Electrochemical Functionalization of Epitaxial Graphene

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I would like to thank several people which have helped me to finish this work. I would like to thank my two supervisors, Martin Bengtsson at LTH and Mikael Syväjärvi at Graphensic AB. Thank you Mikael for giving me the exciting opportunity to work with graphene. I would also like to thank Mikhail Vagin for introducing me to electrochemistry and guiding me through the initial experiments. Thank you Dan Hessman for taking the time to help me with the Raman spectroscopy. For help with the fluorescence I would like to thank Belinda Adler. For tips regarding the activation of the antibodies, thank you Simon Ekström.

Abstract

In this work graphene has been functionalized with antibodies. Different functionalization methods of graphene already found in literature have been evaluated. An electrochemical functionalization process with subsequent immobilization of antibodies have been chosen for this thesis.

The purpose of the thesis has been formulated in collaboration with Graphensic AB. From them a wish to implement an electrochemical cell has been brought up. If graphene could be functionalized it could be used as an electrode in an electrochemical cell to measure many different processes.

Four graphene samples have been used, all of them manufactured by Graphensic AB. The graphene has been grown epitaxially on the silicon terminated surface of 4H-silicon carbide. The samples have been of a high quality, three of them were monolayers, monolayer compromising more than 95% of the surface, and one a bilayer, bilayer compromising more than 50 % of the surface.

The functionalization could be divided into two steps. First the attachment of aryl radicals to the graphene surface. Then the attachment of antibodies to these aryls, both a primary antibody and a secondary. The attachment of aryl radicals were done in an electrochemical cell utilizing a diazonium salt. The decomposition of the diazonium salt into aryl radicals was driven electrochemically in the cell.

The graphene samples were measured electrochemically between each functionalization step so that the changes could be evaluated. The impedance and the peak values of the cyclic voltammograms increased after the diazonium salt step. They then decreased again to values close to the initial value when attaching antibodies. The attachment of the secondary antibody slightly increased the peak values of the cyclic voltammogram.

The secondary antibody contained a fluorescent molecule which was used to evaluate the functionalization. For two of the samples this showed that the functionalization most likely had been successful. For the other two it was hard to distinguish whether or not it had been successful.

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1. Introduction

Graphene, a two dimensional macromolecule consisting of carbon, was for many years only thought of as a theoretical material. No one had been able to produce a single flake of it without it decomposing. Graphene mostly existed as building blocks of graphite, the material used in ordinary pencils, which consists of layers and layers of graphene.

The Nobel Prize in Physics 2010 was awarded *"for groundbreaking experiments regarding the two-dimensional material graphene"* to the two scientists Andre Geim and Konstantin Novoselov [1]. This was for a discovery which led to an article published only six years earlier, in 2004 [2]. They had used adhesive tape to pull graphite apart until only a few layers were left. So few that it could be said to be graphene. These layers were then captured on a silicon oxide substrate and subjected to experiments which led to the Nobel Prize. This is often thought of as the start of graphene research even though they were not the only ones working with graphene at the time.

The initial reports of graphene showed that it has a number of unique properties. It conducts both electricity and heat extremely well, is incredibly strong and has a high optical transparency. Plus being a two-dimensional material it has a very large surface area compared to its thickness. [3]

These properties have made graphene in to an extremely promising material for many applications. The problem have been a reliable method with which to manufacture large amounts of graphene of a satisfactory quality.

The company Graphensic AB, based in Linköping, Sweden, has come up with a method where high quality graphene is grown on silicon carbide. This is done by sublimating the silicon atoms thus leaving carbon atoms which forms graphene on the surface. They have identified this graphene as a possible electrode for biomedical research and electrochemistry. Graphene produced in other ways and then transferred to a substrate often detaches from the substrate when used in electrochemistry. Graphene grown on silicon carbide does not do this. Therefore Graphensic AB has developed an electrochemical cell which uses their graphene as working electrode.

However, to be able to use the electrochemical cell in a meaningful way they need to functionalize the graphene. This is not easy since graphene is a very inert material. If an initial molecule could attach to the graphene surface it would be possible to build on that initial molecule, attaching the wanted molecules. Depending on what type of molecule used for the functionalizion it would make the electrode useful for measuring different reactions. For instance if some sort of receptor molecule could be attached to the graphene it could function as a sensor. This since attaching molecules to a graphene surface should change its electrical properties which then could be detected. Which molecule should be attached depends on the individual research groups that could be interested in this cell. Some very common biomolecule in research are antibodies. These molecules are used by the immune system to detect harmful substances. They are highly specific and only reacts to one sort of molecule [4]. Luckily they come in many versions, each specific to a different molecule.

1.1 Purpose

By attaching antibodies to graphene their specificity could be used to make it into a sensor. But even if it's possible to do this it is not sure that it is enough. They have to be attached in such a way and to such an extent that it would be detectable. This is what this master thesis will try to do. More exactly I will try to functionalize graphene supplied by Graphensic AB with antibodies so that it can be detected by measuring the impedance in their electrochemical cell. First I will have to come up with a method with which the functionalization will be done. Then I will functionalize the graphene and try to detect the functionalization.

2. Theory

2.1 Graphene

Graphene is a two dimensional molecule consisting of carbon atoms coupled in a flat hexagonal lattice. It is said to be two-dimensional since the thickness of one atom is much smaller than the area of the lattice which can be several square micrometers. Graphene has several unique properties such as a high optical transparency, high electron mobility, high thermal conductivity, a large surface area and a high breaking strength. [3]

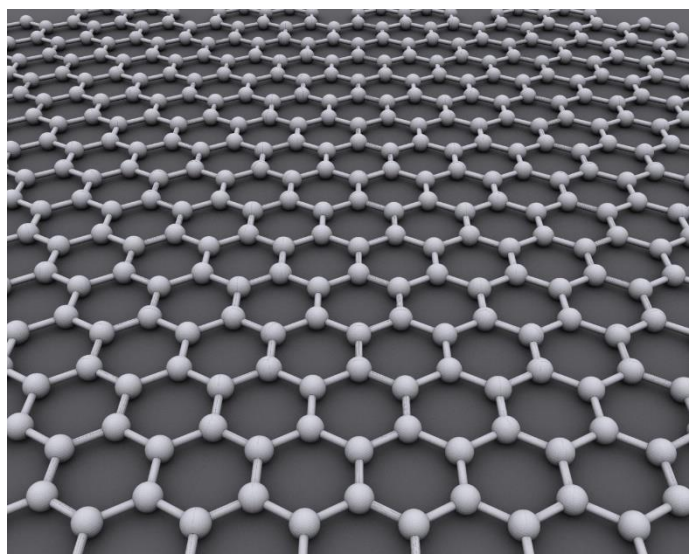

Figure 1: *Structure of graphene. Each circle represents a carbon atom.* [5]

A carbon atom has six electrons with the electron orbital configuration of $1s^2\ 2s^2\ 2p^2$ in its ground state with the four valence electrons in the $2s^2$- and $2p^2$-orbitals. The valence electron orbitals can be combined into orbitals of different shape, called hybrid orbitals. The sp^3-hybridization mixes the 2s-orbital with the three 2p-orbitals into four sp^3-orbitals in a tetrahedral shape around the nucleus each separated by 109.5°. These orbitals form σ-bonds with other atoms. The sp^2-hybridization mixes the 2s-orbital with two 2p-orbitals into three sp^2-orbitals lying in a plane around the nucleus separated by 120°, see *Figure 2*.These orbitals yield σ-bonds as well. This leaves the third 2p-orbital with the fourth valence electron. This orbital lies perpendicular to the sp^2-orbitals forming a π-bond with p-orbitals of other atoms. This is the hybridization that makes up graphene, with the flat

structure coming from the sp^2-orbitals. There is also the sp-hybridization where the sp-orbitals are separated by 180°.[7]

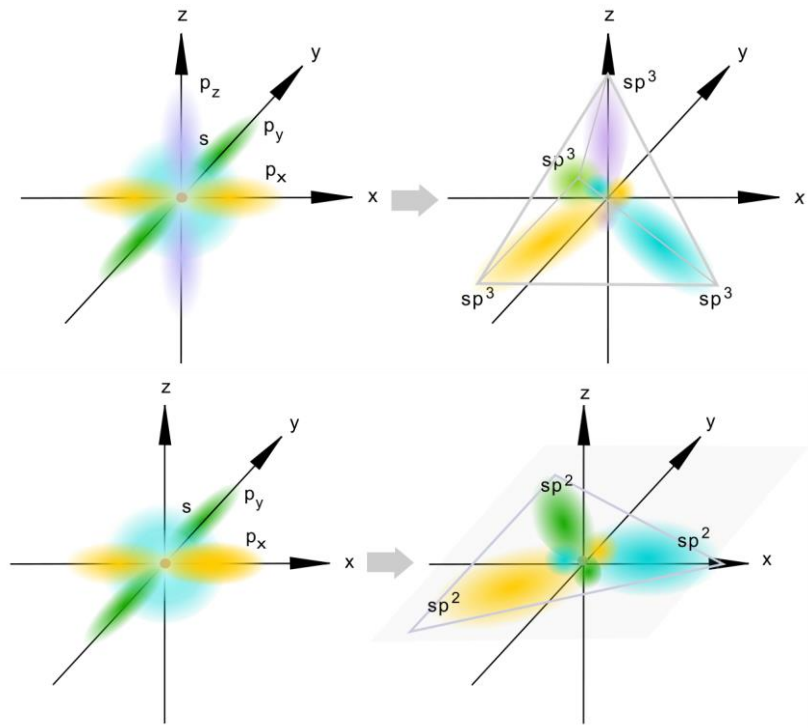

Figure 2: Sp^3-hybridization above and sp^2-hybridization below. The colored areas represent individual electron orbitals. [6]

If several sp^2-hybridized carbon atoms interlink in a lattice, as in graphene, the electrons in the π-bonds will be shared between all atoms, forming an electron gas. It is from the sp^2-hybridization that graphene gets its many unique properties. The strength comes from the three σ-bonds together with the shared π-bonds. The electron mobility comes from the shared electron gas. A carbon lattice of sp^3-hybridized atoms will instead form diamond, which is an insulator. [7]

The intramolecular strength coming from the double bonds also makes graphene very unreactive. If you do manage to chemically bind something to graphene you will most likely have changed the hybridization of the carbon atoms to sp^3. This since you need the electron forming the π-bonds to instead bond with your molecule. Changing the hybridization will change the properties of the graphene. [8]

Since sp^3-hybridized atoms don't have π-bonds they won't contribute to the electron gas and thereby lowering the electron mobility. The strength will also go down since there won't be any extra bonds, the π-bonds, between the carbon atoms in the lattice. Lastly an sp^3-hybridized atom will take on a tetrahedral shape with its bonds, introducing strain in the lattice and thereby weakening it. [9]

Since graphene is a large lattice with sp^2-hybridized carbon atoms the more sp^3-hybridized carbon atoms you have in the lattice the less graphene-like it will be. This will change the properties, decreasing its high electron mobility and strength. However as long as not too many carbon atoms are sp^3-hybridized it won't be a problem. The decrease in its electronic properties could be detectable which could be a way of sensing how many sp^3-hybridized atoms there are in a lattice.

Graphene can be manufactured in a number of ways, each producing graphene of varying qualities. Methods include the mechanical exfoliation of graphene from graphite. The winners of the Nobel Prize in Physics 2010 used the "Scotch tape method" which is a type of mechanical exfoliation. There are also chemical exfoliation methods of graphite, which works by chemically weakening the forces between the layers in graphite, thereby separating them from each other. Graphene can also be made by chemical vapor deposition, converting hydrocarbons into graphene layers on metals. Or it can be grown by thermally treating silicon carbide so that the silicon sublimates and the carbon atoms reorganize into graphene layers on the surface. Another way is to first oxidize graphite, separating its layers and then reducing it to a graphene. [10][11]

Some methods give graphene of a single layer and others of multiple layers. Some give very pure graphene and others contain many defects. Some will have a large surface area of connected carbon atoms and some will instead have many smaller graphene flakes lying next to each other, giving a lower surface to edge ratio. These different properties all influence the chemical reactivity and electrical properties of the graphene. The graphene of highest quality, no defects, one layer and large surface area, is called pristine graphene. However in practice all types of graphene will contain defects.

For pristine graphene the reactivity is very low since the carbon atoms are strongly bound to each other. However at the edges of the graphene layers

the atoms aren't connected to the lattice in the same way, often being sp^3-hybridized. This makes the edges much more reactive than the rest of the graphene. Since graphene manufactured in different ways can have different surface to edge ratios the reactivity will differ. The same holds true for defects such as vacancies. Basically one can say that the easier a carbon atom can sp^3-hybridize the higher the reactivity. For edge atoms and atoms next to vacancies there is space to incorporate the tetrahedral shape without causing much strain. The p-orbital is also not as tightly coupled to other atoms and the double bond is therefore more easily broken. Curvatures in the graphene lattice will exhibit the same behavior since the p-orbital creating the π-bond won't overlap as much with its neighbors. [9]

2.2 Epitaxial graphene on silicon carbide

Graphene can be grown epitaxially on the silicon carbide. This is done by heating the silicon carbide so that the silicon atoms sublimate and leaves carbon atoms on the surface which reintegrate into graphene layers. This is possible since the vapor pressure of silicon is larger than for carbon meaning that silicon will sublimate in greater numbers than carbon thereby creating an excess of carbon on the surface. For the sublimation of silicon to start the silicon carbide needs to be heated to 1150°C. However at this temperature graphene will not form, instead a graphitic layer will. To grow graphene the temperature needs to be much higher. The quality and number of layers can be controlled by altering the inert gas pressure and the temperature. Graphensic AB has developed a method with which high quality graphene with the desired number of layers can be obtained. The silicon carbide is put into a chamber in which both the temperature and pressure can be controlled. It is heated to above 1400°C in two heating steps during which the pressure is kept between 600 and 1100 bar by an inert gas. The silicon carbide is then heated to the growth temperature which is preferred to be 1900°C. The graphene is grown on the silicon terminated surface since it allows for slower reaction kinetics enabling more control over the growth process. By changing the temperature and the time allowing for growth the number of layers can be varied. [12]

Growing graphene on silicon carbide has many advantages. The graphene acquired by this method is mostly of a very high quality. Since it is grown

on silicon carbide and is attached to it, the graphene grown in this way is suitable for electronic applications. At room temperature silicon carbide can be thought of as an insulator since its bandgap is large enough. More than that the silicon carbide can be passivated so that it does not interfere with the electronic properties of graphene. This makes it possible to create graphene electronics directly on silicon carbide. The main disadvantage of the method is that it requires expensive equipment and highly skilled personnel making the manufacturing expensive. [10]

2.3 Electrochemical cell

An electrochemical cell is a device which allows a number of electrochemical measurements to be undertaken. It usually consists of an enclosed container holding a sample solution with an electrolyte containing a redox pair. Connected to the solution are three electrodes, working, reference and auxiliary. The working electrode is where the reaction that is to be investigated takes place. The auxiliary electrode is to where the current will flow. The reference electrode is there so that the potential between the working electrode and the solution can be measured. No current shall flow through the reference electrode. The potential of the working electrode is measured relative to the reference electrode. For this reason it is important that the ohmic potential drop, the potential drop due to the resistance of the solution, between the two electrodes is as small as possible. This can be done by isolating the reference electrode from the solution and connecting it to the working electrode by a channel. [13][14]

2.4 Cyclic voltammetry

Cyclic voltammetry is a common technique used to measure electrochemical reactions. The technique consists of linearly sweeping the potential over an electrode from a start value to an end value and then back again. During this time the current is measured. This process is repeated for a number of cycles. Basically a triangular potential waveform is sent in and the resulting current measured. The plot of current versus the potential for this technique is called a cyclic voltammogram, see *Figure 3*. The cyclic voltammogram is a complicated function which depends on a

number of factors, including time. From this plot information about the electrochemical reaction can be derived.

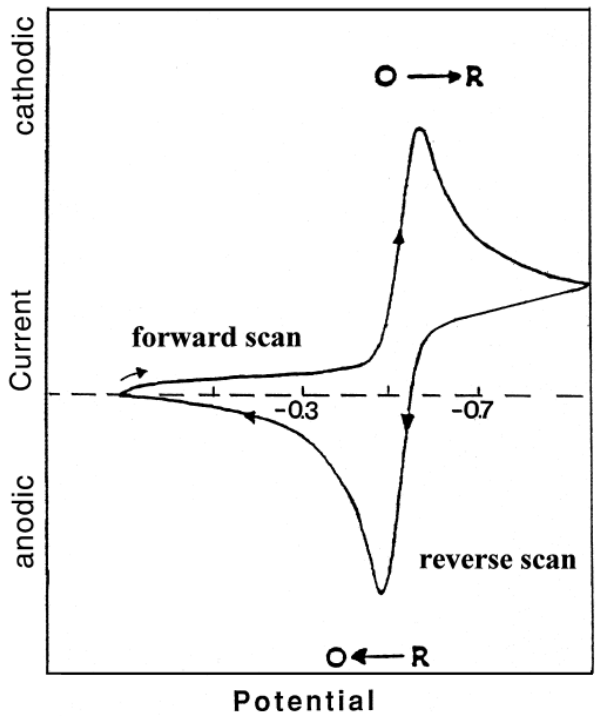

Figure 3: *A typical cyclic voltammogram. The current is plotted against the voltage. Forward scan reduces, reverse scan oxidizes.* [14]

What happens when the voltage increases from the starting value is that the redox pair in the electrolyte will undergo a redox reaction. An increasing voltage will reduce the reactant. This will increase the current exponentially until it reaches a peak from which it starts to decrease. The peak and subsequent decrease comes when all the reactants close to the electrode has undergone the redox reaction. Any new contribution to the current will then have to come from new reactants diffusing towards the electrode which for most reactions is a much slower process than the redox reaction. When the potential has reached the end value it will reverse towards the starting value and the same process will be repeated but mirrored since the redox reaction will be oxidizing the reactants. This however will only happen if the electrochemical reaction is reversible. If the reaction isn't fully reversible some of the product from the first process will not return to its starting form. This will cause the current peaks to

decrease since less and less molecules will partake in the redox reaction for each cycle. Processes can be irreversible, meaning that they only react in one direction, or quasi-reversible, meaning that they are slower in one direction. If a process is not reversible the current peak position will depend on the voltage sweep rate, separating more the faster the voltage sweep is. It is from the current peak positions and values information about the reaction can be gained. [14]

2.5 Impedance spectroscopy

Impedance spectroscopy is an electrochemical method to determine electrode kinetics by measuring the impedance of the electrochemical cell. Impedance is the complex version of resistance consisting of two parts, a real, the resistance, and an imaginary, the reactance.

A sample can be characterized by measuring the impedance of it. In an electrochemical cell the capacitance may be calculated by measuring the impedance. This varies with the type of electrolyte and eventual redox probes. The measurement is done by choosing a voltage around which the measurement will be done. This voltage should be in the middle of the two current peaks of the cyclic voltammogram. The voltage will then oscillate with a small amplitude around the chosen voltage. The oscillation is done with different frequencies and the impedance is measured for each frequency. [15]

2.6 Diazonium chemistry of graphene

Diazonium salts are a group of molecules made up of an anion, which can be of any type, and a cation consisting of an organic group coupled to an N_2-group. The anion is coupled to one of the nitrogen atoms with the other nitrogen atom coupled to the organic group. When the salt splits up into its cation and anion the cation is what is interesting.

The positive charge left from when the anion left will make the nitrogen atoms form a triple bond. The positive charge will then be transferred to the inner nitrogen atom which is still coupled to the organic group, thus having four chemical bonds. This means that the positive charge will be located at the nitrogen atom bound to the organic group. If an electron is

taken up by the diazonium cation the two nitrogen atoms will leave, leaving a radical made from the organic group. [16]

In combination with graphitic materials an aryl diazonium salt is used. The goal of using the diazonium salt is to create a highly reactive aryl radical with which the graphitic materials can be modified. The radical will break the double bond between two carbon atoms, transferring the radical to the surface instead. This surface radical may then react with other aryl radicals. To generate the radical an electron needs to be transferred to the diazonium ion. This can happen spontaneously from the graphene or by supplying extra electrons by applying a potential. This reaction is seen in *Figure 4*. [16][17]

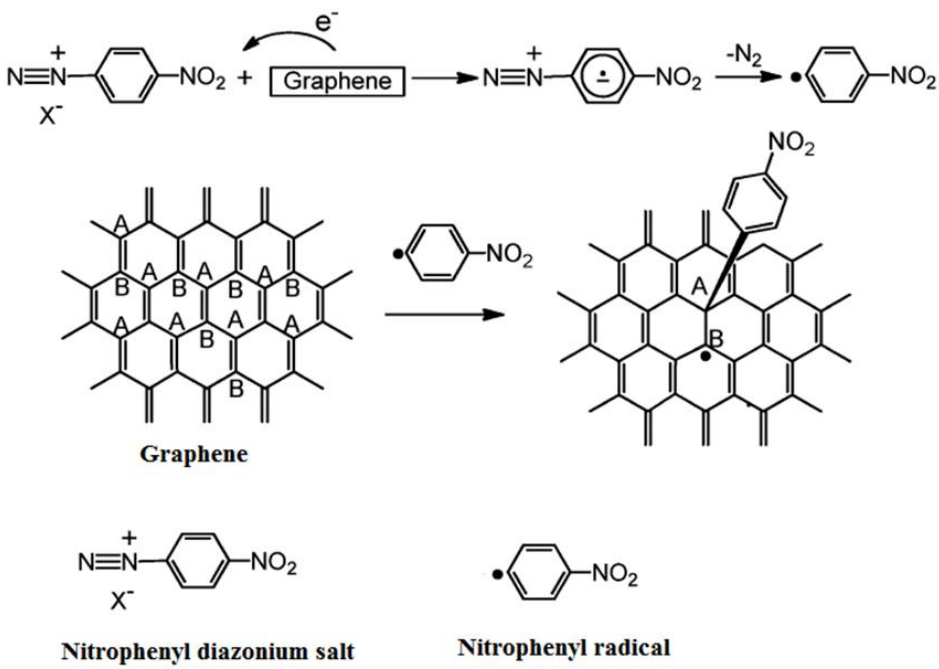

Figure 4: *Aryl diazonium salt in reaction with graphene. Above showing the radical generation from diazonium salt. Below showing the attachment of the radical to graphene. A and B show the two possible positions where the radicals can attach.* [18]

Aryl diazonium salts has several qualities making it useful. They are easily produced from aniline derivatives. They react under ambient conditions, meaning room temperature and normal atmospheric pressure. The leaving

group, nitrogen gas, is very unreactive and does not interfere with the wanted reactions. Another advantage is that the electron transfer reaction needed to form a radical can be driven in an electrochemical cell shortening the reaction time considerably. [18]

2.7 Antibodies

Antibodies are large proteins used by the immune system to detect foreign substances in the bloodstream. Every antibody is specific for one substance, called its antigen. When an antibody come into contact with their antigen it will bind to it. This stimulates the immune system to destroy the antigen.

Antibodies are commonly used in diagnostics because of their relatively easy production and high specificity. Either the level of antibodies can be measured, high levels likely means that the body is infected and has started producing antibodies to combat the infection. Or antibodies can be used to measure the level of its antigen, thereby detecting an illness. Often fluorescent molecules are bound to antibodies so that they can be detected with a microscope. [4]

2.8 Raman Spectroscopy

Raman spectroscopy is a method with which a sample can be characterized by directing a laser on to it and measuring the inelastically scattered photons. The energy shift of these photons will depend on the shape of the molecules. Either the scattered photons can gain energy, called anti-Stokes Raman scattering, or it can lose energy, called Stokes Raman scattering. The two types of Raman scattering is symmetrical around the incident light's energy. Raman scattering is not very common and only about a millionth of the incoming photons give rise to Raman scattering. About a thousandth of the incoming photons give rise to elastic scattering, called Rayleigh scattering.

Since photons are electromagnetic waves they will induce a dipole moment in the valence electrons when they penetrate a substance. This dipole moment depends on the oscillating electric field of the incident electromagnetic wave. It also depends on whatever internal vibrations

already exist in the substance, either ion or crystal lattice vibrations. Since the electric field oscillates the induced dipole moment will oscillate as well. This oscillating dipole will send out electromagnetic waves, some having the same frequency as the incident photons and some with shifted energy. This is the scattered light. The light with the same energy is called Rayleigh scattering and the light with changed energy will be the Raman scattering. The shift in energy comes from the internal vibrations already in the substance. [19]

When using Raman spectroscopy to characterize samples, the energy shift of the scattered electrons is often measured by its wave number in cm^{-1}, the Raman shift.

For graphene three peaks can be expected. A peak at around 1580 cm^{-1}, called the G-peak, which is always visible, a peak at around 1350 cm^{-1} called the D-peak since it comes from defects and a peak at around 2700 cm^{-1} called the 2D (G')-peak since it's the overtone of the D-peak. The D-peak requires a defect to be activated and the amount of defects can be read from it. The 2D-peak does not need a defect for its activation. For pure graphene one can only see the G and 2D (G')-peaks. This means that the presence or absence of a D-peak can be used to evaluate whether there are any defects on the graphene. These defects coming from sp^3-hybridized carbon atoms which have bound to something. [20]

For epitaxial graphene on silicon carbide the Raman spectra is a little different. The peaks for few layer graphene grown in this way will move toward a higher energy shift. The difference being larger the fewer graphene layers there are. This most likely comes from strain induced by the silicon carbide substrate. [21]

3. Method

3.1 Choice of method

The first thing I needed to do in my work was to find a method with which I would try to functionalize my samples. From Graphensic AB there was a wish that I should try to implement an electrochemical cell, which they supplied me with. Other than that they wanted the graphene to be functionalized with a useful molecule and not just something convenient. Also many methods to functionalize graphene are done on reduced graphene oxide which might not be applicable to the graphene I had.

Firstly I chose to functionalize with antibodies. I did this since antibodies are commonly used in research meaning that it probably shouldn't be hard for me to get access to them and many protocols exist for different usages of them. Furthermore graphene functionalized with antibodies might be usable as a sensor for diseases even if this might lie further into the future than my project.

In several articles I found out that it should be possible to functionalize graphene with organic radicals formed from a diazonium salt. This could be done in a matter of minutes using an electrochemical cell compared to hours if done without one. The radical would bind to the graphene covalently thus rehybridizing the carbon atoms. From the attached organic radical further functionalization could take place. The method I chose I initially found in an article written by a group from Swansea University [22].

For me this method was advantageous in many ways. I could quite easily do it myself without much training. Except for using an electrochemical cell it was mostly just putting the graphene chips in different solutions for specific amounts of time. The chemicals used were not very harmful and many of them are standard chemicals and were therefore already available to me. Protective glasses and gloves were in most cases enough. The method was also quite fast. It took less than an hour from the preparation of chemicals to the finish of the electrochemical process. An electrochemical cell and the three electrode setup used is quite simple to assemble and use plus it gives almost instant feedback from the reaction. The fact that the carbon atoms of the graphene would rehybridize was also advantageous since this might be detectable.

Other methods I contemplated included using the Diels-Alder reaction to attach an organic molecule to graphene, directly attaching atoms such as hydrogen or fluorine or functionalizing it with an organometal. These methods were rejected for several reasons but partly because they didn't involve an electrochemical cell. [22][23]

The direct attachment of hydrogen or fluoride was rejected because it involved using plasma and I would need to find a way to attach an organic molecule as well.

The organometallic process is interesting since the organometals binds to the graphene in a way that doesn't change the graphene's electronic properties very much. This since it doesn't form a bond that rehybridizes the carbon atoms of the graphene. Instead it forms a hexahapto-metal bond. This is good in some ways but for me it meant that it probably would be much harder to detect the functionalization. The process was also more complicated than the one I used. It involved heating a solution containing the graphene sample and the organometal for many hours, sometimes for two days, while under a reflux of argon gas.

The Diels-Alder reaction is a reaction between a diene, containing two double bonds, and a dienophile, a molecule reacts with a diene. The many double bonds in graphene enables it to function as both a diene and a dienophile. This is done by simply putting the graphene in the diene or dienophile and heating it for a few hours. This way graphene will form covalent bonds with the chosen diene or dienophile. This method was the second most interesting for me but was rejected because it didn't involve an electrochemical cell.

3.2 Summary

Firstly the unmodified graphene chips were investigated by electrochemical measurements and Raman spectroscopy. The chips were cleaned by putting them in acetone after this first measurement. These electrochemical measurements were done after each functionalization step. After this the diazonium salt functionalization was done. This included converting the accompanying nitro groups into amine groups. Then the chips were once again investigated by electrochemical measurements and Raman spectroscopy. The primary antibodies were then immobilized and

another round of electrochemical measurements done. Lastly the secondary antibodies, with a fluorescent molecule, were immobilized onto the chips. After this last step the chips were looked at with a confocal laser scanning microscope to detect the fluorescence followed by a round of electrochemical measurements.

3.3 Graphene samples

Four, 7x7 mm^2, 4H-silicon carbide chips with graphene on the silicon terminated surface was used in the experiments. These chips were provided by Graphensic AB. Three samples were newly manufactured and one was nine months old. Two of the newer chips had a monolayer of graphene and the third chip a bilayer. The older chip had a monolayer of graphene as well. The quality was such that 95 % of the graphene was a monolayer on the monolayer chips and 50 % of the graphene was a bilayer on the bilayer chip. The graphene samples were kept in plastic containers when not subject to an experiment. The samples were named monolayer A, B, G and bilayer. Monolayer G was the older sample and was named after the Swedish name for old, gammal.

3.4 Electrochemical setup

Electrochemical measurements as well as the electrochemical functionalization were done in a three-electrode setup using an in-house fabricated electrochemical cell, see *Figure 5*. The graphene on silicon carbide was used as working electrode, a platinum wire as auxiliary electrode and an Ag+/AgCl in 3 M NaCl electrode as reference electrode. An O-ring was used to define the electrode area and to seal the cell. Its thickness was 1.5 mm and its outer diameter was 6.3 mm. The measurements was done with a CompactStat.e potentiostat from Ivium Technologies using the software IviumSoft.

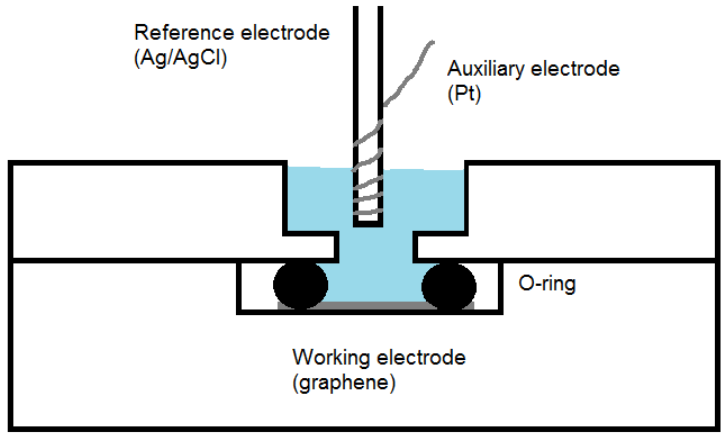

Figure 5: Schematic of the electrochemical cell used.

3.5 Diazonium functionalization

To bind nitro-phenyl molecules to graphene, 4-nitrobenzenediazonium tetrafluoroborate (4-NPD) salt was used, see *Figure 6*. To get it to bind to the graphene the reaction was driven by cyclic voltammetry using graphene as the working electrode. As electrolyte 0.1 M of tetrabutylammonium tetrafluoroborate (NBu4BF4) was used. In this electrolyte 4-NPD was dissolved so that its concentration was 2 mM.

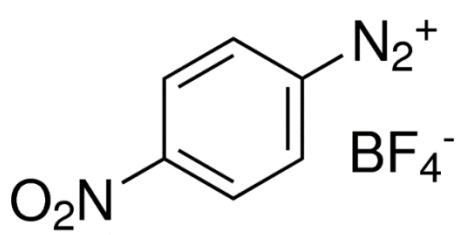

Figure 6: 4-nitrobenzenediazonium tetrafluorobate, the diazonium salt used [24]

The cyclic voltammetry was performed using the same setup as during the cyclic voltammogram measurements. The sweep was performed from 0.5 V to -0.8V with a sweep rate of 100 mV/s and cycles were done until the oxidation peak from the reaction disappeared, see *Figure 7*. This happened

already after the first cycle but in total four cycles were done so that it would be certain that the reaction had taken place. Afterwards, with the graphene chip still in place in the electrochemical cell, the graphene was cleaned first with ethanol and then water. Then the nitro groups on the phenyls were converted into amine groups. This was done by applying a constant voltage of -0.9 V during 100 s so that a stable current was obtained. To do this the same setup as in the electrochemical measurements were used and the voltage was swept from -0.89 to -0.91 V two times with a sweep rate, 0.4 mV/s, so that two cycles would take 100 s. This was then done once more to make sure that the current really was stable.

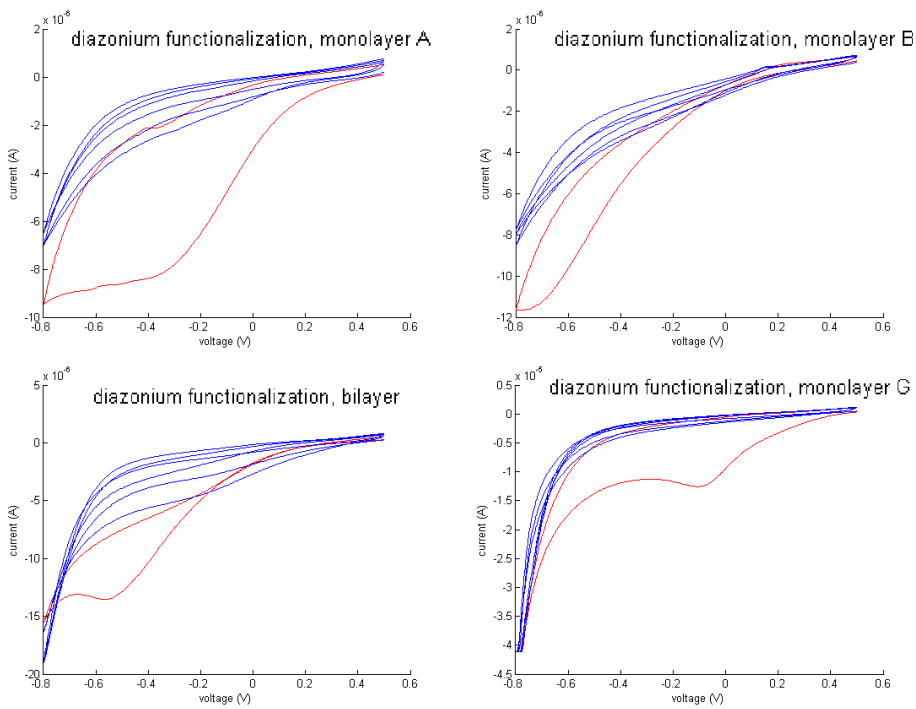

Figure 7: Cyclic voltammograms from the diazonium functionalization. The red curve is from the first voltage sweep. The blue curves are from the following sweeps.

3.6 Primary antibody immobilization

To get the primary antibody to bind to the graphene surface the amine groups of the attached phenyls were used. An antibody solution was made of 990 µl of 100 µg/mL of immunoglobulin G (IgG) from rabbit serum in phostphate buffered saline (PBS) with 5 µl of 8 µg/µl 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) in PBS and 5 µl of 22 µg/µl *N*-hydroxysuccinimide (NHS) in PBS. This solution was incubated for fifteen minutes and then placed on the graphene chips and left there for two hours. After that the chips were cleaned with PBS. Then a PBS solution with 0.5 mg/mL of bovine serum was placed on the chips. This was to inactivate the remaining amine groups. This solution was left on for 30 minutes. Lastly the chips were cleaned with PBS.

3.7 Secondary antibody immobilization

To be able to detect the immobilized primary antibodies, secondary antibodies with a fluorescent molecule, Anti-Rabbit IgG-FITC antibody produced in goat, was coupled to the primary. The chips were first cleaned by placing the chips in PBST and put on a shaker for five minutes. PBST is a PBS solution with 0.05 % tween 20, a detergent, added. This was done three times each time replacing the PBST. After this the solution containing the secondary antibody was put on the chips and left to incubate in the dark for one hour. Then the chips were once again cleaned with PBST on a shaker for three times, five minutes each time, changing PBST each time. The chips were dipped in filtrated water for about five seconds to get rid of excess salt from the PBS and then dried. The chips were covered in aluminum foil to protect the fluorescent molecule from light.

3.8 Electrochemical measurements

Between each functionalization step cyclic voltammetry was done to see if there was a detectable difference. This was done with several voltage sweep rates, ranging from 5 to 500 mV/s, starting with a sweep rate of 50 mV/s to evaluate which start and stop voltage values that should be used. Measurements were done with a pure electrolyte of 1M KCl in water and with two different redox pairs dissolved in the electrolyte so that their

concentration was 1 mM. The redox pairs used were standard redox pairs, ruthenium and ferrocene, from hexaammineruthenium(III) chloride, ($Ru(NH_3)_6Cl_3$) and 1,1'-ferrocenedimethanol, $Fe(C_5H_4CH_2OH)_2$. The electrochemical cell was cleaned with filtrated water before adding or changing electrolyte. Sweeps were done from a negative voltage to a positive voltage for the pure electrolyte and the ferrocenedimethanol and from a positive voltage to a negative voltage for the hexaammineruthenium chloride. This depending on if the redox pairs started in their reduced or oxidized state, with hexaammineruthenium chloride starting in its reduced state. For each sweep rate two cycles were performed with the second cycle saved for evaluation.

An impedance spectroscopy measurement was also done for the same solutions using the same setup. The DC offset which the voltage frequency was varied around was taken from the cyclic voltammograms choosing the voltage which was between the reduction and oxidation current peaks, -180 mV for hexaammineruthenium chloride and 260 mV for ferrocenedimethanol. For the pure electrolyte the voltage frequency was varied around 0 V. The frequencies used for the measurement were between 50 kHz and 0.1 Hz.

3.9 Raman spectroscopy

Raman spectroscopy was done to evaluate the diazonium functionalization of the graphene chips. The ccd camera used was an iXon model from Andor together with a laser with a 532 nm wavelength. This was evaluated by an in-house written LabVIEW program. The exposure time was set to 120 s. All four samples were measured a few times. Some measurements in the middle and some closer to the edges. This so that some measurements would be done where the functionalization would take place, which was in the middle, and some where nothing should be done, close to the edges. Also a sample of pure silicon carbide was measured so that its spectra could be subtracted from the spectra gotten from the graphene chips.

3.10 Fluorescence detection

To detect the fluorescent molecule, FITC, the chips were looked at with a confocal laser scanning microscope. This was done to evaluate where the functionalization had taken place. The microscope was a BX51WI made by Olympus. To it an Olympus Fluoview was connected with the corresponding software. The laser used to activate the FITC had a wavelength of 490 nm. Two lenses were used, one with 4 times magnification and one with 20 times magnification. The chips were placed on a microscope slide, a few drops of water placed on them and then covered with a cover glass. Lastly a few drops of immersion oil was placed on top of it all to connect to the object lens. The chips were investigated and pictures of interesting areas were taken. The photomultiplier tube, PMT, was changed so that clear pictures were gotten. Higher values means it is more sensitive to light.

4. Results

4.1 Raman spectroscopy

The data from the Raman spectroscopy measurements modified compared to the raw data. Firstly the different spectra were lowered so that they would be on the same level of intensity. Then they were normalized so that the highest value would be equal to one. This produced the following figures.

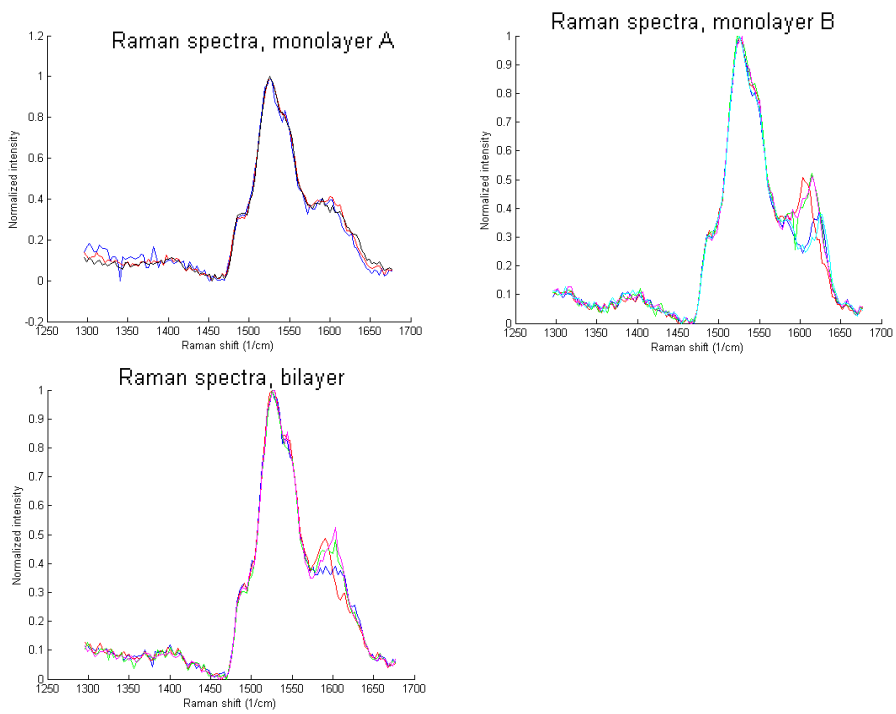

Figure 8: Raman spectra from unmodified graphene on silicon carbide. Measurements were taken from various positions on the chips.

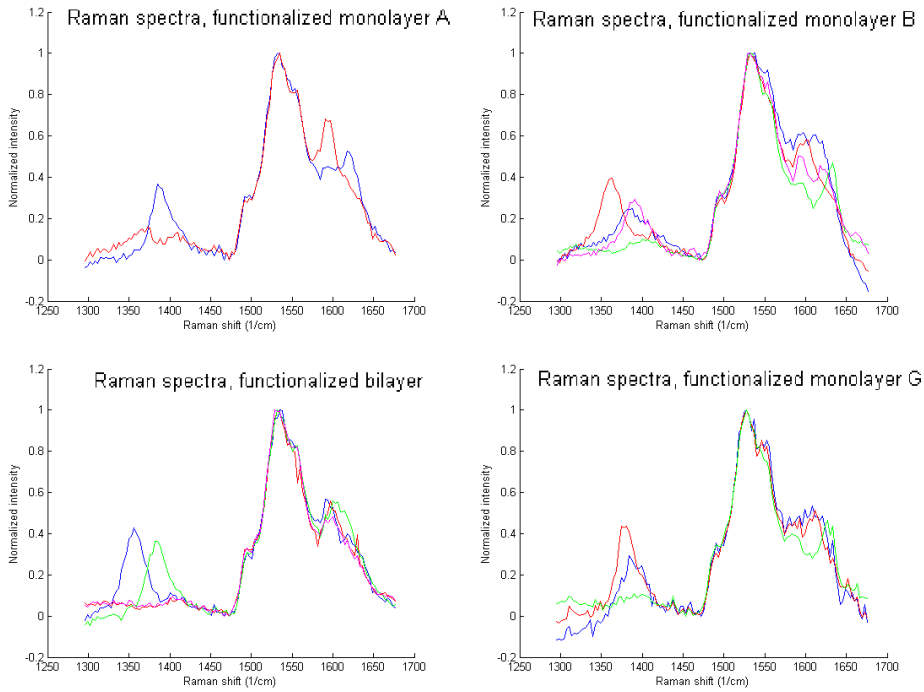

Figure 9: Raman spectra from modified graphene on silicon carbide. Monolayer A, red line is from close to the edge, blue line is from the middle. Monolayer B, green line is from close to the edge, red, blue and purple lines are from the middle. Bilayer, red and purple lines are from close to the edge, blue and green lines are from the middle. Monolayer G, green line is from close to the edge, red, and blue lines are from the middle.

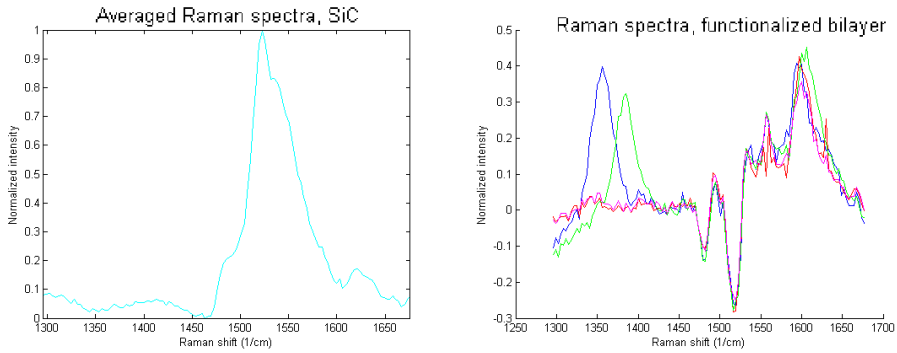

Figure 10: Raman spectra from silicon carbide and Raman spectra from modified bilayer graphene on silicon carbide with the silicon carbide background subtracted.

4.2 Cyclic voltammetry

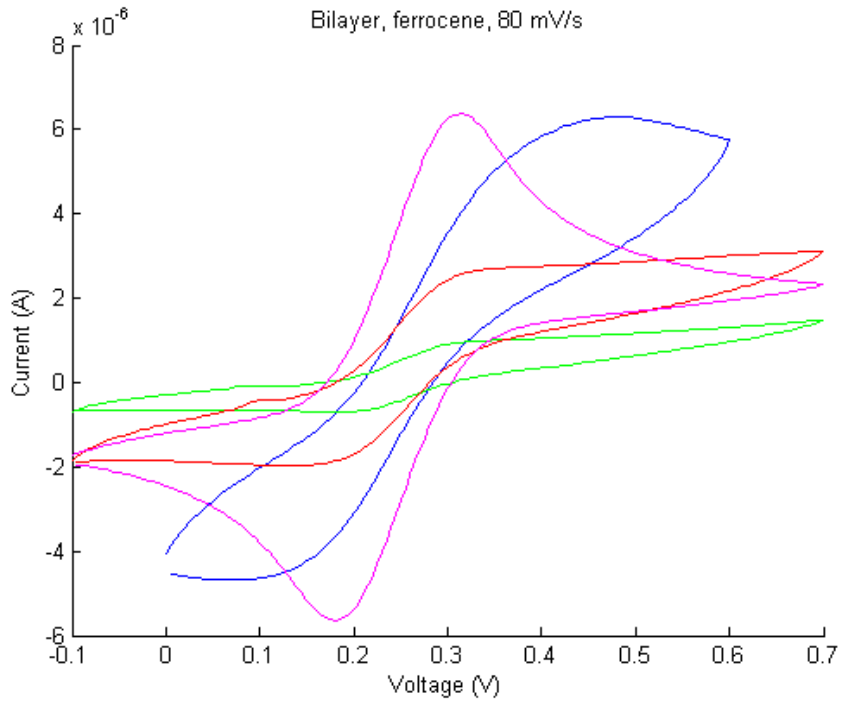

Figure 11: *Example of cyclic voltammograms. These are on the bilayer with a sweep rate of 80 mV/s. The blue curve comes from the unmodified graphene. The green curve from the graphene modified with diazonium salt. The red from the graphene with the primary antibody. The purple from the graphene with the secondary antibody.*

In the figures below the peak values is shown for the different voltage sweep rates against the square root of the voltage sweep rates. Both ruthenium and ferrocene were used for the measurements.

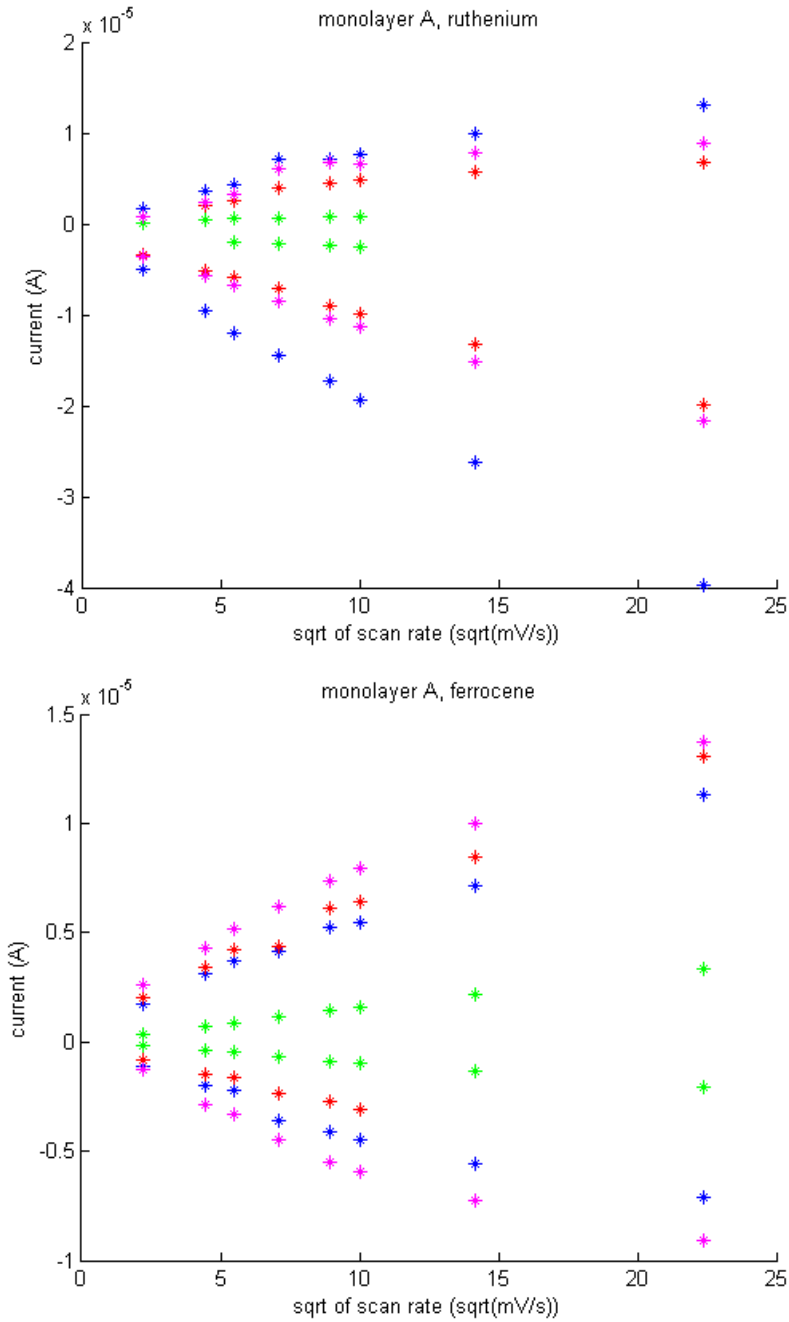

Figure 12: *Cyclic voltammogram peak currents from monolayer A. Blue dots come from unmodified, green from diazonium, red from primary antibody and purple from secondary antibody functionalized graphene.*

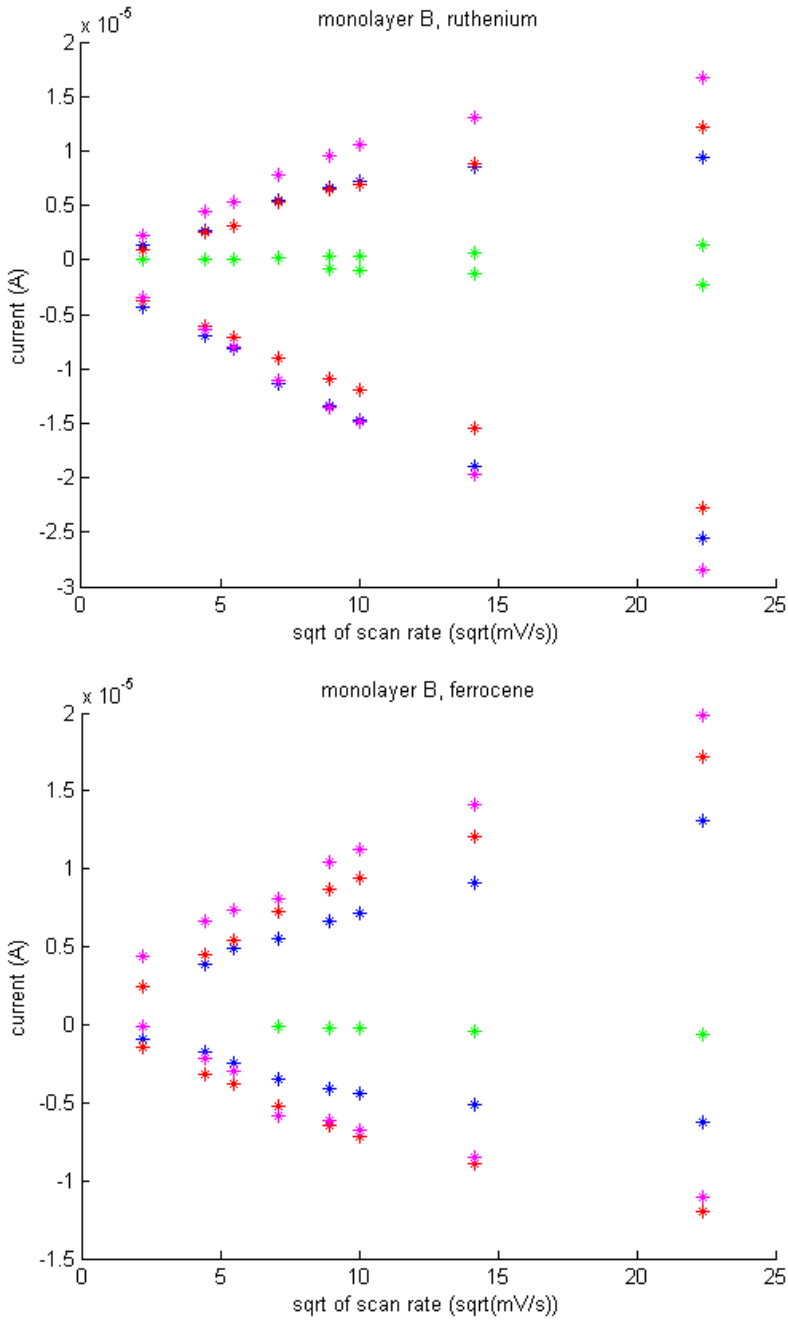

Figure 13: Cyclic voltammogram peak currents from monolayer B. Blue dots come from unmodified, green from diazonium, red from primary antibody and purple from secondary antibody functionalized graphene.

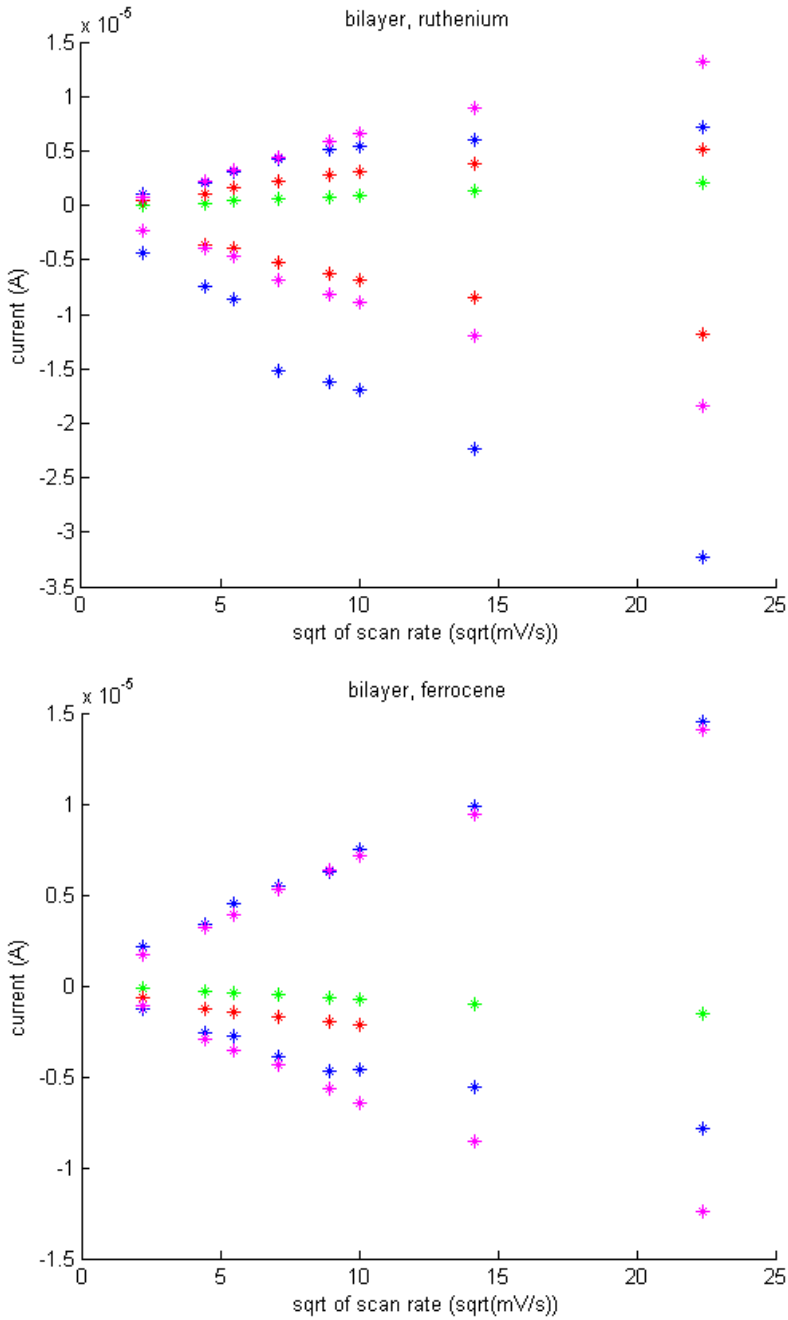

Figure 14: Cyclic voltammogram peak currents from the bilayer. Blue dots come from unmodified, green from diazonium, red from primary antibody and purple from secondary antibody functionalized graphene.

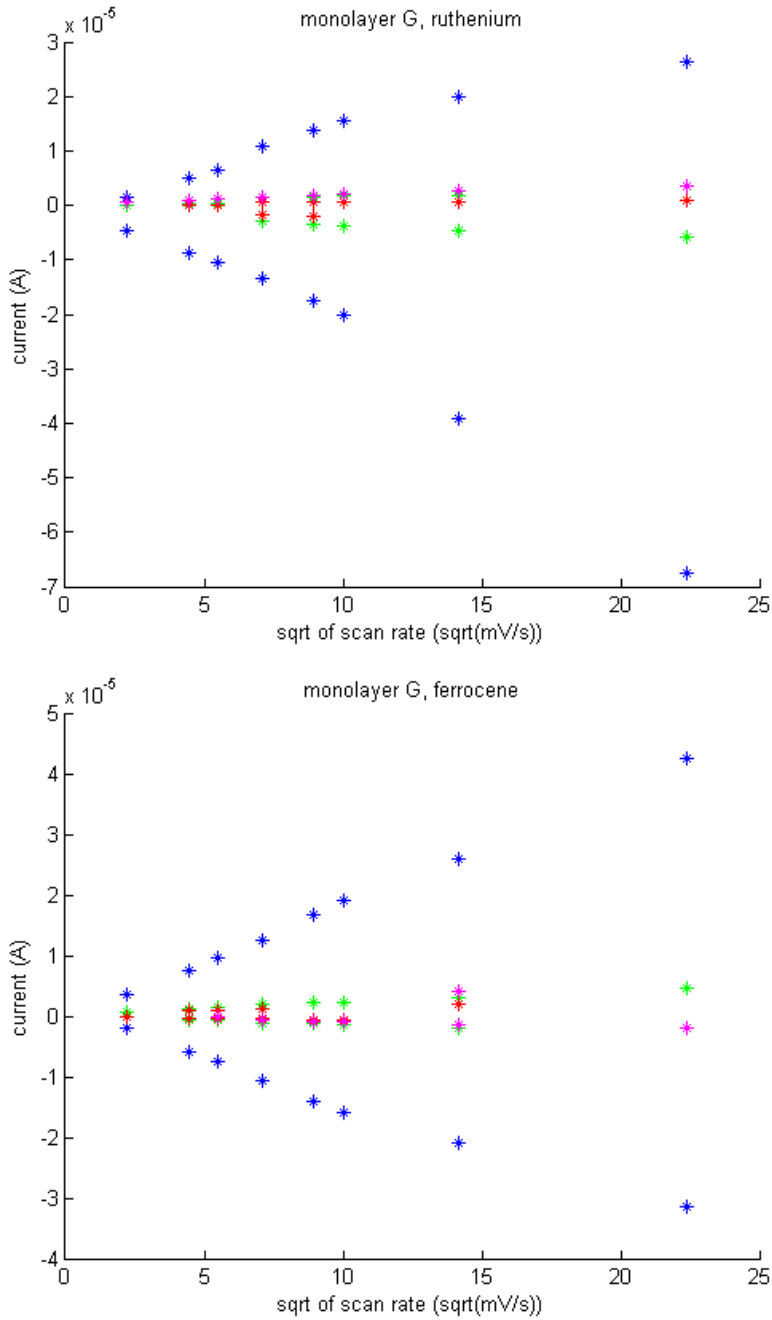

Figure 15: Cyclic voltammogram peak currents from monolayer G. Blue dots come from unmodified, green from diazonium, red from primary antibody and purple from secondary antibody functionalized graphene.

4.3 Impedance spectroscopy

In the figures below the absolute value of the impedance is plotted against the voltage frequency. The values are logarithmic for better representation. Measurements are taken from the different functionalization stages with each stage represented by individual curves. Measurements were done in pure electrolyte, KCl, electrolyte with ferrocene, fc, and electrolyte with ruthenium, ru.

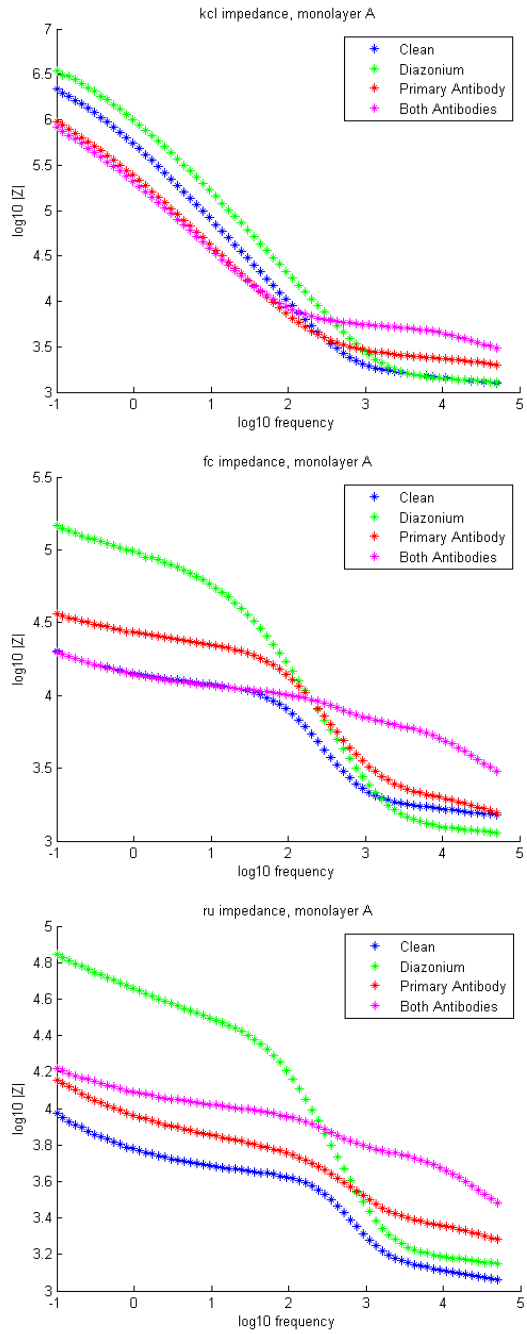

Figure 16: Absolute value of impedance against voltage frequency for monolayer A. After the different functionalization stages using different electrolyte solutions.

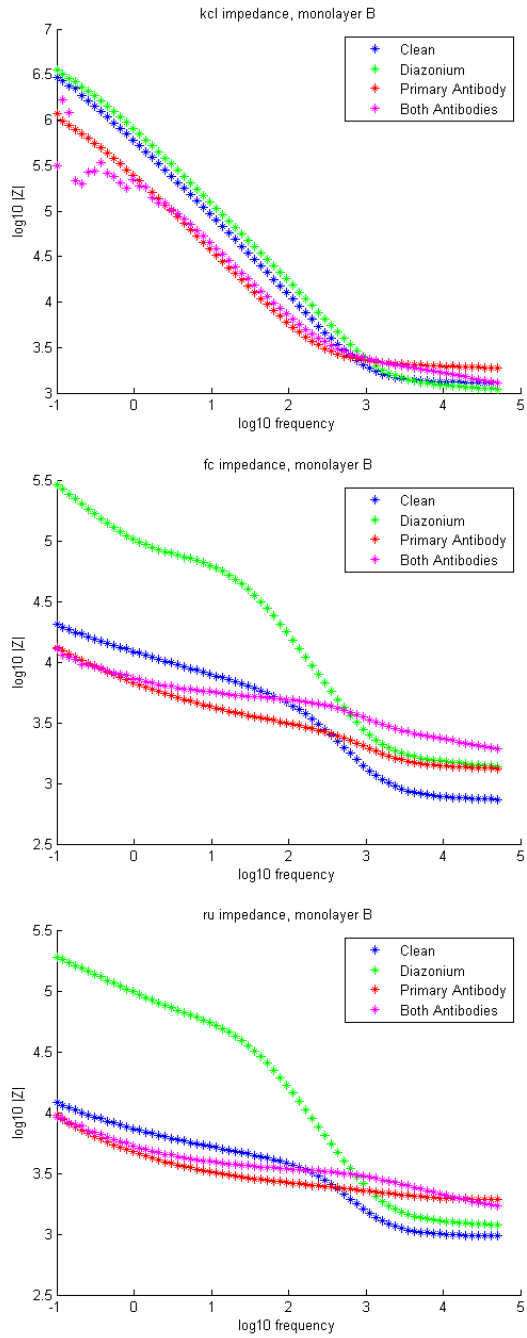

Figure 17: Absolute value of impedance against voltage frequency for monolayer B. After the different functionalization stages using different electrolyte solutions.

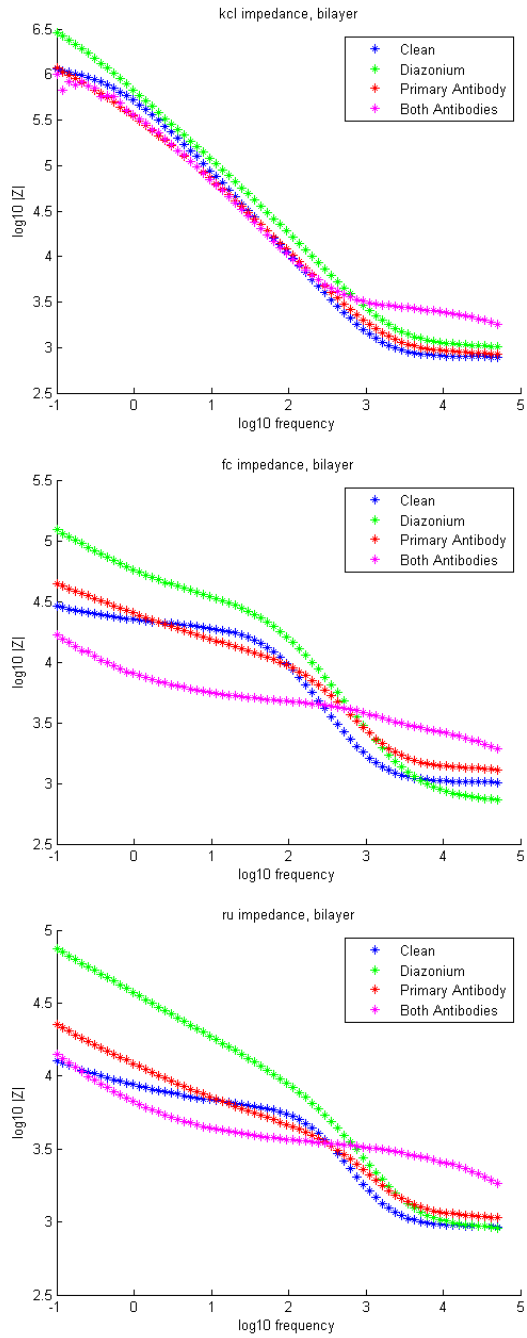

Figure 18: Absolute value of impedance against voltage frequency for the bilayer. After the different functionalization stages using different electrolyte solutions.

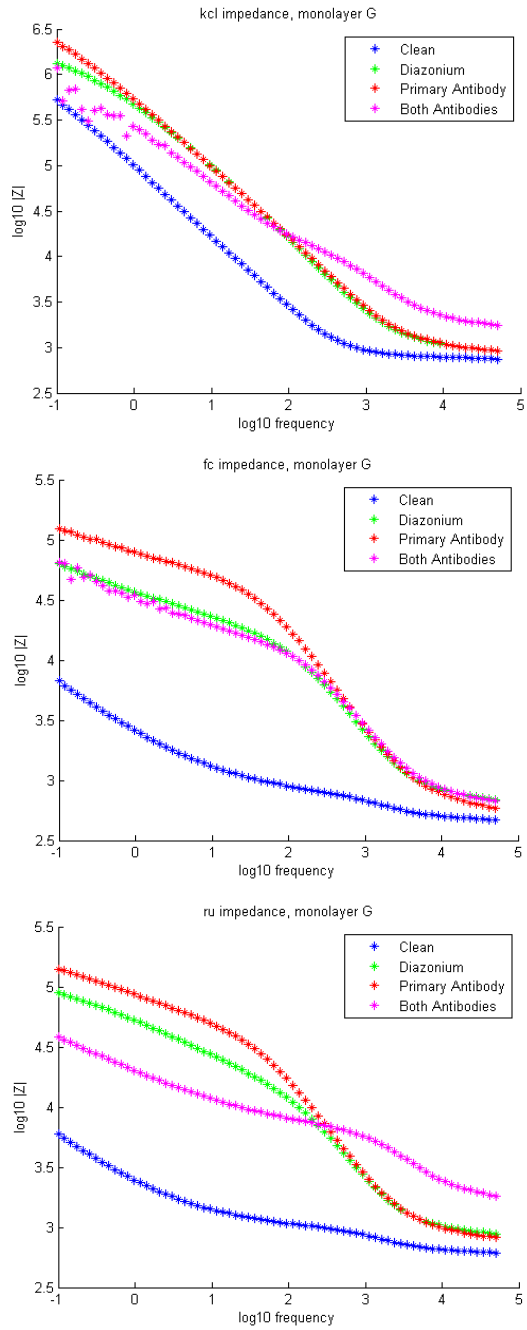

Figure 19: Absolute value of impedance against voltage frequency for monolayer G. After the different functionalization stages using different electrolyte solutions.

4.4 Fluorescence

Below are images taken from the fluorescence detection. The green comes from the fluorescent molecule and thus the secondary antibodies. First a zoomed out picture is shown then two zoomed in pictures. The first zoomed in picture is from the middle and the second from the edge of the sample. This is shown for all four samples.

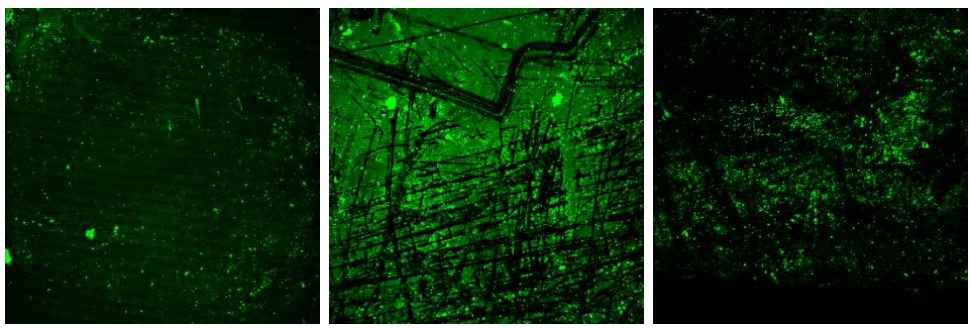

Figure 20: Fluorescence pictures from monolayer A.
From left to right: zoomed out picture (x4 lense) from the middle with PMT set to 595, zoomed in picture (x20 lense) from the middle with PMT set to 595, zoomed in picture (x20 lense) from the edge with PMT set to 595.

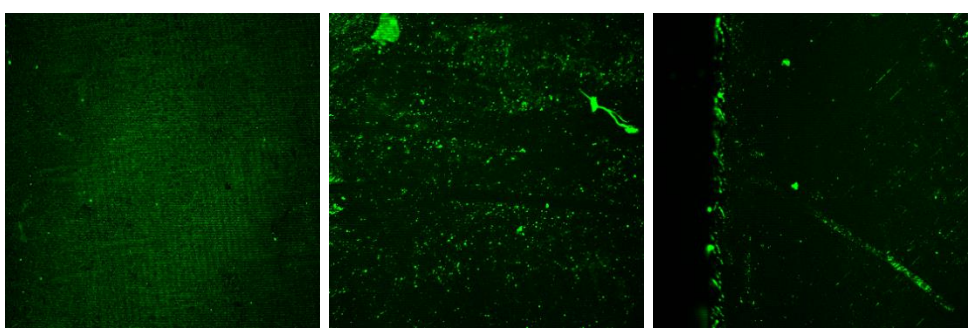

Figure 21: Fluorescence pictures from monolayer B.
From left to right: zoomed out picture (x4 lense) from the middle with PMT set to 700, zoomed in picture (x20 lense) from the middle with PMT set to 595, zoomed in picture (x20 lense) from the edge with PMT set to 595.

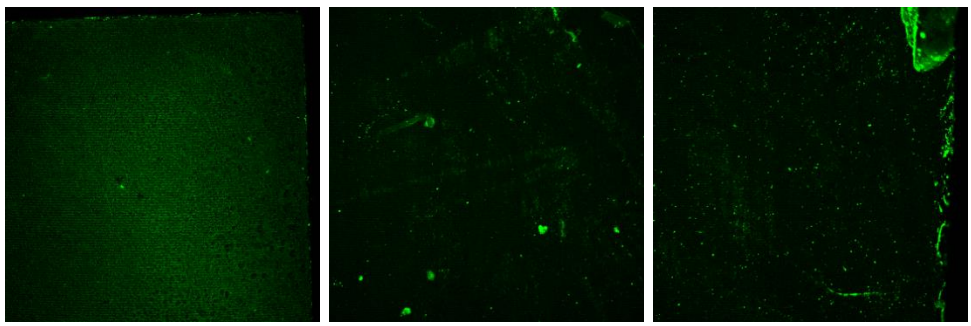

Figure 22: Fluorescence pictures from the bilayer.
From left to right: zoomed out picture (x4 lense) from the middle with PMT set to 700, zoomed in picture (x20 lense) from the middle with PMT set to 595, zoomed in picture (x20 lense) from the edge with PMT set to 595.

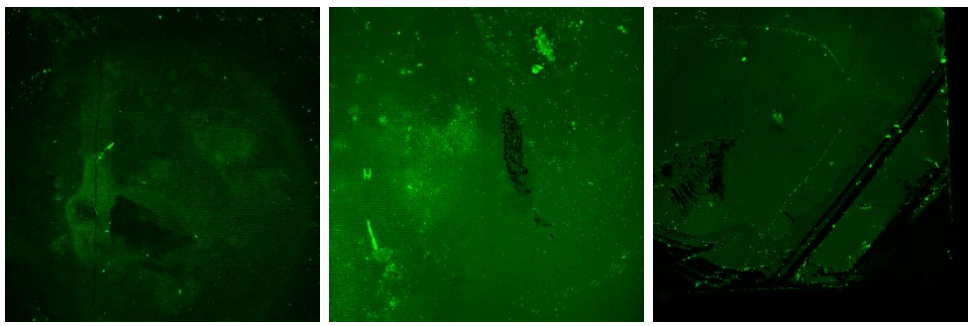

Figure 23: Fluorescence pictures from monolayer G.
From left to right: zoomed out picture (x4 lense) from the middle with PMT set to 595, zoomed in picture (x20 lense) from the middle with PMT set to 400, zoomed in picture (x20 lense) from the edge with PMT set to 400.

5. Discussion

5.1 Raman spectroscopy

When doing the Raman spectroscopy the presence or absence of a D-peak was what was interesting. For the unmodified graphene layers it was expected that no or at least a very small D-peak would be present. From the measurements no D-peak was visible. This indicates that the graphene was of a very high quality and possible defects were very few. After the diazonium salt modification it was expected that the measurements from the middle of the graphene chips, where the functionalization had taken place, would have a D-peak. The measurements from the edges were expected to give a spectra like the unmodified graphene before. This is exactly what happened which indicates that the functionalization was successful.

The D-peak were however not the best looking. The peaks were quite broad but most worryingly the position of them varied. Most of them were found to be centered at a slightly higher shift than 1350 cm^{-1}. Most of them at 1385 cm^{-1}.

The spectra are presented with the background spectra form silicon carbide still present. This was simply because the pictures with the background subtracted looked too messy. This might seem strange that I did not include them just because of that. But the problem was that the spectra from the silicon carbide and the spectra from the graphene samples did not match. The largest peak from the silicon carbide was situated at a slightly lower max value than the largest peak from the graphene chips. This caused the spectra with the background subtracted to look messy. But only at Raman shifts above 1450 cm^{-1}. For lower shifts the background did not have any peaks. Also this lower region is where the D-peak is situated. Since the G-peak at around 1600 cm^{-1} is not important but the D-peak is I could show the spectra with the background left in.

However there is a G-peak visible in the spectra from the graphene, those were the silicon carbide spectra is subtracted. These peaks are situated at a slightly higher Raman shift they should be. But a slightly higher Raman shift can be expected from few layer graphene grown on silicon carbide.

Over all the Raman spectroscopy was an important step because it showed that the diazonium salt functionalization worked. It also did so quite convincingly with clear D-peaks.

5.2 Cyclic voltammetry

The cyclic voltammograms were of mixed results and not too much is visible from them. In general though it seems like the peak currents first decreased with the diazonium salt functionalization and then increased again with the addition of antibodies.

One thing that always was true though was that the addition of the secondary antibody increased the peak currents. Not much but always. Except in one case which was for the negative peaks of monolayer B when ferrocene was used. For those measurements the peak currents were mixed. The increase in peak currents when adding the secondary antibody is really interesting since this might be a way to detect whether the antibody has reacted with its antigen. However since the difference is quite small I expect that the size and type of antigen influences the difference in current. Still it is one of the goals of the work and it is detectable from the produced cyclic voltammograms.

The decrease of current after the diazonium functionalization and the following increase after antibody attachment is in line with previous work. However the increase in current after the addition of the primary antibody was not true for monolayer G. Here the currents of the antibody modified graphene stayed at the same level as after the diazonium salt functionalization. I don't have any idea why this has happened.

One problem that occurred after the diazonium functionalization was that for some of the cyclic voltammograms no peaks appeared. Instead the current continued to rise or decrease. This might be because the reduction or oxidation peak position had simply moved further away. But changing the start and stop voltage did not give rise to any peaks. Instead the current just continued in the same direction. This might be because of something in the electrochemical setup but might also be because of the functionalization itself. I don't think that the current peaks have moved outside of the scan range because some of the peaks are after all there. It was only a few times that an entire series was without peaks. It might be

that for some reason the oxidation does not happen and therefore there aren't anything to reduce. This might happen the other way around as well.

Unfortunately there was a peak at around 0.1 V which was evident in most cyclic voltammograms. This peak is most likely from some kind of contamination which might have influenced all of the measurements with emphasis on might. It is simply not possible to know from my measurements. The contamination peak decreased quite a bit with successive scans and for some scans was hardly visible at all. I suspect that the corresponding oxidation peak is around -0.2 or -0.1 V. So if the scan doesn't reach the oxidation the reduction peak should disappear. But for many scans neither an oxidation or reduction peak were visible even though they should have been in range.

3.3 Impedance spectroscopy

Like the cyclic voltammograms before the results from the impedance measurements gave mixed results and not much is visible. But some interesting things can be gotten from the measurements. Firstly there seems to be two regions in the impedance curves. One for lower frequencies and one for higher with the transition happening at around 1000 Hz.

The curves from monolayer G looks different than the others and are excluded from the analysis in the next two paragraphs.

For the lower frequencies the curves from after the diazonium salt stage were the highest with the others in no particular order. For the measurements using ferrocene and ruthenium the difference was larger than those using KCl. In general the measurements using KCl looks different. The curves are more uniform and the transition between the regions happens quicker.

For the higher frequencies the curves from after the secondary antibody attachment are highest in most of the curves. When it's not the highest it is at least second highest. The curves from the unmodified graphene are often the lowest or close to the lowest. In general it seems that for high frequencies an increase in impedance can be expected when attaching the secondary antibody. But it really can't be said for sure.

Like before monolayer G is different from the others. Also for the impedance it is the stages after the attachment of antibodies that are different. For the other graphene chips the impedance curves after the antibody functionalization is closer to that of the unmodified chips. Here they are closer to impedance curve after the diazonium salt modification. Just like in the cyclic voltammograms. For monolayer G it also seems that the impedance after the attachment of the secondary antibody increases for high frequencies.

3.4 Fluorescence

The fluorescence measurements were done to see where the secondary antibody had attached. What I wanted to see was a green circle in the middle and nothing else. This was unfortunately not exactly what I found. The fluorescence could be found all over the chips and not only in the middle. However there was some features that indicated that the antibodies had at least attached to the chips in a manner resembling what I wanted. Especially for two of the chips.

First of all a ring with much lower intensity could be found when zoomed in which most likely comes from where the O-ring had been lying. However the clearness of this ring varied. Both between the chips and within each chip. Also some difference could be noticed in the intensity of the fluorescence of the edges and in the middle. However this difference was mostly too small to be able to make a clear distinction. The difference for the monolayers A and G was larger than that of the bilayer and monolayer B.

For the zoomed out pictures a green circle in the middle with dark areas beside it was hoped for. The pictures from monolayer A and G also showed this suggesting a successful functionalization. Unfortunately the bilayer and monolayer B pictures did not show much at all.

Also it was visible from the fluorescence that the surface of the samples had been scratched and that there was dirt on them. This of course influenced the pictures and made it harder to see any patterns. Monolayer G though did not have nearly as much scratching and dirt. Probably because it simply had not been outside of the sample holder nearly as much. This sample went through the functionalization process after the

others and under fewer days. Also the intensity of the fluorescence varied, where monolayer G had a much higher intensity which required the PMT to be decreased.

All in all it seemed like the monolayer A and G had been successful and the bilayer and monolayer B less so. It simply was not possible to say very much about the bilayer and monolayer B. When zoomed in some features were there but when zoomed out not much was visible at all. For monolayer A and G it was the zoomed out picture that to me showed that the process had been successful. This was then also backed up by the zoomed in pictures comparing the middle and the edges.

3.5 Conclusions

The most import aspect when evaluating the method used is whether it worked or not. The method I used to functionalize graphene seems to have worked. At least for two of my chips, monolayer A and monolayer G. This is what it looks like from the fluorescence pictures.

The most interesting step in the whole process is the diazonium salt functionalization. This is because after the phenyl-groups have attached to the graphene surface it is no longer the graphene itself that partakes in the reactions. Instead it is a question of organic chemistry.

The diazonium functionalization is an easy and fast process. As long as one has access to an electrochemical cell it should be no problem. Also it is possible to immediately see if the process has finished by looking at the cyclic voltammogram. When there no longer is an oxidation peak the process have finished. Also if there is no difference in the first sweep from the other the reaction has probably not even taken place. Also it is possible to do a Raman spectroscopic measurement to see whether the graphene has rehybridized to sp^3 or not. This is also quite easy and quick to do as long as one has the right equipment, which of course is far from certain. The diazonium salt chosen can be varied but it is important that an aryl radical should be created since it is reactive enough to react with the graphene. But what functional group that is attached to the aryl can be chosen so that the preferred molecule later can be attached to the functionalized surface. A drawback is that it is hard to functionalize the whole chip area. This since the graphene chip is used as the working electrode in an electrochemical

cell. A part of it has to be outside of the electrolyte chamber where it can be contacted. Also leakage has to be prevented which is easily done with an o-ring which then will leave a part of the graphene chip outside.

Using cyclic voltammetry or impedance spectroscopy it is possible to notice some changes between the different stages of functionalization. After the diazonium salt functionalization there is a huge change, increasing the impedance. After adding the primary antibody the impedance decreases again. This however depends on what molecule that is added. An antibody is a large molecule and probably influences the electronic properties more than a small molecule. Also some molecules might increase the impedance even more.

3.6 Improvements

The single most important thing to note about my experiments is that I have only used four samples. This is far from enough to be able to say very much with certainty. More samples would also make it possible to evaluate the different process steps more thoroughly.

Instead of having a fluorescent molecule on the secondary antibody it could have been attached to the primary antibody. That way it would be possible to determine how the primary antibody attaches to the graphene surface. As it is now it is not possible to determine whether it is the primary or secondary antibody that attaches itself to the unfunctionalized area of the graphene surface.

Also the antibody attachment can be done with different concentrations and during different incubation times so that the attachment process can be evaluated. For example when saturation level of antibodies is reached and how the impedance changed by different levels of antibodies.

Since the fluorescence appeared all over the chips and not just where the functionalization had taken place it would be interesting to try the antibody attachment process on an unmodified graphene surface. Depending on how the antibodies attaches to this type of surface a lot can be said of the process I did.

The graphene used in my work has been of very high quality. It consisted of very few layers and with very few edges compared to the surface area.

In general the reactivity of less pristine graphene is higher. The more edges there are and the more impurities there are and the easier it should be to modify the graphene. Therefore it would be interesting to see how the process works on different kinds of graphene. More than just epitaxial graphene on silicon carbide.

Furthermore it would be good to try the electrochemical measurements in a different setup. That way it would be possible to see how the setup I used influenced the cyclic voltammograms. Many of them lacked one or both of the current peaks and I am not sure why. It would be nice to see whether the setup could have been the cause of this.

Also it was impossible to guarantee that the position of the o-ring on the graphene surface was the same every time the chip was placed in the electrochemical cell. If a new electrochemical cell is to be manufactured this is an important aspect in the design. The holder for the chip should be the same area as the chip. Thereby making sure it's kept in the same position every time it's put in the electrochemical cell.

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7. Appendix

7.1 Detailed method

Electrochemical setup

Three-electrode setup.

Working electrode, graphene on silicon carbide

Auxiliary electrode, platinum wire

Reference electrode, Ag+/AgCl in 3 M NaCl electrode

Working electrode area defined by O-ring. 1.5 mm thickness. Outer diameter 6.3 mm.

Potentiostat, Compactstat.e from Ivium Technologies.
Software IviumSoft

Diazonium functionalization

Using the electrochemical setup

Electrolyte, 0.1 M tetrabutylammonium tetrafluoroborate (NBu_4BF_4) dissolved in acetonitrile

2 mM of 4-nitrobenzenediazonium tetrafluoroborate dissolved in the electrolyte

Reaction driven by cyclic voltammetry
From 0.5 V to -0.8 V, sweep rate 100 mV/s
Perform sweeps until oxidation peak disappears

Whilst in cell clean with water then ethanol

Conversion of nitro groups to amine groups

Using the electrochemical setup

Ethanol/water 1:9 mixture in chamber
Voltage of -0.9 V for around 100 s
A stable current should be achieved before stopping

Primary antibody immobilization

To activate the antibodies
Activate by mixing 990 µl of 100 µg/mL antibody solution in PBS with 5 µl of 8 µg/µl EDC in PBS and 5 µl of 22 µg/µl NHS in PBS

EDC: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
NHS: *N*-hydroxysuccinimide
PBS: phostphate buffered saline

Incubate in room temperature for 15 minutes

Place the antibody solution on the graphene chips so that they are covered.
Incubate for 2 hours

Clean the chips with PBS.

To inactivate the remaining amine groups. Place a PBS solution with 0.5 mg/mL of bovine serum on the chips.
Leave this for 30 minutes.

Lastly, clean the chips in PBS

Secondary antibody immobilization

Wash the chips in 10 ml PBST on a shaker for 5 minutes. Do this three times, replacing the PBST each time.

PBST: PBS with 0.05 % tween 20

Put the secondary antibody solution, 1 mg/ml, on the chips for one hour. Protect the chips from light during this time.

FITC: fluorescein isothiocyanate, a fluorescent molecule

Wash the chips in 10 ml PBST on a shaker for 5 minutes. Do this three times, replacing the PBST each time.

To get rid of excess salt from the PBST. Quickly wash the chips in filtrated water, about 5 seconds.

Dry the chips.

Cyclic voltammetry

Using the electrochemical setup

Electrolyte, 0.1 M KCl in water

Redox probes, both diluted to 1mM:

hexaammineruthenium(III) chloride, ($Ru(NH_3)_6Cl_3$)

1,1'-ferrocenedimethanol, $Fe(C_5H_4CH_2OH)_2$

Sweep rates:

5, 20, 30, 50, 80, 100, 200 and 500 mV/s

Choose maximum and minimum voltage values so that both the reduction and the oxidation peaks are included

Measure both redox probes and pure electrolyte, KCl

Perform two cycles per sweep rate and redox probe, use data from second cycle

Impedance spectrocopy

Using the electrochemical setup

Measure both redox probes and pure electrolyte, KCl

Take the DC offset from between the current peaks in the cyclic voltammograms

-180 mV for hexaammineruthenium chloride

260 mV for ferrocenedimethanol.

0 mV for pure electrolyte

Frequencies from 0.1 Hz to 50 kHz

Raman spectroscopy

CCD camera: iXon model from Andor

Laser with wavelength of 532 nm

Evaluated with an in-house written LabVIEW program

Exposure time 120 s

Measure both on areas which were functionalized and outside of those areas

Measure a sample of pure silicon carbide

Subtract the spectra from silicon carbide form the graphene sample’s spectra

Fluorescence detection

Microscope, a BX51WI made by Olympus

Olympus Fluoview connected, corresponding software

Laser with 490 nm wavelength

Two lenses, one with 20 times magnification and one with 4 times

Place chips on microscope slide

Place a few drops of water on them

Place cover glass on chips

Put a few drops of immersion oil on cover glass to connect to the lens