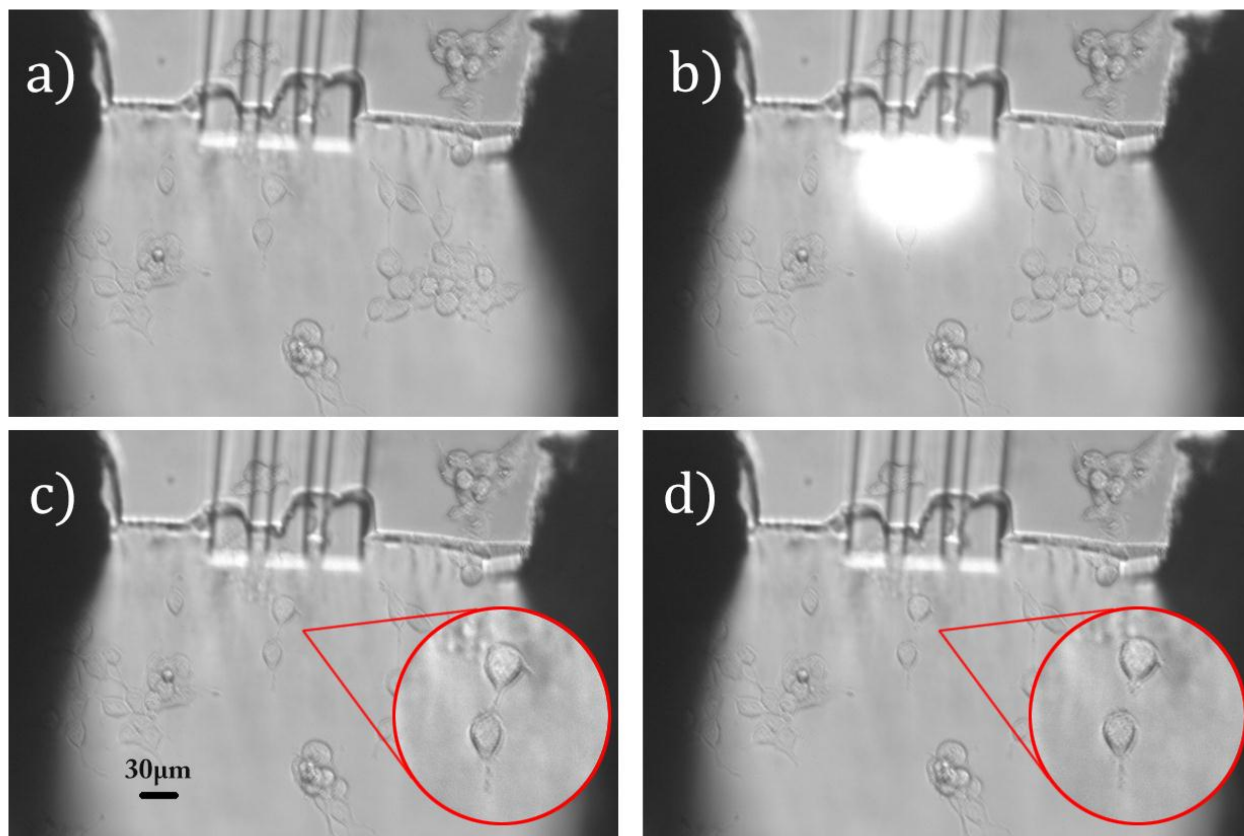


FIGURE 11. CELL EXPOSED TO 10mM EDTA, A) NON-EXPOSED B) FLUORESCEIN C) 2MIN EXPOSURE D) 5MIN EXPOSURE E) 8MIN EXPOSURE AND F) 10MIN EXPOSURE

The above figure describes one of the results, where spherical transformation was observed. However the filopodia were still intact which makes it unclear, whether or not there is a cell detachment being observed or flow induced shear.

A clear example of filopodia contraction is shown in figure 12 when the cell, when exposed to 10mM EGTA, releases the intermediate connection to the other cell. However no sign of full detachment was observed after this intermediate release at 5 minutes of exposure.



FIGUR 12. CELL EXPOSED TO 10mM EGTA, A) NON-EXPOSED B) FLOURESCEIN C) 2MIN EXPOSURE D) 5MIN EXPOSURE

The use of different chelating agents for the purpose of analyzing on demand cell detachment from patterned Teflon AF surface was found to have no or irrelevant effect. This might indicate that the adhesion on the surface might not be calcium dependent or that depletion of calcium is not enough to severe the bond between the cell and surface. For further studies on cell detachment, deeper understanding and studying on the surface composition needs to be conducted.

4.3. Electrical impedance spectroscopy

The method proved to be relatively useful in detecting and differentiating the electrical impedance of radiated and unexposed Teflon AF. The major issue encountered with this method was the high noise ratio which made it, in some cases, difficult to distinguish between the two types.

In the first procedure, the counter electrode was moved across a single pattern. The data from a single experiment was collected and examined with a moving average (period=80) trend line. Two distinctive changes in magnitude were observed which displays an electrical potential difference between unexposed and exposed Teflon AF but whether the change is negative or positive cannot be determined from the data. The two potential changes are detected as the counter electrode crosses the circular pattern moving in and out of it (this procedure is illustrated in figure 13). It should be noted however that although a difference can be observed of the expected transition points, the curve is extremely noisy and quantifications of any degree is not possible. An increase in magnitude shows that patterned Teflon AF has higher impedance than regular Teflon AF. When following the movement path of the counter electrode in figure 13 three areas should comparatively have the same impedance which are the starting point, the area inside the circle and the end point. However a slight decrease in magnitude was also observed towards the end for which the reason is still not clear. This observation could be due to surface disproportion. EIS measures the resistance of the substrate structure, any change on its topography would be detected when moving the counter electrode. A disproportional surface has regions which would increase or decrease in distance between the surface and the counter electrode. A decrease in distance would occur if there is a convex curvature on the surface, the electrons would have a smaller distance to travel which means that they will face less resistance and as a result a lower impedance is detected. The opposite is true for an increase in distance. Usually this type of behaviour would be observed as a symmetrical curve. If the counter electrode is brought closer to the convex curvature a decrease in impedance would be detected but when the counter electrode has passed the curvature the plot should decrease and stabilize.

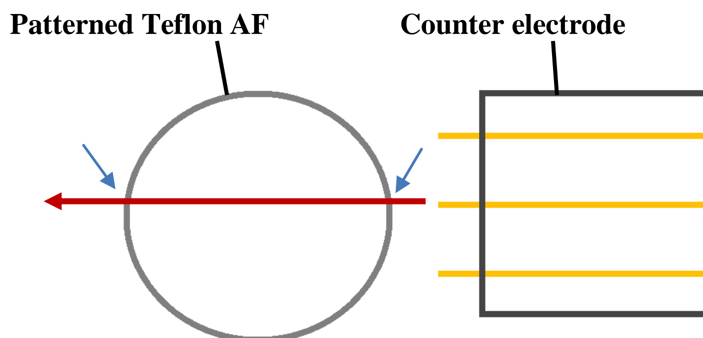


FIGURE 13. THE TWO SMALL ARROWS SHOW THE CROSSING POINTS ON THE PATTERNED TEFLON AF STRUCTURE AT WHICH CHANGE IN ELECTRICAL POTENTIAL IS DETECTED

The second experiment was conducted moving the pipette across three patterns. The result is plotted below in figure 14. As the movement path includes three patterns the figure should show 6 changes however distinguishing these changes were found to be quite difficult considering that there are more than 6 possible potential differences detected. The difficulty lies in the increase and decrease in impedance with time. The noise for this experiment was significantly reduced compared to the previous experiment through better calibration of the reference electrode but it still contributed to the problem. Looking at the figure, it could to certain extent be considered a prime example regarding disproportional

surface and symmetrical appearance of the curve as discussed previously where the increase in magnitude indicates an increase in distance. The curve seems to stabilize towards the end as the counter electrode has passed a certain point which is at time 35s. A notable feature of the curve is that the bumps are unevenly spread looking at the x-axis and the reason for this has to do with manual movement of the counter electrode. The changes at the end are detected within much shorter time interval as the counter electrode was moved much quicker across while the former changes show the opposite.

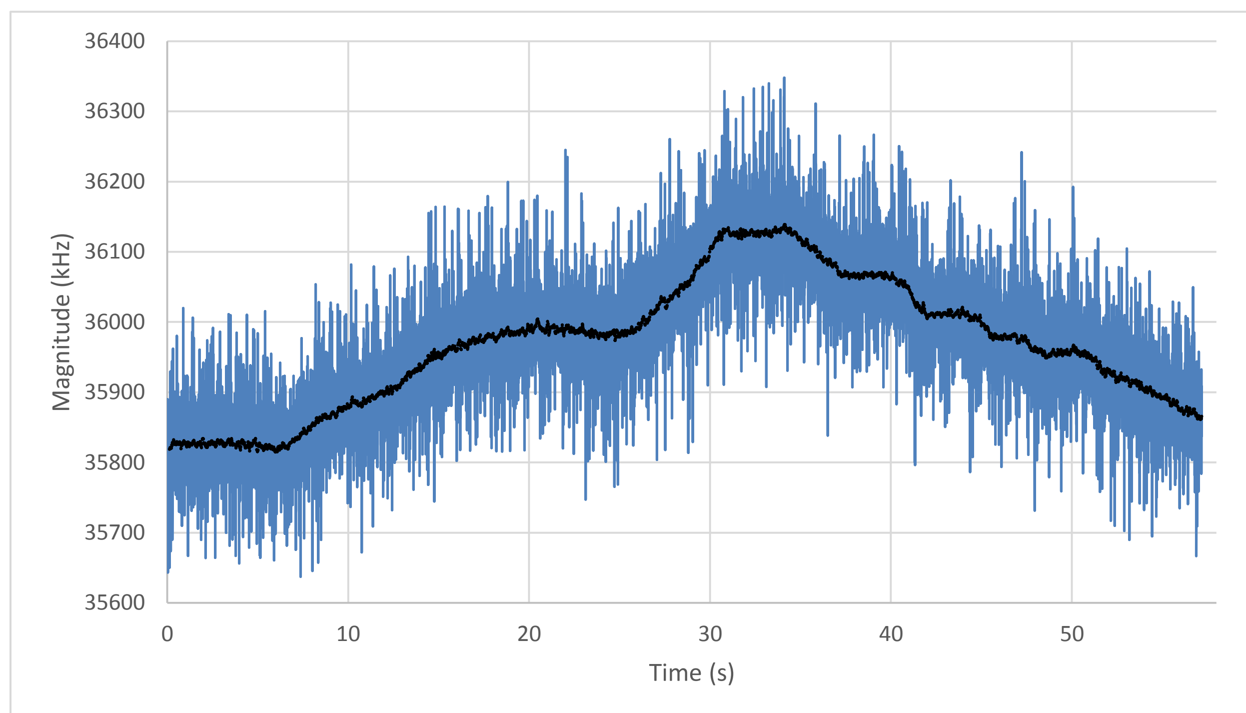


FIGURE 14. CHANGE IN MAGNITUDE OVER TIME MOVING ACROSS THREE PATTERNS AT FREQUENCY 61 kHz

The distinctive potential changes on the curve differed when looking at the data from the same experiment at different frequencies. For the first experiment, moving the pipette across a single pattern, presenting a variance between regular and patterned Teflon AF, was more visibly clearer at lower frequency (10 and 17 kHz). Distinguishing between the two types of Teflon AF was more difficult at higher frequencies and the decrease observed at the end of the curve in figure 10 became much steeper. In contrast the potential changes in the second experiment, moving the pipette across three patterns was more distinctive at mid frequency while higher or lower frequencies became less distinctive. (Figures on how the magnitude changes over time at different frequencies is shown in appendix 2 and 3 for both experiments.)

It is known that patterning of Teflon AF using e-beam lithography radically changes the composition of the surface, but the appearances of this composition is still unknown. An interesting assumption that can be drawn after having observed potential difference using EIS is that the e-beam when striking the substrate creates a positively charged surface. Eukaryotic cells which consist of an outer phospholipid bilayer are known to be negatively charged. These opposite charges would attract each other and this

could explain the favourable adhesion of cells on the patterns. Further studies need to be conducted on the composition and electrical properties of the surface to certify the assumption.

In addition, the scanning nature of the e-beam, making multiple passes to construct the patterns, may form some topological patterning, in addition to chemical structure disturbances. This nano patterning may equip the surface with additional anchoring sites, allowing the cells to strongly seat to the surface.

5. CONCLUSION

This study has focused on characterizing the Teflon AF substrate and its performance regarding cell adhesion and detachment mechanism as well as its electrical properties, using multifunctional microfluidic pipette and electrical impedance spectroscopy. The controlled cell adhesion using e-beam lithography was found impressive where HEK 293 cells adhered, covering between 70-90% of the patterned regions on primary used surfaces. However cleaned and reused surfaces showed much lower attachment to the circular patterns and higher density of adhesion on unexposed Teflon AF. The reason for this undesirable adhesion whether it is due to bio fouling, Teflon AF contamination or removal of Teflon AF in the cleaning procedure, is still unknown. Experiments for on demand cell detachment from the substrate surface using EDTA, EGTA or BAPTA was found to have no or irrelevant effect which leads to say that the adhesion to the surface might not be calcium dependent or that only depleting calcium is not enough to detach cells from the surface. A difference in the electrical potential between patterned and regular Teflon AF has been observed using electrical impedance spectroscopy.

In summary a difference in electrical potential was observed through the use electrical impedance spectroscopy however further studies needs to be conducted to understand more about the composition and the structure of Teflon AF to further outline its major means of control and to establish an on demand cell detachment procedure.

6. FUTURE WORK

For future work on Teflon AF surfaces, further studies on its chemical composition after e-beam patterning needs to be conducted. On the other hand disregarding the Teflon AF surface, a few other experiments can be conducted to possibly establish an on cell detachment procedure. A few studies have shown the use of bilayer lipids to coat a glass surface.[27, 28] The use of microfluidic pipette has, in a different study, been applied to pattern the bilayer lipid into different shapes and patterns. It has also been presented that a detergent can be used to lift certain part of the bilayer off the surface. This can be performed with high precision using the microfluidic pipette. The unknown factors here would be if the cells adhere to the surface, and if it is possible to detach them afterwards using the detergent without killing the cell or destroying their membrane.

Another study has shown the possibility of using alginate for cell adhesion. Alginate is a polymer which is mostly distributed in brown algae. It has a gel type chemical composition which is strengthened by bonding to calcium ions however removing calcium would result in weakening of the gel composition making it more fluidic. When alginate is bonded to calcium it can be used to either coat or pattern a surface. Using the microfluidic pipette, alginate on the surface can afterwards be exposed to chelating agent removing calcium which would weaken the composition and lead to a release. The unknown factor in this study would be if the cell, when exposed to chelating agent, would detach from the gel or if the gel would release from the surface, or indeed if it would be possible at low sheer stress.

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APPENDIX

APPENDIX 1 DIFFERENCE IN CELL ADHESION BETWEEN PRIMARY AND SECONDARY USED TEFLON AF SURFACES

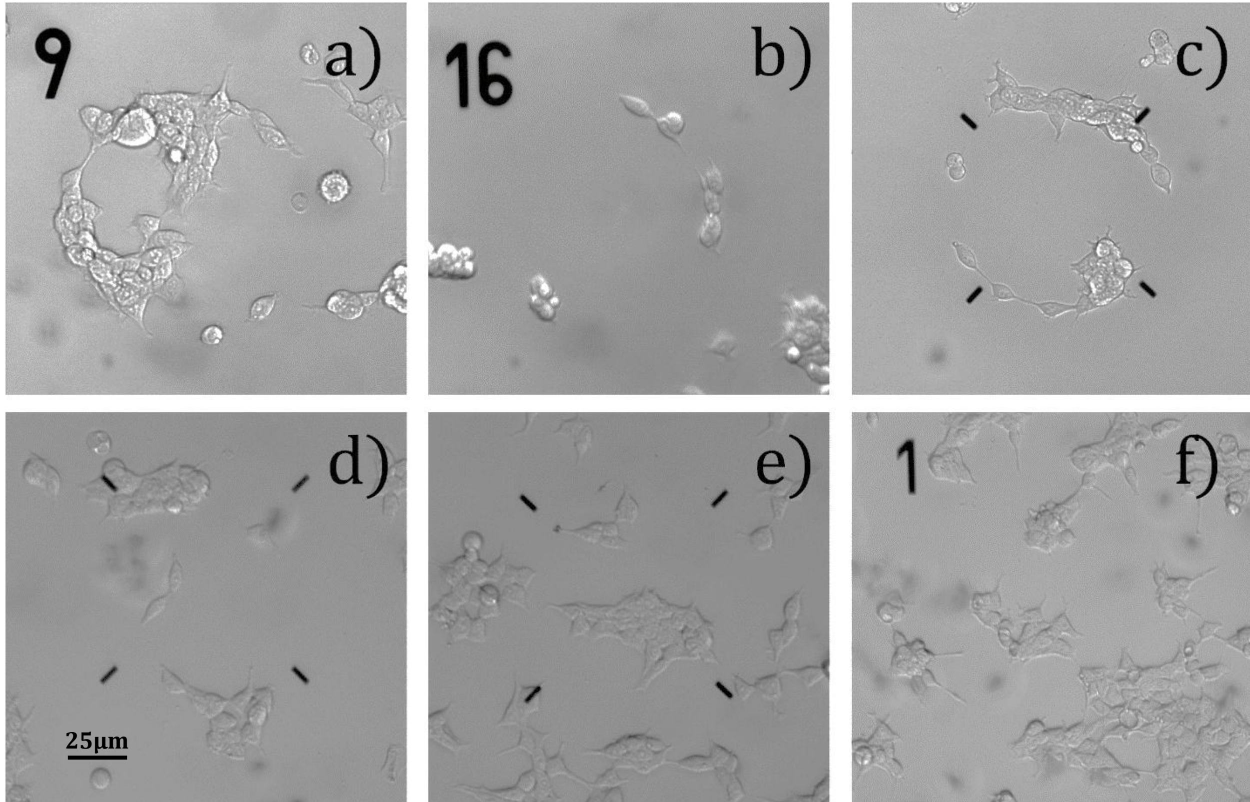
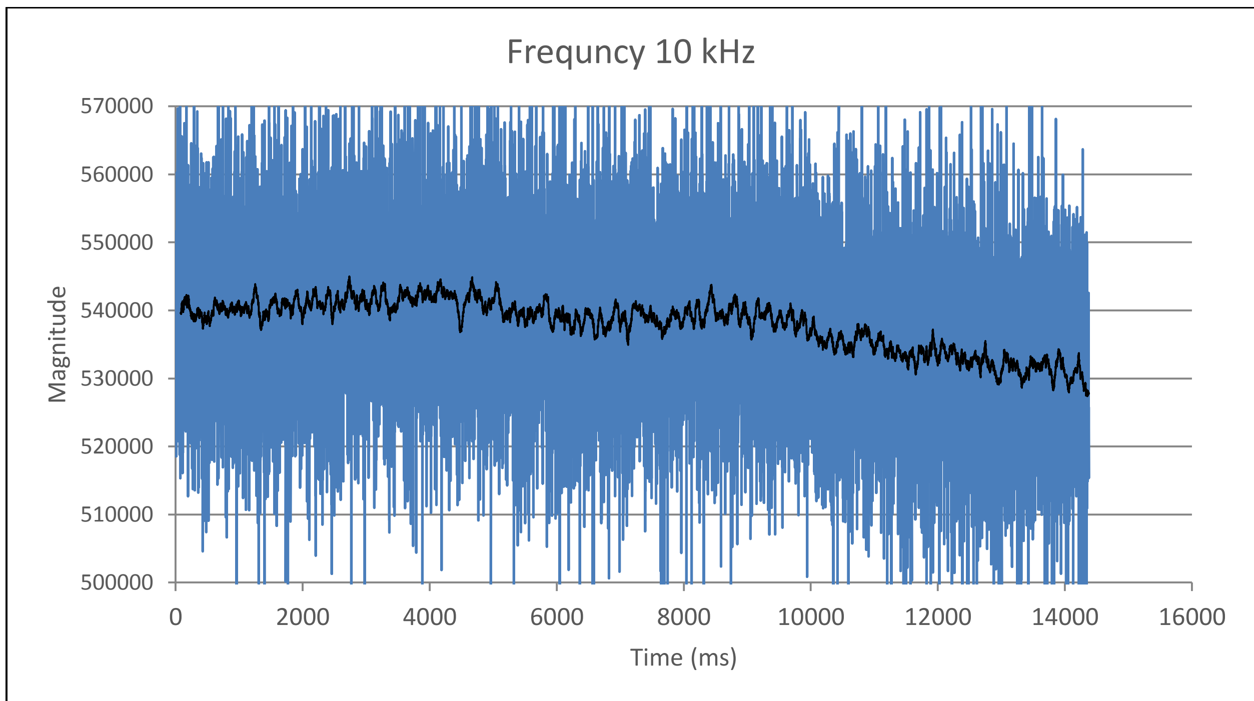
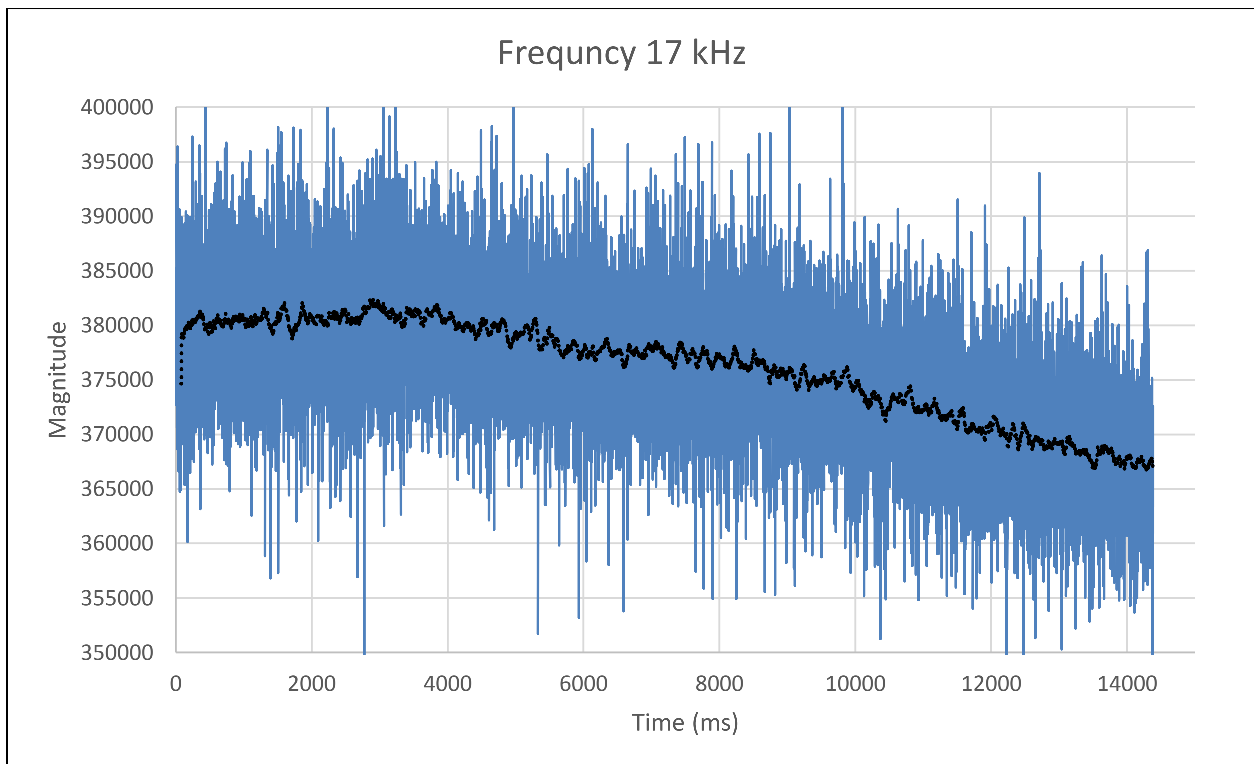
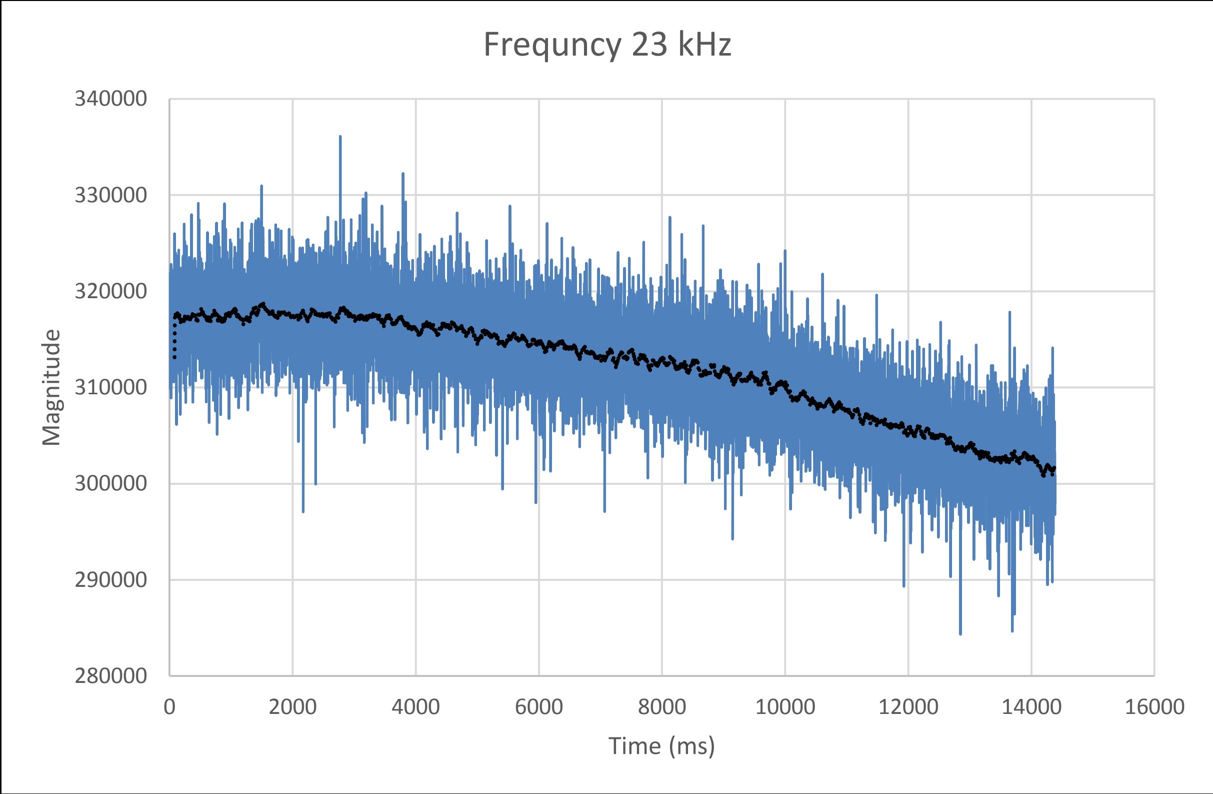


FIGURE 14. COMPARING CELL AHDEISON ON PRIMARY AND SECONDARY USED OF TEFLON AF SURFACES. A), B) AND C) ARE PRIMARY USED SURFACES WHILE D), E) AND F) ARE SECONDARY.

APPENDIX 2. ELECTRICAL IMPEDANCE SPECTROSCOPY, MOVING ACROSS A SINGLE PATTERN. CHANGE IN MAGNITUDE OVER TIME OBSERVED IN DIFFERENT FREQUENCIES.



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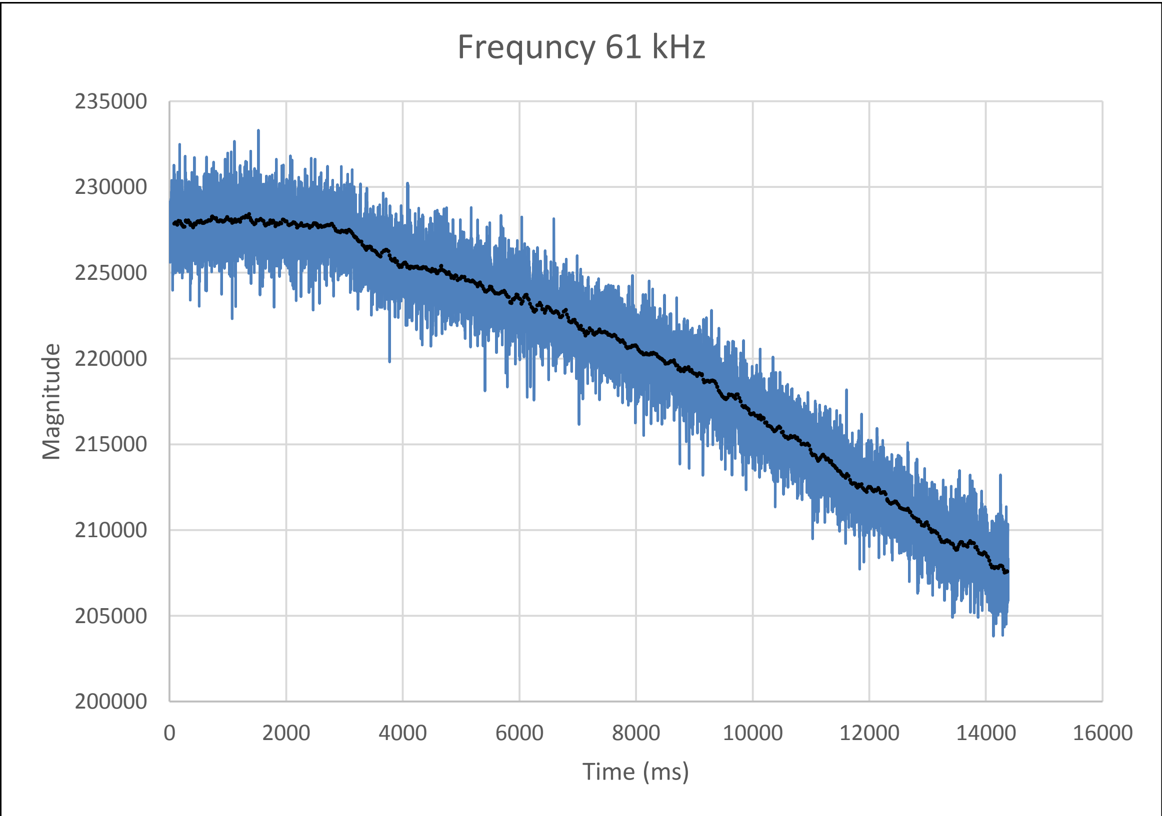
Time (ms)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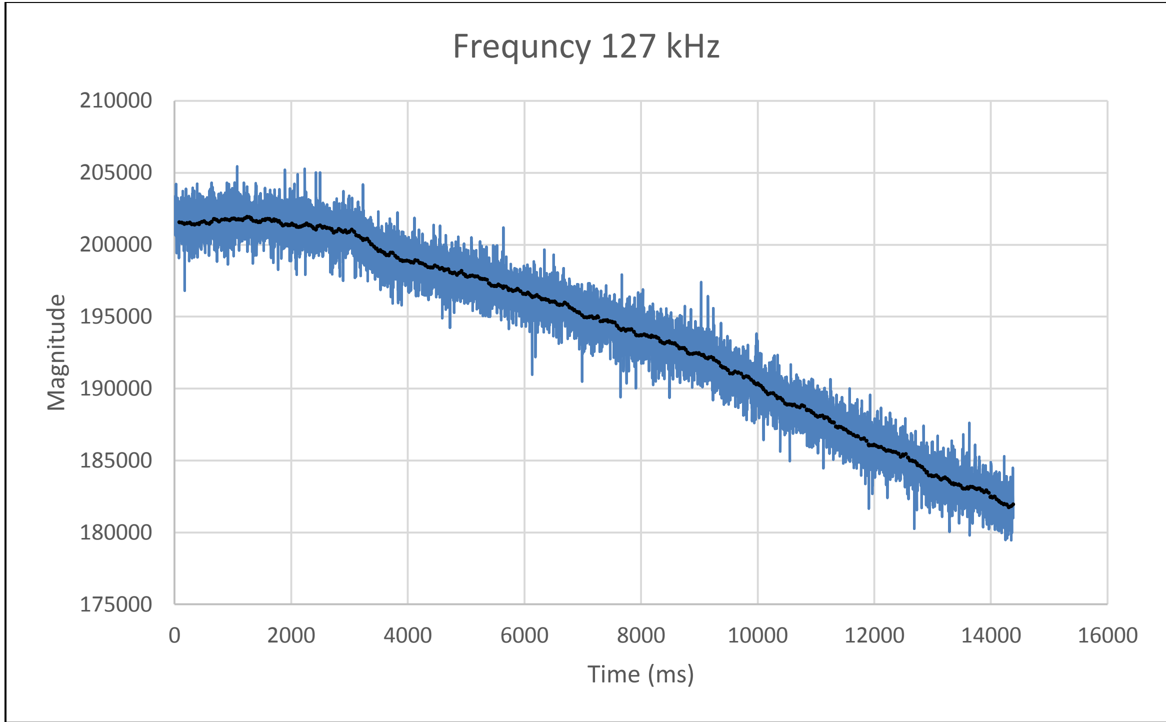
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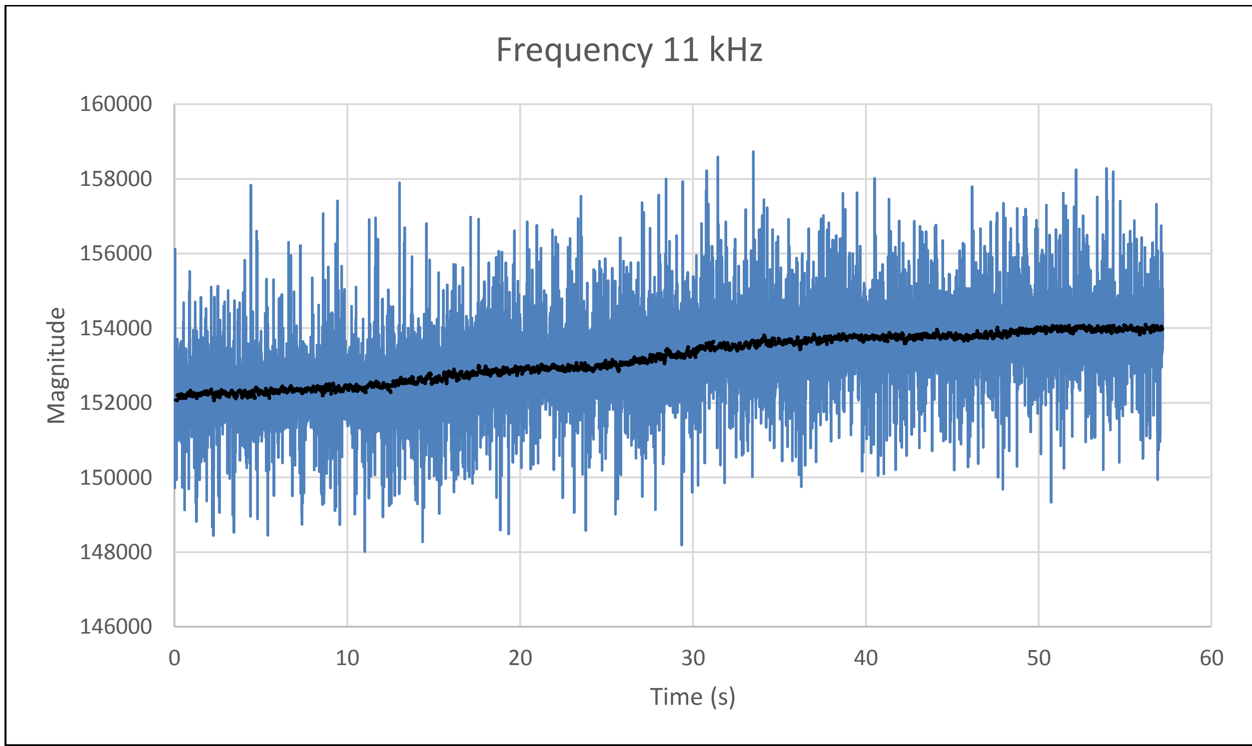
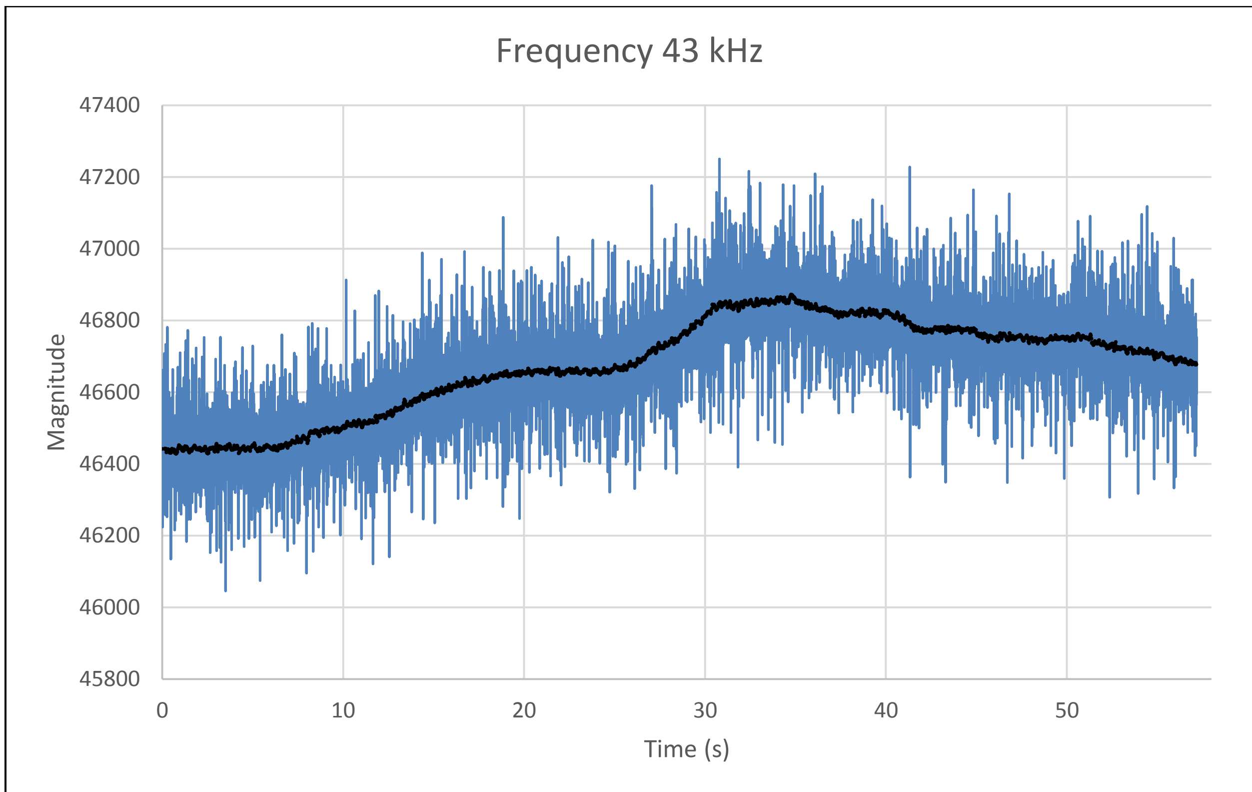
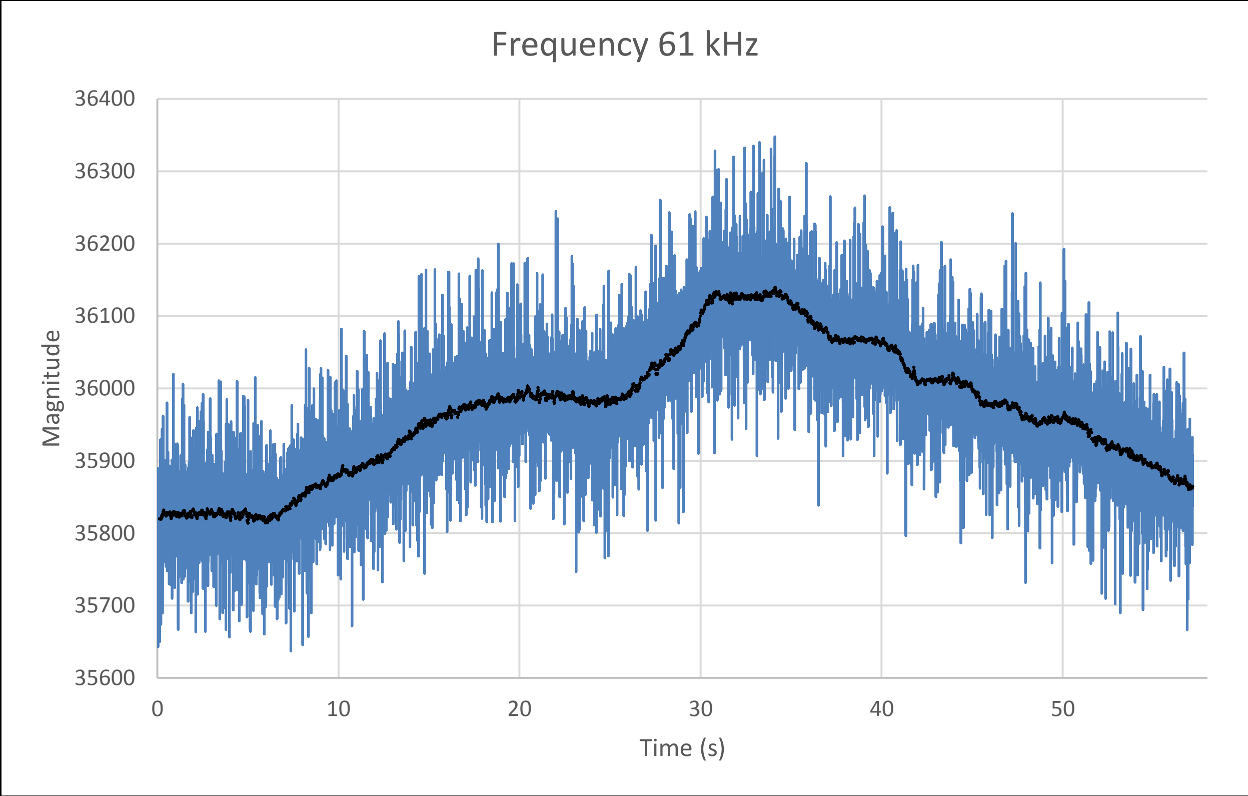
Time (ms)

APPENDIX 3. ELECTRICAL IMPEDANCE SPECTROSCOPY, MOVING ACROSS THREE PATTERNS. CHANGE IN MAGNITUDE OVER TIME OBSERVED IN DIFFERENT FREQUENCIES.



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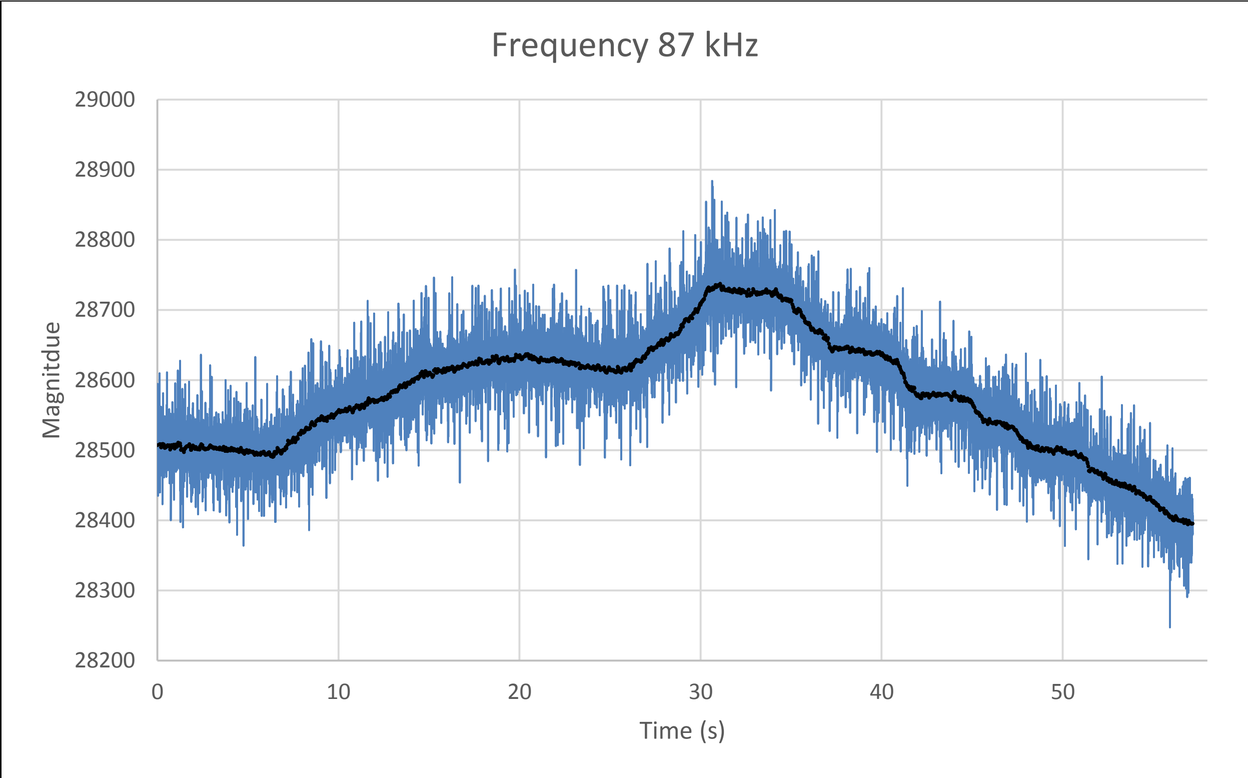
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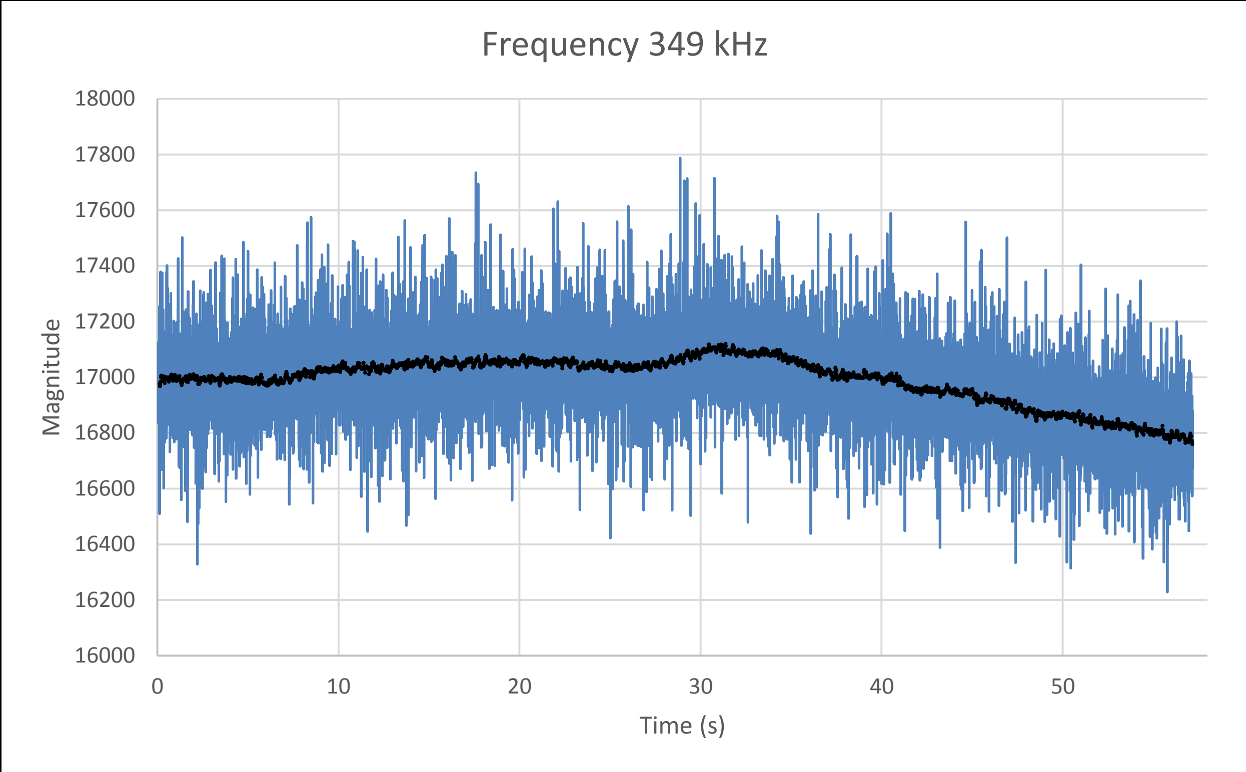
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