Characterization of a reduced graphene oxide based biosensor via infrared spectroscopy

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CHARACTERIZATION OF A REDUCED GRAPHENE OXIDE BASED BIOSENSOR VIA INFRARED SPECTROSCOPY

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To my family and friends for their endless support

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Declaration

I herewith declare that I have produced this thesis without the prohibited assistance of third parties and without making use of aids other than those specified. Notions taken over directly or indirectly from other sources have been identified as such. This thesis has not previously been presented in identical or similar form to any other Austrian or foreign examination board.

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Abstract

The fabrication of a small and user-friendly sensor, which has a high dynamic range in which it is sensitive and specific for certain analytes is a major topic in research in the last decades. The first fabrication of a graphene monolayer in 2004 caused a boom in material research because its unique electrical properties make it a potential candidate to replace materials like copper (a conductor) and silicon (a semiconductor). However, many preparation steps have to be optimized. Furthermore, diverse factors which could potentially effect the functionality of the device have to be determined. Graphene is often used in biosensors which utilise biomolecules like DNA, aptamers or proteins. Those can specifically interact with the analyte which is further detected by the device. A graphenebased field-effect transistor (GFET) was developed in our group which uses a protein as a sensing molecule. The protein is the odorant binding protein 14 from the honey bee (*Apis mellifera*). It binds specific odorants and can consequently be used for an artificial nose.

The first part of the thesis analyses the fabrication of the FET step by step via surface sensitive methods. Beside static measurements, dynamic measurements were done for critical steps. This data was used for optimization of the setup. The graphene product of Freicht et al. showed the best result for optimal surface coverage. Hydroiodic acid showed the best reduction properties. Measurements, done via infrared spectroscopy, showed that OBP14 adsorbs stable via the PBSE linker on the surface.

Further experiments were done via polarized attenuated total reflection (ATR) infrared spectroscopy to determine the orientation of certain vibrations and the surface concentration of the protein and linker. The protein concentration was determined to be $9.68 \pm 3.03 \text{ pM/cm}^2$ or $157 \pm 50 \text{ ng/cm}^2$ respectively. This is equivalent to a surface concentration of approximately 60%.

The last part deals with the effect of pH and ligand binding on the sensor protein. Therefore, the protein was immobilized via His-tag on the surface of an ATR-crystal and the environment changed periodically. The decrease in pH showed a significant spectral change which is attributed to the weakening of H-bridges in the protein and rearrangement of helix 7, which causes opening of the binding pocket. Additionally, a theoretical mechanism for ligand uptake and release was proposed. The ligand modulation showed only a very weak signal. Due to the low intensities and poor signal-to-noise ratio it was not possible to obtain clear information on structural changes or the kinetics of ligand binding.

Kurzfassung

Die Herstellung eines Sensors, der sowohl in einem großen Konzentrationsbereich sensitiv und spezifisch ist, dabei aber auch möglichst klein und einfach zu bedienen ist, ist schon lange ein großes Thema in der Analytik. Die erste Herstellung von Graphen im Jahre 2004 leitete ein neues Zeitalter in der Elektronik ein da es aufgrund seiner Eigenschaften bisherig verwendete Materialien wie Kupfer (als Leitermaterial) und Silizium (als Halbleiter) ablösen kann. Allerdings müssen dafür diverse Präparationsschritte optimiert werden. Weiters müssen Faktoren untersucht werden, welche die Funktionalität des Sensors beeinflussen. Graphen wird oft in Biosensoren genutzt in welchen Biomoleküle wie DNA, Aptamere oder Proteine als Rezeptor dienen. Diese interagieren mit den Analyten und führen zu einem Signal im Messgerät. In unserer Gruppe wurde ein auf reduziertem Graphenoxid basierender Feldeffekttransistor entwickelt, der als Rezeptormolekül ein Protein hat. Dieses Protein ist das Odorant-binding-protein (OBP14) der Honigbiene (*Apis mellifera*), welches spezifische Duftstoffe bindet und somit für eine elektronische Nase genutzt werden kann.

Im ersten Teil der Arbeit wurde jeder Schritt der Feldeffekttransistorpräparation mittels oberflächensensitiver Methoden untersucht und optimiert. Bei kritischen Schritten wurden nicht nur statische Informationen erhoben sondern auch Veränderungen im Laufe der Zeit beobachtet. Dabei konnte gezeigt werden dass das Graphenprodukt von Feicht et al. die besten Ergebnisse für eine optimale und gleichmäßige Abdeckung der Oberfläche dient. Iodwasserstoff erzielte die besten Ergebnisse bei der Reduktion des Graphenoxids. Mittels Infrarotspektroskopie konnte gezeigt werden, dass OBP14 stabil über den Pyrenelinker auf an der Graphenoberfläche adsorbiert bleibt.

Im zweiten Teil wurde mittels polarisierter abgeschwächter Totalreflexion (ATR) Infrarotspektroskopie sowohl die Orientierung als auch die Oberflächenkonzentration von Protein und Linkermolekülen bestimmt. Die Proteinkonzentration konnte für den FET auf $9.68 \pm 3.03 \text{ pM/cm}^2$ bzw. $157 \pm 50 \text{ ng/cm}^2$ bestimmt werden. Dies entspricht in etwa einer Oberflächenbelegung von 60%.

Im letzten Teil wurde der Effekt von unterschiedlichen pH und Ligand auf das Rezeptorprotein untersucht. Hierbei wurde das Protein auf der Oberfläche eines ATR-Kristalls mittels His-tag immobilisiert und die Umgebungsbedingungen periodisch geändert. Die pH-Senkung zeigte eine signifikante Änderung im Spektrum welche auf eine Abschwächung der Wasserstoffbrücken innerhalb des Proteins und Aufklappen der Helix 7 zurückgeführt wird. Zusätzlich wurde ein theoretischer Mechanismus zur Ligandaufnahme und -abgabe beschrieben. Die Ligandmodulation konnte ein schwaches Signal der Strukturänderung zeigen. Aufgrund der schwachen Ausprägung war es allerdings nicht möglich genauere Informationen über diese Änderungen oder deren Kinetik zu bestimmen.

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Chapter 1

Introduction

In the course of history, famine caused by crop pest pathogens has struck humanity several times. However, the use of new technologies (industrialisation, fertilizer) and more efficient artificial irrigation increased the yield of crop production to reduce the threat of famine. With increasing population and monoculture during industrial and agricultural revolution, this problem became again more important as a single disease could destroy tons of crop. One famous example was the great famine of 1845 in Ireland. It was caused by the oomycete *Phytophthora infestans* which infected the potatoes and further led to the destruction of a large portion of the crop yield and approximately a million deaths caused by starvation. More recently, the bacterium *Xylella fastidiosa* spreads in Italy (2013 till now) which cause a massive death of olive trees and consequently a massive decrease of olive yield in this area. Although this is more an economic problem than a problem with food supply, it shows the danger of pest in our time.

Figure 1.1: (A) Total global crop production in 2014 (in million t). 7.6 billion t of crop was produced. Approximately 75% of the production is from the top 10 most harvested crops (5.8 billion t). (B) Global loss of five mayor crops due to pest and their mayor contributors in 2001 (adapted from [1]). The first column represents the estimated loss of crop worldwide without any physical, biological or chemical pest control, while the second column shows the actual loss although pest control measures are taken.

Today, according to Food and Agriculture Organisation of the United Nations, the world produc-

tion of the top ten most important crop commodities was approximately 5.8 billion t with a total global crop production of more than 7.6 billion t in the year 2014 (Fig. 1.1A). Including meat, fish, egg and milk, which sum up to additional ≈ 1.1 billion t, approximately 45% (3.9 billion t) of the total food production is for human consumption, while the rest is for livestock raising. This is an equivalent of approximately 1.4kg/person/day . However still more than 800 million people (11% of world population) starve because of inadequate nutrition. One cause of the insufficient food supply are weather phenomena like drought, cold periods and flooding. Another reason for pre-harvest loss of up to 35% of the world crop production is crop pest [1, 2]. According to a work of Oliveira et al., insect pests alone generate an annual loss of 7.6% (equivalent of 25 mio t) and a economic loss of 17.7 billion US\$ in Brazil only [3]. Common crop pests are insects like desert locust, fungi like *Aspergillus flavus* (produces aflatoxin), black stem rust (wheat), coffee wilt disease (coffee), viruses like the mosaic virus (causes the loss of high yields of tomatoes, potatoes and other plants) and bacteria like *Xanthomonas oryza* (rice) [4]. Additional to the starvation in the developing countries, globalisation and global warming increases the risk that some crop diseases spread to the industrial countries [5].

In order keep the spreading of these at bay, massive amounts of pesticides are used in agriculture. This is especially effective against insects and weed. However, beside the environmental issue of using pesticides there are also health issues of people living on or nearby farmland. The protection of plants and stopping of pest spreading is more difficult for fungal, virus and bacterial infections. It is necessary to identify the sick plant as soon as possible and isolate/quarantine or destroy it. Fig. 1.1B illustrates the effectiveness of crop protection. The application of biological, chemical or physical crop protection shows significant effect on the crop yield. However, it also shows that the crop loss is still unacceptable high and the potential of early detection systems for crop pest. This is especially important since this data does not include any post harvest loss (e.g. storage, transport, processing, packaging, waste) which range from 10-40% [6].

Plants are not defenseless in the fight against parasites and infections. Many different mechanisms were developed to counter parasites and pest. One way is the development of genetical resistances [4]. In this case, proteins which are essential e.g. for viral infection are altered so that the virus is not able to infect the cell. An indirect defense mechanism is the attraction of enemies of the attacker. Plants are known to change their volatile organic compound (VOC) pattern in consequence of attack of herbivores or infections. These odorants attract predators and parasites of the herbivore [7–12]. Corn seedlings attract the parasitic wasp *Cotesia marginiventris* by releasing terpenoid volatiles after being fed to caterpillars [10]. Those attack the herbivores. Furthermore, the VOC have an antimicrobial effect. Lima beans use E-2-hexenal and Z-3-hexenol after infected by *Pseudomonas syringae* which causes the stopping of bacterial growth [13]. This growth inhibiting effect was also reported after the infection of cotton leaves by the fungus *Aspergillus flavus* after release of E-2-hexenal [14].

These special volatiles which are an indication of a disease are of special interest for sensor research. The main goal is to make an “artificial nose” to detect these VOC. It could be used for a non invasive screening platform to detect infected or sick plants in a fast and easy way. This screening method could enable us to remove them before the disease can spread over the plant/crop-population.

Currently, there are several different methods available to detect infections. Most important ones are immunochemical methods like enzyme linked immunosorbant assay (ELISA) or immunostrip lateral flow devices and polymerase chain reaction (PCR) based methods [15–17]. All provide

fast and easy high throughput detection of over 700 different pathogens [18]. However, there are some drawbacks. First of all, the plants have to be screened, which is very challenging for a large area as the sample collection needs to be done invasive. Secondly, if a plant is infected, pathogens are localized in different parts of the plant (e.g.: leaves, roots, periderm) which makes the collection of samples more difficult. Additionally symptoms for diseases can be very different and are not unique for one specific disease. Those symptoms are often shown in a later stage of infection. So the pathogen could already have spread to many other plants in the surrounding area. This makes it more important, that a sensor system detects these infections in the early stage and covers a large area. Further analysis about the kind of infection could be done with previous mentioned methods.

The “artificial” or “electronic nose” is a sensor platform which mimics the nose by using electronic devices and/or biological component (DNA, proteins,...) to detect odorants. Several groups already found different approaches to detect VOC [19] and distinguish between different pathogens [20]. However, this detection was always done with highly complex machines like gas chromatography-mass spectroscopy coupling. Those methods are highly sensitive, provide quantitative and qualitative information, but require highly skilled and trained user and are not usable in the field [21].

Consequently research focused onto the development of a sensor which has following specifications:

- small size device with cheap and single use sensor
- high specificity and sensitivity, with large dynamic range
- user-friendly equipment
- works in complex media (e.g. blood) without additional preparation
- simultaneous detection of different analytes
- provides quantitative and qualitative information within minutes

In recent years, first detection systems with focus on a so called lab-on-a-chip (LOC) devices are in focus of research to fulfill these goals [22, 23]. LOC means, instead of using large and expensive equipment to determine the quantity of an analyte, the analysis is done on a single small device. Instead of using a few ml of sample only a drop is needed. Additionally there is no need for professionally trained persons.

1.1 Biosensors

Biosensors are an alternative for the detection of complex analytes. Generally speaking, they consist of three parts: a bioreceptor, a transducer for sensing and an electronic system for signal processing and display. The key component of a biosensor is the bioreceptor. These are biomolecules like DNA, proteins or aptamers which have an affinity to certain ligands. So instead of using a big and complex electronic instrument for sensing, a small biological compound (e.g. protein) can be used. The transducer is composed of the general setup eg. a LOC. It can consist of channels, electrodes, optical elements or nanoparticles. It detects the interaction

between the receptor and the ligand and transforms it into a measurable signal (eg. electric, fluorescence,...) [24, 25]. The electronic system further amplifies, processes and displays the data.

The setup and sensing technique of a biosensor is highly diverse. One very common biosensor, which is widely used, is the pregnancy test. This lateral flow test is a very simple device which uses labeled antibodies as receptor and a chromatographic matrix as transducer. The detection is done by bare eye which makes it very simple easy to handle. However, most of these instruments work purely qualitative.

Another method is the utilization of enzymes to catalyze a reaction with the analyte. There are commercially available products which use proteins like hexokinase/ NAD^+ -systems or glucose oxidase to detect glucose in blood. The formed NADH and peroxide are detected which are directly proportional to the glucose concentration [26].

Beside the usage of proteins and DNA, whole organelles or cells can be used for sensing. Mitochondria are sensitive to calcium concentrations and were used for calcium sensing [27]. In an approach of Védrine, microalgae (*Chlorella vulgaris*) are placed into a microfluidic cell to detect herbicides in water [28]. Therefore an optical fiber was placed over the algae to shine light on the bioreceptors and further collect the fluorescence of the chlorophyll, which is increasing with increasing herbicide concentration. Cells, especially microbes, are much cheaper than enzymes/proteins, can survive harsh conditions and are able to perform more complex reactions. Additionally, it is easier to get high yields of cells than of proteins [29].

One example of a transducer is the field effect transistor (FET). This electronic device consists of three electrodes (source, drain, gate), separated by a semiconductor. Between the electrodes is a channel whose conductivity can be controlled by the electric field (via the gate). However, it can also be used for sensing. In this setup instead of changing the conductivity by controlling the electric field, the difference in conductivity is measured as a result of the change in electric field.

One new material which could be incorporated into a FET and used to fulfill the above mentioned specifications is graphene. Graphene is a single-atom thick material of carbon atoms which are bonded via sp^2 -orbitals. Its most important features for sensors are its properties as a zero-gap semiconductor, its exceptional electric properties and transparency. In the last decade, research focused on the usage of graphene as a platform material for labeled or label-free biosensors for various ligands. The FET detects the change of the charge density (in the electric field) on the surface due to binding of an analyte to a receptor [30, 31]. This change is due to the structural change of a protein or DNA. Both consist of many charged residues (amino acids in proteins and the phosphate backbone in DNA).

Another approach is the usage of the same graphene setup but instead of measuring the change in current, the change of resistance is measured. The interaction of a molecule with the graphene surface changes the resistance. Yagati reported a limit of detection of 10pM for his impedimetric biosensor in which he measured c-reactive protein [32].

Graphene is a very efficient fluorescence quencher which is used in an optical approach. Aptamers are labeled with fluorescent dyes and bond on the graphene surface. In absence of a ligand, the fluorescence is quenched. The binding of the analyte causes the fluorescent dye to get moved out of the quenching region of graphene. The fluorescent signal can be detected and quantified. Using a fluorescent-dye-quencher system has the advantage of low background. This system was

1.2. REDUCED GRAPHENE OXIDE FIELD EFFECT TRANSISTOR

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Figure 1.2: Measuring platforms based on graphene. **(A)** A ampere-metric reduced graphene oxide (rGO) field effect transistor measures the change of current as a function of antigen concentration which is binding on a antibody on the surface of the chip (adapted from [35]). **(B)** A imedimetric biosensor changes its resistance due to the binding of the ligand. The grey line shows the impedance of graphene, the red line the impedance of a graphene layer with a receptor and the blue line shows the impedance of a bond ligand at the receptor. (adapted from [36]) **(C)** In a different setup a FRET signal is used to detect a ligand. The fluorescence is quenched due to the quenching-effect of graphene. After the binding of the ligand to the aptamere, the fluorophor is removed from the surface and the fluorescent signal can be detected. (adapted from [33])

already used in several studies in aqueous solution and showed a limit of detection of down to 31pM. [33, 34]

1.2 Reduced graphene oxide field effect transistor

A reduced graphene oxide field effect transistor (GFET) was developed in our group, which uses proteins or aptamers as active sensor [35, 37–39]. The used protein was either specific to only one analyte by using an antibody or specific to a range of ligands with different binding affinities by using an odorant binding protein. The advantage of the usage of proteins as sensor is the flexibility of the system. In order to produce a sensor for a different analyte only the protein, which is able to bind specifically the molecule has to be exchanged, without changing anything in the rGO-FET. In consequence, many different analytes can be measured in parallel as the fabrication of the sensor is for all parts equal except the sensor protein/aptamer. An other big advantage is the high sensitivity of the system. Even small changes in the surface charge, which can be introduced e.g. by the structural change of a protein due to binding of a ligand can

be detected. This sensor platform could be used to detect the odorants which are released by infected or attacked plants. So it can be used as a cheap device for monitoring food safety, environment but also for disease monitoring.

1.3 Aim of the thesis

In this thesis an odorant binding protein is used as receptor for the FET. It is a small 16kDa protein which is located in the sensillum lymph in honey bee antenna. Its main task is to transport VOC from the sensillum wall to the lipophilic odorant receptors located at the neurons. Two of the ligands of the protein are eugenol and methyl eugenol which are released when a plant is infected. Both also show a significant ability to kill microbes [40–43]. The detection of those VOC could help to build up a monitoring network to detect pathogen infections in an early stage and enable to stop the spreading of diseases before it becomes endemic.

The fabrication of a FET with highly reproducible resistances is a major task to create a commercial product. Predominantly, there are two problems. First of all, the graphene products have various qualities. Secondly the surface coverage of graphene. The goal of the first part of this thesis was to examine the setup of the rGO-FET and characterize each layer with spectroscopic methods. The gained data is used to improve the preparation steps. Till now there is predominantly information about the electrical characteristics of the system available [35, 37–39]. However in order to get low resistance and high yields of working FETs the fabrication has to be optimized. Therefore surface characterization methods like infrared and Raman spectroscopy, scanning electron microscopy and QCM-D were used.

The second goal was to determine the surface concentration of the protein and pyrene-butylsuccinimidyl-ester via infrared spectroscopy. This method uses the band absorbance at parallel and perpendicular polarisation to calculate the surface concentration. It was introduced by the group of Fringelli and Baurecht in Vienna [44–48]. However the method was never applied to a complex system such as the GFET.

The third goal deals with elucidating the conformational and structural changes which are caused by environmental changes of the protein. Therefore, modulated infrared spectroscopy was applied. Our group used this special method for modulation of potential of cytochrome *c* oxidase [49–52] but it was also used in other groups for ligand-modulation [53] and temperature modulation [54]. This method is able to provide kinetic and spectral/structural information about the protein-ligand interaction. The spectral changes caused by three different ligands were monitored. The measured data had to be analyzed to obtain information about structural changes and the kinetics of OBP14. Therefore techniques like second derivative analysis, 2D-correlation spectroscopy and phase sensitive detection were applied.

Chapter 2

Theoretical Background

2.1 Olfaction

Olfaction is one of the five senses and is an essential way for animals and humans to interact with the environment. The perception of odorants enables the detection of food, toxins, finding of sexual partner, orientation and identification of hostile organisms [55, 56]. The odorants are low molecular weight molecules which can be water-soluble (water organisms), lipophilic or gaseous (land organisms). The general structure is either an aromatic ring or an alkane, which is functionalized with carboxyl acids, ketones, aldehydes, alcohols or alkenes. The olfactory system is a very complex mechanism which binds the odorants, conduct the information and discriminates between thousands of different odorants.

2.1.1 Interaction between plants and insects

The interaction between plants and insects plays a major role in nature. Plants attract insects like bees or bumblebees to fertilize the blooms. This exchange of food against transport of pollen (symbiosis) is the basis for most of our food supply. However there are not only useful animals. Many animals eat parts of the plants without providing a benefit for the plant. As plants are stationary and can not flee and also can not defend themselves in a physical way, they have to use different ways of defence.

The first line of defense is the production of volatile organic compounds (VOC) to attract the natural enemies of the pathogen [7–12]. These attack the herbivore and save the plant from further damage. Genetic resistances also play a big role against viral, bacterial and fungal infections. Another way is the production of toxic substances to keep pathogens off the plants. So caffeine and its derivatives is used by plants like coffee or tea to paralyse and/or kill insects [57]. VOC have also antimicrobial activity [40–42]. Highly volatile molecules like eugenol or methyl eugenol are produced and released in order to stop the growth bacteria. The exact mechanism is not completely understood but it is suggested that eugenol/methyl eugenol interacts with the membrane and causes enhanced fluidity and permeability. This causes the leakage of ions and proteins and consequently to the death of the bacterial cell [58, 59].

2.1.2 Odorant binding protein

The OBPs are small water soluble proteins which are located extracellular in the mucus layer (vertebrates) or lymph (insects). They belong to the superfamily of lipocalins, which include transport proteins like retinol-binding protein or β -lactoglobulin and can further be divided into 3 subfamilies: [55, 60, 61]

- pheromone binding proteins (PBPs)
are postulated to bind female pheromones and are only found in male insects
- general odorant-binding proteins (GOBPs)
are expressed in male and female antennae and are subdivided into GOBPI and GOBPII
- antennal specific proteins (ASPs)
are highly expressed in antennae and have no specific role

Figure 2.1: 3D structure of different binding proteins: (A) OBP14 [62] , (B) ASP1 [63] (C) OBP5 [64] of *Apis mellifera* and (D) pheromone binding protein 1 of *Bombyx mori* [65]. Though the amino acid sequence of the proteins have a low homology, the 3D-structure shows high similarity in secondary and tertiary structure.

The odorant binding proteins exhibit a high structural similarity (Fig. 2.1) [66]. OBPs are small globular proteins and have a typical molecular weight of 15-20kDa, where six α -helices are the leitmotiv [67]. They normally contain six cysteins whereat there are two highly conserved cyteine pairs which have a distinctive sequence [68]. Between the second and the third cysteine are always three amino acids and between the fifth and the sixth cysteine are always eight amino acids [55]. Those cysteine pairs are linked via disulfide bridges and are important for the 3D-structure

2.1. OLFACTION

of the protein [69]. Though their high similarity in 3D-structure, the amino acid sequence has an average similarity of 10-15% [55].

OBPs can be classified by their length of the C-terminus:

Table 2.1: Insects OBP classification. adapted from [67, 70]

Classification	Cysteines	Description	Examples
Classical OBPs	6	3 disulfide bonds formed by 6 cysteine residues; ~ 14kDa	<i>Bombyx mori</i> PBP1
Plus-C OBPs	6+2/3	1 highly conserved proline residue at least 2 conserved cysteines (17-25 kDa)	<i>A. gambiae</i> OBP48
Minus-C OBPs	<6	-	<i>Apis mellifera</i> OBP14
Atypical OBPs	≥6	a long C-terminus up to 38 kDa, only found in blood sucking insects	<i>A. gambiae</i> OBP35

Most odorants for land organisms are small lipophilic volatiles which spread though the air. In order to “smell” the odorant, a huge system of proteins and mediators has to be activated. The target for odorants is the odorant receptor (also called olfactory receptor). Most odorant receptors are G-protein coupled receptors, which are localised in the membrane of dendritic cells. Due to the aqueous environment around the cells, a carrier is needed to transport the hydrophobic odorant through the extracellular matrix to the receptor. This task is done by odorant binding proteins (OBPs). Those are loaded with the odorant and carry the ligand to the nearest receptor [71]. There either the odorant itself or the odorant and the OBP trigger the signal cascade inside the nerve cell (Fig. 2.2D).

2.1.3 Olfaction in Vertebrates

In vertebrates the odorants enter the olfaction sensors via the nose for warming and humidification. The sensory cells are located in a main olfactory epithelium (MOE) which is covered with a mucus layer. Each neuron in the MOE expresses only one single receptor gene [72]. Olfactory receptors (OR) can be grouped into nine classes. Two of them (OR class I and II) are the most abundant receptors in tetrapods which are assumed to be responsible for binding airborne odorants [73, 74]. Due to the high diversity of odorant molecules and comparative low diversity of OR (300-1,300), it is believed that not all OR are specific to recognize molecules but some are able to distinct structures like alcohols, alkenes or aromates [75]. The signals are further processed in the brain and associated with a smell.

Once an odorant binding protein delivers an odorant which binds to the OR, the conformational change causes the release of a G-Protein $G_{\alpha_{olf}}$ (2.2C). The G-protein activates the adenylyl cyclase III (ACIII) [76] which produces cyclic adenosine mono phosphate (cAMP) and activates the cyclic nucleotide-gated channels (CNG). The increase of intracellular Ca^{2+} leads to the opening of Ca^{2+} -activated Cl^{-} channels (CaCCs). The Cl^{-} efflux causes a further depolarisation of the cell membrane and the action potential is transmitted to the brain [77, 78].

Figure 2.2: Signal cascade, triggered by ligand binding to OR: **(A) insect ionotropic receptor:** The binding of an odorant leads to a dimerisation of the OR with Or83b and form an ion channel. The opening causes an increase of Ca^{2+} . **(B) insect metabotropic receptor:** The binding of a ligand to the OR causes the activation of an adenylate cyclase via a G-protein and triggers an opening of the Or83b ion channel. **(C) Vertebrate receptor:** An odorant is carried via OBP to the receptor and triggers the release of a G-protein which activates an adenylate cyclase. The cAMP opens a Ca^{2+} -channel and the increased Ca^{2+} -level activates the Cl^- -channel (adapted from [73]). **(D) Magnification of the antenna of an insect:** The odorant penetrates the sensillum wall in the antenna and gets into the lymph where it gets bound by OBPs which transports the odorant to the OR in the ORN (adapted from [79]).

2.1.4 Olfaction in Insects

The insect olfactory perception and signal transduction differs in anatomy as well as in signal transduction from the vertebrate [80]. The antennae contain most of the chemosensory proteins, used for olfaction [81]. Sensillar lymph covers the olfactory epithelium tissue which contains the olfactory receptor neuron (ORN). Those receptor neurons are organized along the antenna where every ORN expresses only one OR [82, 83]. The number of ORN differs in species and type of insect. *Drosophila melanogaster* (fruit fly) has 1,200 ORN [84], a male honey bee drone has about 300,000 ORN while a female worker has 65,000 ORN [85]. Inside the lymph, a high concentration (up to 10mM [86]) of OBPs transports the airborne odorants to the OR in the ORN.

The OR in insects aren't only different in number (60-80 OR) or structure but also in function compared to vertebrate ORs. Additionally, almost all OR are localized to the basiconic and trichoid sensilla [73]. It is postulated, that insect OR are ion channels [80]. Additionally, a second highly conserved universal co-receptor Or83b is always co-expressed with the OR [87]. Or83b does not bind any odorant but interacts with OR to modulate them. This type of signaling is suitable to track rapid changes in concentration which is an important feature for insects [73].

Figure 2.3: Effect of pH on the structure and affinity of PBP1: (A) A schematic drawing of the structure of an sensilla of *Bombyx mori*. (B) The pH near the sensillum wall is 4.5. The PBP1 binds the ligand (e.g. bombykol) and diffuses into the sensillum bulk, which has a pH of 6.5. This pH shift causes a conformational change and a change of affinity to the ligand. When the PBP reaches the cell membrane of the ON, the pH decreases again to pH 4.5 which results in a conformational change and changes affinity. (C) The percentage of unbound ligand dependent on the pH of the environment. Ligands of PBP1 like bombykol show to bind to PBP1 at lower pH than non-physiological ligands. adapted from [88]

Two different signal-cascade models are under discussion. In the first (ionotropic) model [89], the binding of the odorant to the OR causes a dimerisation of the OR and Or83b and form an ion channel. This channel opens and Ca^{2+} gets into the cell (2.2A).

The second study proposed a metabotropic mechanism [90] in which the Or83b itself is a CNG. An OR (G-protein coupled receptor) binds the odorant and the conformational change causes the release of a G-protein. The adenylate cyclase which produces cAMP gets activated. This opens the Or83b and causes to the penetration of Ca^{2+} and the neuron depolarization (2.2B). The axons of ORNs which express the same OR innervate into the same glomeroli in the antennal lobe, where the signal is further processed.

The binding affinity of odorant binding proteins depends on the pH of the environment [91–99]. Several in vitro studies used PBP1 (pheromone binding protein 1) from *Bombyx mori* to examine the structural changes. The porous sensillum wall [100] has a negative charge which causes the pH near the wall to be two pH units lower than in the bulk [56, 101]. The low pH causes a low affinity of PBP1 to ligands. As the protein moves through the bulk sensillum lymph to the receptors, the pH changes to pH 6.5 [102]. This causes a conformational change which affects the affinity. The affinity increases for primary ligands, but low binding ligands are released and degraded by enzymes [103]. So, some kind of pre-selection is performed. As the PBP1 reaches the receptor, the pH decreases due to negatively charged lipids in the neuron membrane, the affinity is reduced and the ligand released to the receptor (Fig. 2.3).

This property of PBP1 has also been found for other OBPs like PBP1 of *Antheraea polyphemus*

Figure 2.4: Conformational change of OBP14 due to eugenol binding. The golden protein represents OBP14 without eugenol while the blue protein represents OBP14 with eugenol.

[104] or PBP1 of *Amyelois transitella* [105] but also in odorant proteins of *Apis mellifera* as for instance ASP1 [63, 106].

2.1.5 Odorant binding protein 14 of *Apis mellifera*

Odorant binding protein 14 is one of 21 OBPs found in the honey bee, *Apis mellifera* Linnaeus (*A. mellifera* L.). It is composed of six α -helices plus one short C-terminal seventh helix at the C-terminus and has a molecular weight of 16kDa. The full protein consists of 119 amino acids, where 5 cysteines form two disulfide bonds (AA 17-49 and AA 88-106). The isoelectric point (pH where the protein has a net charge of zero) is between 5.71 (calculated) and 5.45 (experimental) [107], which is comparable high to other OBPs (pH 4.5-5).

In comparison to other OBPs, OBP14 can be classified as C-minus OBP as it has no peptide chain after the seventh helix. Sequence analysis showed a 35% identity with OBP13. The helices form a cavity with a volume of $415 \text{ \AA}^3$ which is not accessible for the solvent. This cavity binds different odorants e.g. eugenol, α -Methyl-cinnamaldehyde, geraniol. The binding of eugenol (which is the main ligand) causes a decrease of volume to $360 \text{ \AA}^3$ [62].

2.1.6 OBPs as biosensors

OBPs have been shown to have high temperature stability [108], while insect OBPs have an even higher stability than vertebrate OBPs due to the disulfide bridges which stabilize the protein. Infrared spectroscopy and cyclic dichroism were used to analyse the temperature stability of OBP14. This showed a higher stability to high temperatures when a ligand is bound to the protein. The denaturation temperature correlates with the binding affinity of the ligand, where high affinity means higher stability [109, 110].

These properties combined with high stability against denaturation, as well as proteolytic degradation make these proteins potential receptor-elements for biosensor devices or other biotechnological applications.

Since OBPs are water soluble proteins, they can easily be immobilized on a surface via reactive ester or FLAG/his-tag technology. Inserted as a receptor into a sensor platform like a graphene based field effect transistor they can be used to detect low amounts of odorants like eugenol or methyl eugenol. As this protein is evolutionary selected to bind only specific molecules and in combination with a graphene based field effect transistor it has the potential to be used for the first hand-held smelling device to detect infected plants.

2.2 Graphene

Graphene is an allotrope (same element but different structure) of carbon which was synthesized first by Novoselov and Geim at the University of Manchester in 2004 [111]. Its unique properties have put it into focus of material research.

Graphene is a two-dimensional monolayer of graphite. It consists of sp^2 bounded carbon atoms in a hexagonal lattice. A carbon atom forms 3 σ -bonds (in plane) to its neighbors and the 4th bond is a pi-bond, which is oriented in z-direction (out of plane). The pi-bond of each carbon atom hybridize to form a π -band and a π^* -band. Those π -bands lead to the unique electronic properties. So graphene is a polycyclic aromate, which forms a π -electron cloud [111–113]. The two-dimensional structure is not totally planar, but has many foldings and waves which lead to thermodynamic stabilization.

A monolayer of graphene has exeptional properties (summarized in Table 2.2):

Table 2.2: Properties of graphene monolayers compared with copper (conductor) and silicon (semiconductor) (adapted from [114] unless stated otherwise)

Property	Graphene	Copper	Silicon
Density (g/m ² or g/cm ³)	$7.57 \cdot 10^{-4}$	8.96	2.33
Thickness of monolayer (m)	$3.35 \cdot 10^{-10}$	-	-
Electrical resistance (Ω m)	31	$2 \cdot 10^{-8}$ [115]	3000 [115]
Tensile strength (GPa)	125 [116]	0.21	0.7
Thermal conductivity (W/(m·K))	5000 [117]	401	149
Fermi velocity (m/s)	10^6 [118–120]	$1.65 \cdot 10^5$	
Electron mobility (cm ² V ⁻¹ s ⁻¹)	$2.5 \cdot 10^5$ [121]	-	1,350
Optical absorption	2,3 % [122]	-	-
Bandgap (eV)	0 [111]	-	1.12 [123]
Charge carrier density (m ⁻³)	$1 \cdot 10^{17}$	$9 \cdot 10^{28}$ [115]	$1 \cdot 10^{16}$ [115]

In contrast to the strong σ bonds, the electrons in π -bonds are weakly bound and consequently the electron mobility is high. The mobility is dependent of the number of graphene layers. A monolayer has the highest electron mobility ($2.5 \cdot 10^5$ cm² V⁻¹ s⁻¹) [121]. The increase to a trilayer leads to a decrease in mobility and the behavior of a semimetal [124]. Further increase to 10-12

layers changes the electric properties fundamentally and it becomes an insulator (graphite) [125].

According to electron band theory, materials can be divided into:

- **conductor:** the valence band and the conducting band of these materials (silver, gold, copper,...) are overlapping and the fermi level lies inside this overlapping area.
- **semiconductor:** the valence band and the conducting band are separated by a small gap (0 - 4eV) in which the fermi level is located. Energy (potential, heat, light,...) has to be applied to raise the electrons into the conducting band and make the material (silicon, germanium,) conducting.
- **semimetal:** In a semimetal, valence band and the conducting band are in contact but aren't overlapping and the fermi level lies in one of the bands.
- **insulator:** the valence band and the conducting band are separated by a gap $>4\text{eV}$. The material (air, rubber,...) is not conducting.

From this perspective, a graphene monolayer is a zero-gap-semiconductor and a multilayer of graphene sheets is a semi metal. Another property of graphene is the ambipolar behavior when used as a semiconductor in a field-effect transistor. This means that current can flow either via electrons or via holes depending directly on the sign of the applied field.

2.2.1 Graphene preparation

In 2004, Novoselov et al. were the first who produced monolayers of graphene. In order to use the graphene for commercial purposes, the synthesis route has to produce flakes with reproducible high quality, in large quantity and by an easy method. In general, graphene exhibits defects like impurities, oxidation, foldings and rolled up edges, which is also a consistency problem. Although there are many different ways to produce graphene, each has its advantages and disadvantages but until now there is no synthesis route that covers all requirements adequate. In general there are four main methods:

- **Scotch tape method**

The classical "scotch tape method" was invented by Novoselov et al. and was the first fabrication method. Highly oriented pyrolytic graphite (HOPG) was dry-etched by oxygen plasma. The created mesas were covered by a photoresist and baked. Scotch tape was used to peel off layers of graphite. Afterwards, the photoresist was dissolved by acetone and the flakes were put on silicon wafer [111]. This method results in the lowest yield.

- **Chemical vapor deposition**

Chemical vapour deposition (CVD) is used to produce monolayers of graphene on a metallic substrate. Therefore a precursor gas (mixture of different ratios of H_2 , CH_4 and Ar) is injected into a chamber. Under high temperatures (800-1400°C) and high pressure (80 Torr), monolayer and multilayer of graphene are formed on substrates like Ni [126], Si or Cu [127] (Fig. 2.5a).

2.2. GRAPHENE

• Graphene on SiC

An alternative to chemical vapour deposition is the thermal decomposition of silicon carbide. A 6H-SiC surface is heated to high temperatures (1250-1450°C) for 1-20 minutes. This results in the formation of 1-3 layers of graphene [128].

• Wet chemical methods

One way of wet graphene synthesis is the intercalation of metals like potassium in the graphite structure to destabilize the Van-der-Waals interactions between the graphene sheets in graphite. The synthesis is done in an organic solvent like ethanol and result in graphite sheets with about 40nm thickness.

Another synthesis route is via the production of graphite oxide, on the basis of the work of Brodie [129]) and Hummers [130]. Graphite is oxidised in a solution of sulfuric acid and potassium permanganate (Fig. 2.5b). The exfoliation of the graphene is done via ultrasonication/mechanical force. The produced graphene oxide sheets (GO) are reduced with a reducing agent to obtain reduced graphene oxide (rGO). The disadvantages are the defects in the structure because of the oxidation and reduction and the consequential poor electric properties. Various reducing agents and their conductivity are presented in table 2.3.

Figure 2.5: Graphenesynthesis via (a) oxidized graphite and (b) chemical vapor deposition.; [31]

Hummers provided the basis for many other variations for GO synthesis which differ in flake size, purity and concentration [39, 131].

Table 2.3: Common reducing agents and their effect on graphene oxide (adapted from [132]).

Reducing agent	conductivity	C/O-ratio	Doping	Conditions	Ref.
NaBH ₄	17 S · m ⁻¹	-	-	80°, 2h	[133]
NH ₃ BH ₃	19300 S · m ⁻¹	14.2	B/N-doped	80°C, 12h	[134]
Hydrazine	2420 S · m ⁻¹	10.3	N-doped	100°, 24h	[135]
Hydroiodic acid	29800 S · m ⁻¹	12	I-doped	100°, 1h	[136]
Ascorbic acid	800 S · m ⁻¹	-	adsorbed	RT, 48h	[137]

Three different GO synthesis products were examined to study their composition and binding kinetics. All three examined products base on the graphite oxide synthesis route introduced by Hummers in 1958 [130]. The general concept is to mix graphite flakes with sodium nitrate (1:3) and stir it in sulfuric acid. An excess amount of potassium permanganate is added for oxidation of graphite, while cooling the reaction to 0°C. Hydrogen peroxide reduces remaining permanganate and mangan dioxide and after purification steps, grahite oxide was obtained. The Marcano synthesis was chosen as it is one of the most frequent cited GO fabrication methods. GO production according to Feicht et al. is distinguished by the other methods by the fast and easy purification method.

- Feicht et al. prepeare GO according to Hummers but modify the GO with 1-dodecylamine and the purification is done in a single step extraction in organic solvents [138].
- Marcano et al. use H_3PO_4 instead of $NaNO_3$ in combination with H_2SO_4 and K_2MnO_4 for for graphite oxidation to increase the oxidation efficiency. Additionally, multiple centrifugation and washing (dd H_2O) steps were performed with filtration steps to remove impurities like graphite oxide flakes, ions and side products [131].
- Larisika et al use two oxidation steps for GO preparation. Graphite flakes were stired in H_2SO_4 conc. for 5h and after a purification step stirred in a solution of K_2MnO_4 and H_2SO_4 . For purification, filtration (200nm pore size) and washing with 10%HCl and dd H_2O was carried out [39].

2.2.2 Graphene oxide

The structure of graphene oxide (GO) as well as graphite oxide is still under discussion [139]. There are many different models which describe the structure but the two most important are illustrated in Fig. 2.6. The Lerf-Klinowski model suggests a planar hexagonal lattice like graphene, with defects in form of hydroxylic, carboxyl and epoxide groups [140]. The Dekany model proposes GO to consist of two domains. One domain is the hexagonal aromatic lattice which is interrupted by cyclohexan species. The cyclohexan groups are oxidized and contain hydroxyl- or ether groups [141, 142].

Figure 2.6: Proposed models of the structure of GO: (A) Lerf-Klinowski-model proposes a planar lattice with hydroxides at the edges and epoxies in the lattice [140]. (B) Dekany-model suggests the introduction of cyclohexanes into the lattice which are oxidized [141]. Both more recent models are extended and also contain C=O compounds.

2.2.3 Field effect transistor

A transistor is an electronic element to control voltage and current. Transistors are divided in two subclasses: bipolar transistor and field effect transistor (FET). In this work, only the field effect transistors are relevant.

The classical transistor consists of a semiconductor like germanium or silicon. The properties of the semiconductor are used to control the source-drain current. As a consequence of the miniaturisation of electric elements, the classic materials reach their limitations. Due to the good conductive properties and unique characteristics as a semiconductor, graphene can be used as a semiconductor material.

A FET consists of three parts: source, drain and gate. The gate is used to control the current by applying a voltage. The voltage is used to regulate the charge density and band gaps of the semiconductor via field effect [31, 143, 144]. A graphene based field effect transistor in biosensor configuration measures the dynamic change on the surface. The electrons on in the π -band are affected by the binding of molecules on the surface and hence change the conductivity [113].

One big advantage of graphene based field effect transistors (GFETs) is the simple modulation of the Fermi energy. The applied voltage to raise the energy level is much lower than for comparable traditional silicon based transistors. The potential is in the range of a few 100mV, which is similar to biological systems. This makes a GFET interesting as a biosensor. [111]

2.2.4 Graphene-based FET as biosensor

In consequence of the monolayer structure, graphene is an excellent material for sensors. The two dimensional structure creates a high surface area on which the analytes can be adsorbed. In 2007, Schedin et al. built a gas sensor that was able to detect gases like CO, H₂O, NH₃ and NO₂ [145].

Many graphene based sensors followed: The group of Fowler showed the detection of DNT (dinitrotoluol), a compound of explosives [146], Shan et al. were able to sense glucose via glucose oxidase [147] and Alwarappan et al. sensed catecholamines via a graphene based device [148].

Figure 2.7: (A) The GFET-Cell, used in the group with the graphene chip inside. (B) Titration of eugenol on OBP14 measured with a GFET. The measured current increases with increasing eugenol concentration. The titration measurements can be used to calculate the dissociation constant of a receptor-ligand-system. (C) A schematic figure of the GFET setup. The contacted electrodes are used to measure the change in current, caused by the binding of eugenol to the OBP14 (adapted from [149]).

In our group, Reiner-Rozman et al. used a reduced graphene oxide field effector to sense bovine serum albumine (BSA) with anti-BSA antibodies [37, 150].

In an alternative setup, small molecule detection was done by the work of Larisika et al. They used a GFET with OBP14 from the honey bee (*Apis Mellifera*) immobilized on the surface to detect odorants in aqueous solution [149]. Fig. 2.7 illustrates the setup of the flow cell and measured titration of eugenol on OBP14.

The graphene based field effect transistor setup is also used in other groups for detection of analytes. Liu et al. used micro-patterned reduced graphene oxide for the detection of rotavirus down to a concentration of 10^2 pfu/ml (plaque forming unit) [151]. Other groups are working on a rGO-FET for next generation sequencing [152].

2.3 Vibrational Spectroscopy

In order to examine the structure of molecules, spectroscopy offers a broad range of methods in different wavelength ranges. Nuclear magnetic resonance spectroscopy (NMR, radio frequency), electron paramagnetic resonance spectroscopy (microwave frequency), vibrational spectroscopy (infrared), UV/Vis spectroscopy and X-ray spectroscopy are widely used for structural analysis. NMR and X-ray spectroscopy are able to give us information of the 3D-structure of the macromolecule of interest at a specific state. Infrared (IR) and UV/Vis spectroscopy also offer information about the dynamic changes during an excitation. This makes these techniques very valuable for the study and understanding of the function of proteins. Vibrational spectroscopy can be divided into three wavelength ranges:

Table 2.4: Wavelength and wavenumber ranges of vibrational spectroscopy [153]

	Wavelength	Wavenumber	Frequency
Near infrared (NIR)	0.78-2.5 μm	40000-4000 cm^{-1}	400-120 THz
Mid-infrared (MIR)	2.5-50 μm	4.000-200 cm^{-1}	120-6 THz
Far-infrared (FIR)	50-1000 μm	200-10 cm^{-1}	6-0.3 THz

Figure 2.8: Spectrum of radiation [50].

Each of the wavelength ranges contains different vibrational modes. The near infrared region contains overtone vibrations, the far infrared region contains weak vibrations and the mid infrared region contains stretching and bending modes of intramolecular transitions [154].

2.3.1 Molecule vibrations

Infrared spectroscopy is the measurement of rotation- and vibration transitions of a molecule. The interaction of radiation with a molecule causes the absorption of a photon, which has the same energy (frequency) to stimulate the ground state to the first overtone. The absorption requires the change of the dipolemoment of the molecule during the vibration. The band intensity is direct proportional to the change of the dipole moment. Molecules like O₂ or H₂ are not

absorbing IR radiation, whereas molecules like HCl, H₂O or CO₂ are absorbing IR radiation [155].

Covalent bonds are not rigid but flexible like springs, which can be bent, clinched or stretched. These vibrations can be assigned to quantum levels of the molecule. The harmonic oscillator (Fig. 2.9) is a model for the energy transfer of the vibration states of a two atomic molecule. It consists of two masses m_1 and m_2 which are connected via a spring with the length r . The change their distance (Δr) leads to stretching and compression of the spring. Hooks law describes the restoring force:

$$F = -k \cdot \Delta r \quad (2.3.1)$$

The reduced mass is used for simplicity:

$$\mu = \frac{m_1 \cdot m_2}{m_1 + m_2} \quad (2.3.2)$$

Figure 2.9: Function of harmonic and enharmonic oscillator. [49].

The vibration frequency is defined as:

$$\nu = \frac{1}{2\pi} \cdot \sqrt{\frac{k}{\mu}} \quad (2.3.3)$$

The potential of the harmonic oscillator can be described as:

$$V(x) = \frac{1}{2} \cdot kx^2 \quad (2.3.4)$$

In a harmonic oscillator, the energy in the potential increases to infinity. In a bond, massive stretching leads to a break of the bond and massive compression leads to the repulsion of the atoms. Therefore, the Morse potential can be used as a model for the anharmonic oscillator:

$$V(x) = \frac{\alpha}{2\pi} \sqrt{\frac{2D_e}{m}} \quad (2.3.5)$$

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where D_e is the dissociation energy and α is a constant.

In general, two kinds of vibrations can be distinguished: valence vibrations and deformation vibrations. Both can be symmetric and asymmetric, whereby the symmetric vibrations leads less often lead to a change in dipole moment and tend to be weaker [155].

Figure 2.10: C-H stretching and bending vibrations at different wavenumbers [49].

2.3.2 Principle of infrared spectroscopy

Infrared spectrometers can be grouped into two categories: dispersive infrared spectrometers, which are the older form, and the modern Fourier transform (FT) IR spectrometer, which are based on a Michelson - interferometer. In FT-IR spectrometers, the light beam is generated in a source like a globar and shines to a semi transparent mirror (beam splitter). One beam with half intensity is reflected by a static mirror. The other beam is guided to a moving mirror. Both beams are reflected and meet at the semitransparent mirror and interfere whereby the interference can be constructive or destructive. In order to know the exact position of the mirror, a laser with known wavelength is also passing the interferometer. The radiation is passed through the probe and reaches the detector (like mercury cadmium tellurium (MCT), deuterium-triglycin sulphate (DTGS) detectors), where an interferogram (intensity as a function of mirror position) is recorded [156, 157].

As shown in Fig. 2.11 the interferogram of a monochromatic light (e.g. laser) shows a sinusoidal wave pattern and after Fourier transformation a peak at the wavelength of the incoming beam is shown. Polychromatic light shows a more complex pattern as it consists of many overlapping wavelengths. The interferogram can be described as:

$$B(\delta) = \int_a^\infty I(v) \cos(2\pi\delta v) dv \quad (2.3.6)$$

where $B(\delta)$ is denoted as the intensity of the beam at the detector with mirror retardation δ and $I(v)$ is the intensity at wavenumber v .

The recorded interferogram is transformed into a single channel spectrum ("reference" I_0) via Fourier transformation:

$$I(v) = \int_{-\infty}^{+\infty} B(\delta) \cos(2\pi\delta v) d\delta \quad (2.3.7)$$

Figure 2.11: Principle of infrared spectroscopy: A light beam gets split up by a semi-transparent mirror, where one beam gets reflected to a standing mirror, one to a moving mirror. Both beams interfere at the beamsplitter and are directed to the sample. The detector records the interferogram. After fourier transformation, monochromatic light results in a single peak in the spectrum. Polychromatic light results in a spectrum with many peaks. [49].

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In order to get information about a change, an additional measurement (“sample” I) has to be taken and the absorption can be calculated by:

$$A = -\log \frac{I}{I_0} \quad (2.3.8)$$

Infrared radiation is absorbed when the frequency of light and the vibration are equal and the vibration causes a change of dipole moment. The location of the band of a vibration in the spectrum depends on the mass of the vibrating atoms, the type of bond and inter- and intramolecular interactions. Accordingly, the vibrations of methyl groups are at high wavenumbers and the vibrations of carbon-halogen bonds are at low wavenumbers. Since the intensity of the band is directly proportional to the change in dipole moment, very polar bonds (like carbonyl or carboxyl) generate high intensities and less polar bonds like methyl-groups generate lower intensities [154].

2.3.3 Infrared spectroscopy of Proteins

The absorbance of light is characteristic for each vibrational mode of a functional group in molecules. The bands in an infrared spectrum can be easily identified for small molecules, though it gives only information about the functional group of the molecule. In order to know the identity of the molecule, the spectrum has to be compared with spectra from a database. For larger molecules like proteins it is very difficult to obtain single bands of single functional groups because many bands are overlapping.

Infrared spectroscopy has been applied to study proteins since the last couple decades. It can provide useful information about protein structure and gives insight into protein dynamics because of its advantages of high time resolution up to the ns range. One strength of IR spectroscopy is the universality, so it is able to characterize soluble proteins, as well as membrane proteins. The infrared spectrum of a protein is characterized by nine bands which are called amide bands [154]. Those bands are formed by the protein backbone and contain different vibrations shown in Table 2.5.

Table 2.5: Amide bands and their components [154]

Amide Band	Wavenumber	Assignment
A	3300 cm^{-1}	N-H stretching
B	3100 cm^{-1}	N-H stretching
I	1653 cm^{-1}	80% C=O stretching 10% C-N stretching 10% N-H bending
II	1567 cm^{-1}	60% NH-bending 40% C-N stretching
III	1299 cm^{-1}	30% C-N stretching 30% N-H bending 10% C=O stretching 10% O=C-N bending 20% other vibrations
IV	627 cm^{-1}	40% O=C-N bending 60% other vibrations
V	725 cm^{-1}	N-H bending
VI	600 cm^{-1}	C=O bending
VII	200 cm^{-1}	C-N torsion

The most interesting protein bands are the amide I, amide II and amide III band. They provide information about the secondary structure of the protein (Table 2.6).

Table 2.6: Assignment of different components of the amide I band to secondary structure in literature

Structure	Literature ν (cm^{-1})			
	Bart [153]	Stuart [154]	Goormaghtigh [158]	Byler [159]
Random	1642-1657	1640-1645	1637-1645	≈ 1645
α-helix	1648-1657	1648-1657	1645-1662	≈ 1654
β-sheet	1623-1641		1613-1637	1624-1637
	1674-1695		1682-1689	≈ 1675
β-turn			1650-1686	
Turns and bends	1662-1686			1663-1694
Intermolecular		1670-1695		
β-structure		1610-1625		
Intramolecular		1680-1690		
β-structure		1630-1640		
3-turn helix		1666-1659	≈ 1656	

The amide I band provides most information because it is the most intense of the three and highest sensibility to conformational changes [160, 161]. One major drawback of it is the O-H bending vibrations of water, which absorbs at about 1640cm^{-1} . Water is not only the solvent of the protein, but provides H-bridges and is also inside the protein [158, 162, 163]. So the analysis of secondary structures can become difficult due to incomensation of water absorbance.

The amide I band can be divided into different absorbance ranges and their assigned secondary structures (Table 2.6, Fig. 2.12). The ranges are not strict and overlap and vary in literature. The band position of amide I and amide II bands depend strongly on the strength of H-bonds between C=O and N-H. As different secondary structures show different H-bond lengths it is possible to make an assignment. Longer/weaker H-bond is localized at higher wavenumbers, while stronger/shorter H-bonds are at lower wavenumbers. In order to determine the components of the amide I band, the second derivative is calculated. The minima of the spectrum correspond to the peak positions [159, 164].

Figure 2.12: Secondary structure components of the amide I band. The amide I band (black line) consists of several elements (colored lines, Lorentzian line-shape) which can be attributed to secondary structures of a protein, depending on their position. In order to get information about the position of the particular bands, second derivative can be used to deconvolute the amide I band (grey line).

2.3.4 Absorption of amino acid side chains

The absorption of amino acid side chains of proteins can provide information about the reactions inside a protein. This is a very important property to examine the changes in the protein structure during catalytic activity, binding of ligands or change of pH. The finding and assignment of specific amino acid side chains is difficult due to the overlapping of bands. These bands are predominantly found in the range between 1750cm^{-1} and 1000cm^{-1} (Table 2.7) [153, 161, 165].

One feature of infrared spectroscopy is the high sensitivity to detect protonation and deprotonation. This makes it an efficient tool to study conformational changes, kinetics or pK-values of acidic or alkaline groups [49].

2.3.5 Reaction induced infrared difference spectroscopy

The investigation of the structural alteration of a protein is still a challenge due to the low extent of conformational change. Changes in the spectrum occur in the range of about 0.1% of the maximum intensity. In order to detect those small changes spectra of “ground state” A

Table 2.7: Wavenumber, molar extinction coefficient, FWHM and pK of important amino acid side group vibrations. (adapted from [158, 165])

	Vibration		ν (cm ⁻¹)	ϵ (l · mol ⁻¹ cm ⁻¹)	FWHM (cm ⁻¹)	pK
Asp	-COO ⁻	(ν_{as})	1574 ± 2	380 ± 20	44 ± 2	≈ 4.5
	COOH	(ν)	1716 ± 2	280 ± 20	50 ± 2	
Glu	-COO ⁻	(ν_{as})	1560 ± 2	470 ± 30	48 ± 2	≈ 4.4
	COOH	(ν)	1712 ± 2	220 ± 10	56 ± 2	
Arg	CN ₃ H ₅ ⁺	(ν_{as})	1673 ± 3	420 ± 40	40 ± 2	≈ 12.1
		(ν_s)	1633 ± 3	300 ± 20	40 ± 2	
Lys	-NH ₃ ⁺	(δ_{as})	1629 ± 1	130 ± 10	46 ± 2	≈ 10.2
		(δ_s)	1526 ± 3	100 ± 10	48 ± 2	
Asn	-C=O	(ν)	1678 ± 3	310 ± 20	32 ± 2	
	-NH ₂	(δ)	1622 ± 2	160 ± 15	44 ± 2	
Gln	-C=O	(ν)	1670 ± 4	360 ± 20	32 ± 1	
	-NH ₂	(δ)	1610 ± 4	220 ± 20	44 ± 2	
Tyr	ring-OH	-	1518 ± 1	430 ± 20	8 ± 1	≈ 10.7
	ring-O ⁻	-	1602 ± 2	160 ± 20	14 ± 2	
His	ring C-N	(ν)	1596 ± 1	70 ± 10	14 ± 1	≈ 6.0
Phe	ring C-C + C-H	($\nu + \delta$)	1494 ± 1	80 ± 10	6 ± 1	

and “excited state” B are taken. The spectra are subtracted to find the differences (D) in the spectra.

$$D = B - x \cdot A \tag{2.3.9}$$

Where x is a variable factor to compensate concentration fluctuations (e.g. water). Positive bands represent a development during excitation and negative bands represent a decrease during excitation [153]. Attenuated total reflection IR spectroscopy provides an easy way to change the surrounding conditions in situ and measure the differences.

2.3.6 Attenuated total reflection infrared spectroscopy

The standard IR-measurement is done by measuring the absorption of transmitted light. Therefore the sample is injected into an IR-transparent cell and positioned directly between the light source and the detector. This technique is fast and easy to handle.

A more advanced technique is called attenuated total reflection infrared spectroscopy (ATR-spectroscopy) which was introduced by Harrick in 1960 [166]. This technique takes advantage of the phenomenon of total reflection, which can take place when a light beam is passing through a medium with high refractive index (n_1) and gets to a medium with lower refractive index (n_2). If the angle of incidence is lower than the critical angle, the beam gets refracted from the normal. At the critical angle, the beam is parallel with the interface. If the angle of incidence is higher than the critical angle, the beam is totally reflected. The critical angle is defined by Snell’s law:

$$\sin(\Theta) = \frac{n_1}{n_2} = n_{12} \tag{2.3.10}$$

In 1890, Otto Wiener investigated the interaction of a light beam on a metall mirror with a photographic emulsion [167]. He discovered a standing wave pattern, normal to the reflecting surface. The propagating wave was damped and had a penetration depth of about 100nm.

A ATR-crystal, also called an internal reflection element (IRE) also generates this evanescent wave at its surface. The crystal is made of a material with a high refractive index (Table 2.8). The IR beam passes through the crystal and gets reflected at the boundary between the sample material and the IRE. At the position where the beam hits the edge of the IRE, a standing wave and an evanescent field is created orthogonal to the reflectance area (Fig. 2.13).

The standing wave can be mathematically described as:

$$E = 2 \cos \left(\frac{2\pi z}{\lambda_e} + \phi \right) \quad (2.3.11)$$

where z is the coordinate on the axis perpendicular to the interface and $\lambda_e = \frac{\lambda_1}{\cos(\Theta)}$ with $\lambda_1 = \frac{\lambda}{n_1}$ and ϕ is the phase angle [166].

This evanescent wave interacts with the sample material in the lower refractive index material and is decaying exponentially. The time averaged field intensity can be described by:

$$E = E_0 e^{-z/d_p} \quad (2.3.12)$$

where E_0 is the time averaged electric field intensity at the interface. The penetration depth d_p of the evanescent field depends on the wavelength (λ), angle of incidence (Θ) and refractive index of the two media (n_1, n_2) [166].

$$d_p = \frac{\frac{\lambda}{n_1}}{2\pi \sqrt{n_1^2 \sin^2(\Theta) - n_2^2}} \quad (2.3.13)$$

The interaction in the optically less dense medium leads to partial absorbance of the light and consequently to attenuation.

Another effect associated with total internal reflection was the dislocation of the reflection (Goos-Hänchen displacement) [169, 170]. The theory indicates that the displacement accrues due to energy transfer in the light beam. This causes an energy flow parallel to the surface and consequently to a displacement [171].

The IRE has specific requirements like chemical inertness, high refractive index, hardness and it should not adsorb in the wavenumber range of interest. The element can also have different kind of shapes like hemicylinder, dove-prism, achromatic prism, different number of reflections (single and multiple reflections) and single or double pass of the beam.

Figure 2.13: The beam hits the surface and gets reflected. An evanescent wave is generated into the medium with lower refractive index and standing wave into the medium with higher refractive index. The evanescent wave can interact with molecules on the surface. [168]

Table 2.8: Spectral range, refractive index and critical angle of materials, used as ATR-crystals [154, 171, 172]

Material	Spectral range (μm)	Refractive index	Critical angle (θ_{cr})
Si	1.1-6	3.5	15.6°
Ge	2-12	4	14.5°
Al ₂ O ₃	0.22-4.5	1.7	34.5°
Quartz	0.3-2.3	1.43	44.5°
ZnSe	0.5-20	2.4	-
KRS-5	0.6-40	2.4	-
Diamond	0.4-100	2.4	-

2.3.7 Modulated excitation infrared spectroscopy

One major problem in studying proteins and their conformational changes is the small spectral change. This problem increases drastically when working with monolayers. Consequently, many bands are not seen because of high noise. One way to overcome this problem is differential spectroscopy. Another more sophisticated method is modulated excitation spectroscopy (ME). Modulated excitation spectroscopy is a technique in which periodic excitation of the system is used to increase the SNR by compensating the background. The system is altered periodically via change of for example potential, concentration (e.g. ligand), light or mechanic force Furthermore, it is can be used detect highly overlapping bands via phase sensitive detection (PSD).

Figure 2.14: Schematic illustration of modulated excitation measurements. A parameter (like temperature) is changed periodically. The observed system reacts and after a short adaptation time, the system changes periodically around a median absorbance with the frequency of the excitation. The phase lag ψ can be determined as the time/phase angle, which the system needs to react on the stimulation. Adapted from [173]

In the experiment, a species is adsorbed on a ATR-element. The modulation of an external parameter causes a periodic change of concentrations of the species c_i , which can be described as:

$$\eta(t) = \eta_0 + \Delta\eta_0 + \Delta\eta_1 \cos(\omega t + \Theta) \quad (2.3.14)$$

where $\eta_0 + \Delta\eta_0$ denotes the average value of the excitation over the modulation, η_0 is the unmodulated ground state and Θ denotes the phase of stimulation. As shown in Fig. 2.14 the absorbance changes periodically with the modulation. After a few modulation cycles, the absorbance reaches a stationary state, which is the average of the modulated excitation absorbance. The system reacts with a time lag to the stimulation, which is defined as a phase lag ϕ [54, 174, 175]. Phase sensitive detection uses this phenomenon to increase the signal-to-noise ratio. Therefore it extracts only the parts of the signal which are modulated with the frequency of the modulation. Additionally, it can also be used to deconvolve overlapping bands as long as they have different phase lags [175].

Modulated IR spectroscopy has been shown to be a useful tool to study the function and kinetics of proteins [51, 52, 175, 176]. Cytochrome *c* oxidase of *rhodobacter spheroides* was used in SEIRAS (surface enhanced infrared absorbance spectroscopy) experiments to determine, structural change and the pK_a values of the prosthetic groups due to electrochemical modulation under anaerobic [51] and aerobic conditions [52]. Modulated measurements without a surface enhancement were done to study the conformational change of RNase A or temperature modulation [54] and the binding and rinsing of cholate on BSA [53].

2.3.8 Time resolved IR spectroscopy

Time resolved Fourier transform infrared spectroscopy (tr-FTIR) is used to analyse the dynamic change of systems. There are two major different techniques. The choice of technique depends on the time scale of the observed reaction. In all cases, spectra are taken during the perturbation of the system.

2.3.8.1 Rapid scan

The rapid scan technique is the easiest form of tr-FTIR measurements (Fig. 2.15A). The interferogram data points are measured along the retardation axis. The spectra are measured consecutively. This leads to the dependence of the time resolution from the mirror velocity [172].

Figure 2.15: Schematic illustration of the sequence of a (A) rapid scan and step (B) scan measurement. In a rapid scan experiment the interferograms are measured consecutively along the retardation axis. For step scan measurements, the interferograms are measured consecutively along the temporal axis. The numbers indicate the chronological sequence of data point acquisition; Handbook of vibrational spectroscopy [172]

2.3.8.2 Step scan

In contrast to the rapid scan technique, the step scan technique measures the interferogram along the time axis (Fig. 2.15B). The moving mirror is stopped at discrete positions and after a short waiting time, the interferogram data point is measured. The mirror stays at this position and measures the same retardation data point of all interferograms before changing position. The data points of each interferogram are collected and put together by the computer. Due to the independence of the time resolution from the mirror velocity, the time resolution can be as fast as 1ns. This makes the technique very the method of choice for very fast systems [172].

2.3.9 Quantitative IR spectroscopy

In order to get information on the surface concentration and orientation of a species on a ATR-crystal, several models have to be introduced.

2.3.9.1 Thin film approximation

Equation 2.3.13 shows the penetration depth of the evanescent field. In case of protein adsorption, the protein layer has a thickness of some nanometer. This comparative small layer (about 1% of d_p) plays a small role compared to the penetration depth. In this case, Harrick introduced the thin film approximation in which the thin layer can be neglected and the refractive index of the bulk n_3 is used instead of the refractive index of the layer n_2 . [171]

2.3.9.2 Relative electric field components

In ATR-IR-spectroscopy, the IR beam gets guided into the multiple reflection element and it hits the interface between the MIRE and the bulk solution and generates an evanescent wave. This evanescent wave can be divided into three relative electric field components:

$$E_{0x}^r = \frac{E_{0x,2}}{E_{0pp,1}} = \frac{2 \cos \Theta \sqrt{\sin^2 \Theta - \frac{n_3^2}{n_1^2}}}{\sqrt{1 - \frac{n_3^2}{n_1^2}} \cdot \sqrt{(1 + \frac{n_3^2}{n_1^2}) \sin^2 \Theta - \frac{n_3^2}{n_1^2}}} \quad (2.3.15)$$

$$E_{0z}^r = \frac{E_{0z,2}}{E_{0pp,1}} = \frac{2 \cos \Theta \sin \Theta}{\sqrt{1 - \frac{n_3^2}{n_1^2}} \cdot \sqrt{(1 + \frac{n_3^2}{n_1^2}) \sin^2 \Theta - \frac{n_3^2}{n_1^2}}} \quad (2.3.16)$$

$$E_{0y}^r = \frac{E_{0y,2}}{E_{0vp,1}} = \frac{2 \cos \Theta}{\sqrt{1 - \frac{n_2^2}{n_1^2}}} \quad (2.3.17)$$

In the coordinate system of the evanescent field, the x-component is in-plane in direction of the light beam, the y-component is in-plane perpendicular to the light beam and the z-component is pointing out of plane perpendicular to the light beam. The electric field of parallel (pp) polarized light consists of E_x and E_z components, and the electric field of vertical polarized light results in the E_y component (Fig. 2.16).

$$E_{pp} = \sqrt{E_{0x}^2 + E_{0z}^2} \quad (2.3.18)$$

$$E_{vp} = E_{0y} \quad (2.3.19)$$

Figure 2.16: Scheme of the coordinate system of the evanescent field on a ATR-crystal.[177]

According to Harricks thin film approximation, these equations can be modified by using n_3 (bulk solution) instead of n_2 (thin film).

$$E_{0x}^r = \frac{E_{0x,2}}{E_{0pp,1}} = \frac{2 \cos \Theta \sqrt{\sin^2 \Theta - \frac{n_3^2}{n_1^2}}}{\sqrt{1 - \frac{n_3^2}{n_1^2}} \cdot \sqrt{(1 + \frac{n_3^2}{n_1^2}) \sin^2 \Theta - \frac{n_3^2}{n_1^2}}} \quad (2.3.20)$$

$$E_{0z}^r = \frac{E_{0z,2}}{E_{0pp,1}} = \frac{2 \cos \Theta \sin \Theta}{\sqrt{1 - \frac{n_3^2}{n_1^2}} \cdot \sqrt{(1 + \frac{n_3^2}{n_1^2}) \sin^2 \Theta - \frac{n_3^2}{n_1^2}}} \quad (2.3.21)$$

$$E_{0y}^r = \frac{E_{0y,2}}{E_{0vp,1}} = \frac{2 \cos \Theta}{\sqrt{1 - \frac{n_3^2}{n_1^2}}} \quad (2.3.22)$$

Table 2.9: Relative electric field components for a protein film (amide II, $n_2 = 1.41$) in aqueous environment ($n_3 = 1.32$) on a Si ($n_1 = 3.5$) and ZnSe ($n_1 = 2.4$) ATR crystal (angle of incidence of 45°).

Surface	E_{0x}^r	E_{0y}^r	E_{0z}^r
Si	1.395	1.527	1.445
ZnSe	1.274	1.693	1.777

2.3.9.3 Effective thickness

The attenuation of the reflection due to the interaction of the evanescent wave does not follow a simple law. Therefore the low absorption approximation is introduced.

In transmission measurements with low absorbance, Lambert Beer's law can be simplified to:

$$\frac{I}{I_0} = e^{\alpha \cdot d} \longrightarrow \frac{I}{I_0} \approx 1 - \alpha \cdot d \quad (2.3.23)$$

where α is the absorption coefficient and d is the thickness. In order to apply Lambert Beer's law to ATR-spectroscopy, the effective thickness d_e has to be introduced:

$$R^N = (1 - \alpha \cdot d_e)^N \quad (2.3.24)$$

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“Effective thickness is defined as the thickness of a material required to give a spectrum of the same contrast in a transmission measurement as that obtained via internal reflection” [168].

$$d_e = \frac{n_2}{n_1 \cdot \cos \Theta} \cdot \frac{d_p}{2} - (e^{-\frac{2z_i}{d_p}} - e^{-\frac{2z_f}{d_p}}) \cdot (E_0^r)^2 \quad (2.3.25)$$

where the isotropic layer extends from z_i to z_f on the z -axis resulting in $d = z_f - z_i$ [174, 178]. If the thickness of the rarer medium (n_2) is much thicker than the penetration depth (d_p), the effective thickness is directly related to the penetration depth:

$$d_e = \frac{\frac{n_2}{n_1}}{\cos \Theta} \cdot \int_0^\infty E_0^2 \cdot dz = \frac{\frac{n_2}{n_1} \cdot E_0^2 \cdot d_p}{2 \cos \Theta} \quad (2.3.26)$$

In case of a very thin layer ($d \ll d_p$), the electric field intensity throughout the layer can be assumed to be constant. Consequently, d_p can be replaced by d and the equation is not wavelength dependent.

$$d_e^{th} = \frac{\frac{n_2}{n_1} \cdot E_0^2 \cdot d}{\cos \Theta} \quad (2.3.27)$$

d_e^{th} can be separated into parallel and perpendicular polarized light, where the ratio is:

$$\frac{d_{e,pp}}{d_{e,vp}} = \frac{(E_{0x2r})^2 + (E_{0z2r})^2}{(E_{0y2r})^2} \quad (2.3.28)$$

If the rarer medium is an isotropic medium and $\Theta=45^\circ$ the ratio is 2. In order to use Lambert Beers law on ATR spectroscopy, polarized light has to be used because the effective thickness of polarized light is a linear combination of $d_{e,pp}$ and $d_{e,vp}$ [47].

$$d_{e,vp} = d_{e,y} = \frac{3}{2} \cdot (1 - \langle \cos^2 \Theta \rangle) d_{e,y}^{iso} \quad (2.3.29)$$

$$d_{e,pp} = d_{e,x} + d_{e,z} = \frac{3}{2} \cdot (1 - \langle \cos^2 \Theta \rangle) d_{e,x}^{iso} + 3 \langle \cos^2 \Theta \rangle d_{e,x}^{iso} \quad (2.3.30)$$

The axial effective thickness $d_{e,x}^{iso}$, $d_{e,y}^{iso}$ and $d_{e,z}^{iso}$ is direct proportional to the real sample thickness d (Harricks thin film approximation) [168, 171].

2.3.9.4 Dichroic Ratio

Polarized light can also be used to get information about the orientation of the molecules e.g. lipids [179], but also the secondary structure of proteins [180]. The integrated absorbance of a band is directly proportional to the square of the scalar product of the transition dipole moment and the $\vec{M}$ electric field $\vec{E}$ of radiation (responsible for excitation). The intensity of absorption depends on the orientation of the vectors.

$$\Delta I \propto (\vec{M} \cdot \vec{E})^2 = |\vec{M}| \cdot |\vec{E}| \cos^2(\vec{E}, \vec{M}) = (E_x M_x + E_y M_y + E_z M_z)^2 \quad (2.3.31)$$

E_i and M_i ($i = x, y, z$) denote the components of the electric field and the transition dipole moment in the coordinate system.

In order to work with dimensionless relative intensities, the dichroic ratio is calculated via the ratio of the integrated bands measured with perpendicular and parallel polarisation:

$$\langle R \rangle = \frac{\int A_{pp}(\tilde{\nu}) d\tilde{\nu}}{\int A_{vp}(\tilde{\nu}) d\tilde{\nu}} = \frac{d_{e,pp}}{d_{e,vp}} = \frac{E_x^2}{E_y^2} + 2 \cdot \frac{E_z^2}{E_y^2} \cdot \frac{\sum_{i=1}^n \langle \cos^2 \Theta \rangle}{\sum_{i=1}^n \langle \cos^2 \Theta \rangle} = \quad (2.3.32)$$

$$= \frac{E_x^2}{E_y^2} + 2 \frac{E_z^2 \overline{\langle \cos^2 \Theta \rangle}}{E_y^2 (1 - \overline{\langle \cos^2 \Theta \rangle})} \quad (2.3.33)$$

$\overline{\langle \cos^2 \Theta \rangle}$ is defined as “the average over the mean squares of the cosines of the angle between the transition moments of a given vibration and the z-axis of the laboratory coordinate system” [181].

$$\overline{\langle \cos^2 \Theta \rangle} = \frac{E_x^2 - \langle R \rangle \cdot E_y^2}{E_x^2 - \langle R \rangle \cdot E_y^2 - 2E_z^2} \quad (2.3.34)$$

This equation can be transformed to:

$$\Theta = \arccos \left(\frac{E_x^2 - R \cdot E_y^2}{E_x^2 - R \cdot E_y^2 - 2E_z^2} \right)^{\frac{1}{2}} \quad (2.3.35)$$

Θ is the average angle of the dipole transition of a vibration. The order parameter S can also be used to gain information about the orientation. The value of S lays between $-\frac{1}{2}$ and 1 (Fig. 2.17). If the vibration is oriented parallel to the surface ($\Theta = 90^\circ$), or normal to the z-axis respectively, $S = -\frac{1}{2}$. If $S = 0$, there is no specific orientation with respect to the z-axis. If $S = 1$, the vibration is oriented parallel with the z-axis ($\Theta = 0^\circ$).

$$S_z = \frac{3}{2} \cdot \overline{\cos^2 \Theta} - \frac{1}{2} \quad (2.3.36)$$

The parameters S_z and Θ provide valuable information about the system. However it is limited by the exact knowledge of the refractive index, especially for lipid-protein-complexes. Another limitation concerns the assumptions of the angle of the dipole moment of the selected mode with respect to the molecular axis (β). This parameter varies for helices in proteins between an angle of 24° and 40° for amide I band and 73° and 88° for amide II band [183]. Nevertheless it is still used as approximation for oriental measurements of the orientation of α -helices in proteins [47, 181, 184–186].

Figure 2.17: The C=O vibrations of the amide backbone in an α -helix contribute 80% of the intensity of the Amide I band. This enables the calculation of the angle of the helix with respect to the z-axis (normal to the surface). β is the angle between the C=O vibrations and the helix. In measuring the orientation of the C=O vibrations of the helix, it is possible to measure the orientation of the helix. Three cases are shown: In the first case Θ_1 the helix is oriented orthogonal to the surface. The average angle of the C=O vibrations is 0° and $S=1$. In the second case Θ_2 , the angle is increasing and the vibrations are oriented at an angle of 54.7° with respect to the z-axis ($S=0$). If the helix is oriented parallel to the surface (Θ_3), the angle is 90° and $S = -\frac{1}{2}$. The angle β represents the angle between the C=O vibration and the orientation of the helix (helix adapted from [182])

As additional tool, dichroic spectra (D^*) provide information on whether a vibration is oriented parallel or perpendicular to the surface. A negative band is assigned to a vibration parallel to the surface where a positive band means the vibration is oriented perpendicular to the surface. If no peak appears, the vibration is oriented neither parallel nor perpendicular to the surface.[44, 181]. It is calculated via

$$D^*(\tilde{\nu}) = A_{pp}(\tilde{\nu}) - R_{iso} \cdot A_{vp}(\tilde{\nu}) \quad (2.3.37)$$

where R_{iso} is defined as the dichroic ratio for a isotropic distributed analyte: $R_{iso} = \frac{E_x^2 + E_z^2}{E_y^2}$.

2.3.9.5 Active total reflections

The number of reflections in a multiple internal reflection element can be calculated via:

$$N = \frac{l}{t \cdot \tan \Theta} \quad (2.3.38)$$

where l is the length and t is the thickness of the MIRE.

2.3.9.6 Surface concentration

In transmission experiments, the concentration of an analyte in solution can be easily calculated via Lambert-Beers law:

$$E_\lambda = \log \left(\frac{I}{I_0} \right) = \epsilon_\lambda \cdot c \cdot d \quad (2.3.39)$$

where ϵ_λ is the molar extinction coefficient at wavelength λ , d is the thickness of the cell and E_λ is the extinction at wavelength λ .

The volume concentration c and the surface concentration Γ are related to each other:

$$\Gamma = c \cdot d = \frac{A_{pp} \cdot d}{\epsilon \cdot d_{e,pp}} = \frac{\int A_{pp} d\tilde{\nu} \cdot d}{\int \epsilon d\tilde{\nu} \cdot d_{e,pp}} \quad (2.3.40)$$

$$= \frac{A_{vp} \cdot d}{\epsilon \cdot d_{e,vp}} = \frac{\int A_{vp} d\tilde{\nu} \cdot d}{\int \epsilon d\tilde{\nu} \cdot d_{e,vp}} \quad (2.3.41)$$

Including multiple reflections and number of functional groups, the equation is modified to:

$$\Gamma = \frac{\int A_{pp}(\tilde{\nu}) d\tilde{\nu} \cdot d}{N \cdot v \cdot d_{e,pp} \int \epsilon(\tilde{\nu}) d\tilde{\nu}} \quad (2.3.42)$$

$$= \frac{\int A_{vp}(\tilde{\nu}) d\tilde{\nu} \cdot d}{N \cdot v \cdot d_{e,vp} \int \epsilon(\tilde{\nu}) d\tilde{\nu}} \quad (2.3.43)$$

where $\int A_{vp}(\tilde{\nu}) d\tilde{\nu}$ is the integrated absorbance of a band, measured with perpendicularly polarised light, N is the average number of active internal reflections and v is the number of equal functional groups. $\epsilon(\tilde{\nu}) d\tilde{\nu}$ (Table 2.10) is the decadic integrated molar absorption coefficient of the band and $d_{e,vp}$ is the effective thickness [44–47, 53, 181, 187].

Table 2.10: Optical Parameter for surface quantification of proteins via integral of the band area. [181].

Band	Wavenumber (cm ⁻¹)	$\epsilon(\tilde{\nu}) d\tilde{\nu}$ (l · mol ⁻¹ cm ⁻¹)	Local refractive index n_2
$\nu(\text{NH})$	≈ 3300	$2.09 \cdot 10^7$	1.4
$\nu(\text{CH})$	2800-3000	$6.60 \cdot 10^5$	1.47
Amide I	≈ 1650	$2.74 \cdot 10^7$	1.4
Amide II	≈ 1540	$8.25 \cdot 10^6$	1.4

2.3.10 2D Infrared spectroscopy

An external perturbation is applied to a system which causes the system to change in a unique and selective manner, as explained in chapter 2.3.7. The obtained spectra often consist of highly overlapping bands, which makes it difficult to find and assign individual components of the bands. There are several methods like second derivative, curve fitting, fourier self deconvolution, phase sensitive detection and 2D correlation spectroscopy to deconvolute those bands [188–190].

2D spectroscopy has been applied in NMR spectroscopy for several decades. Two dimensional correlation spectroscopy is applied to obtain information, which is not available from 1D spectra.

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2.3.10.1 Theory of 2D-infrared correlation spectroscopy

The perturbation induced absorbance (dynamic spectrum $\tilde{y}(v, t)$) can be expressed as a combination of static absorbance spectrum (reference spectrum) and an absorbance spectrum $y(v, t)$ which is dependent on the modulation.

$$\tilde{y}(v, t) = y(v, t) - \bar{y}(v) \quad (2.3.44)$$

The reference spectrum $\bar{y}(v)$ is not strictly defined and can be set as the averaged spectrum during perturbation which is given as

$$\bar{y}(v) = \frac{1}{T_{max} - T_{min}} \int_{T_{min}}^{T_{max}} y(v, t) dt \quad (2.3.45)$$

where T_{min} is defined as the beginning and T_{max} as the end of the period of perturbation. An other definition is the absorbance which is without any external perturbation ($t = T_{min}$).

2D correlation spectroscopy compares the spectral intensity variations over an external variable t at two spectral variables v_1 and v_2 . The intensity of 2D correlation spectrum $X(v_1, v_2)$ can be expressed as

$$X(v_1, v_2) = \langle \tilde{v}(v_1, t) \cdot \tilde{v}(v_2, t') \rangle \quad (2.3.46)$$

$X(v_1, v_2)$ can be converted into a linear combination of synchronous $\Phi(v_1, v_2)$ and asynchronous $\Psi(v_1, v_2)$ 2D-correlation intensities.

$$X(v_1, v_2) = \Phi(v_1, v_2) + i\Psi(v_1, v_2) \quad (2.3.47)$$

The 2D correlation function is expressed as

$$\Phi(v_1, v_2) + i\Psi(v_1, v_2) = \frac{1}{\pi(T_{max} - T_{min})} \int_0^\infty \tilde{Y}_1(\omega) \cdot \tilde{Y}_2^*(\omega) d\omega \quad (2.3.48)$$

where $\tilde{Y}_1(\omega)$ is the forward Fourier transform of the spectral intensity variations $\tilde{y}(v_1, t)$ and $\tilde{Y}_2(\omega)$ is the conjugate of the Fourier transform at v_1 and time t .

$$\tilde{Y}_1(\omega) = \int_0^\infty \tilde{y}(v_1, t) e^{-i\omega t} dt = \tilde{Y}_1^{Re}(\omega) + i\tilde{Y}_1^{Im}(\omega) \quad (2.3.49)$$

$$\tilde{Y}_2(\omega) = \int_0^\infty \tilde{y}(v_2, t) e^{-i\omega t} dt = \tilde{Y}_2^{Re}(\omega) + i\tilde{Y}_2^{Im}(\omega) \quad (2.3.50)$$

The $\tilde{Y}^{Re}(\omega)$ and $\tilde{Y}^{Im}(\omega)$ are the real and imaginary components of the Fourier transform.

If a sinusoidal perturbation with frequency Ω is applied to the system the dynamic spectrum can be expressed as:

$$\tilde{y}(v, t) = A'(v) \sin(\omega t) + A''(v) \cos(\Omega t) \quad (2.3.51)$$

where $A'(v)$ and $A''(v)$ are amplitudes of two orthogonal components of the sinusoidal function. The introduction of the wavenumber-dependent magnitude $\hat{A}(v)$ and phase angle of the signal $\beta(v)$

$$\tilde{y}(v, t) = \hat{A}(v) \sin(\Omega t + \beta(v)) \quad (2.3.52)$$

where

$$\hat{A}(v) = \sqrt{A'(v)^2 + A''(v)^2} \quad (2.3.53)$$

and

$$\beta(v) = \arctan \frac{A''(v)}{A'(v)} \quad (2.3.54)$$

Substitution of the Fourier transformation of equation 2.3.51 into equation 2.3.48 obtains the analytical expression for the intensities of corresponding 2D correlation spectra:

$$\Phi(v_1, v_2) = \frac{1}{2} [A'(v_1)A'(v_2) + A''(v_1)A''(v_2)] \quad (2.3.55)$$

$$\Psi(v_1, v_2) = \frac{1}{2} [A''(v_1)A''(v_2) - A'(v_1)A'(v_2)] \quad (2.3.56)$$

Using the terms $\hat{A}(v)$ and $\beta(v)$ the equation can be converted to

$$\Phi(v_1, v_2) = \frac{1}{2} \hat{A}(v_1) \hat{A}(v_2) \cos[\beta(v_1) - \beta(v_2)] \quad (2.3.57)$$

$$\Psi(v_1, v_2) = \frac{1}{2} \hat{A}(v_1) \hat{A}(v_2) \sin[\beta(v_1) - \beta(v_2)] \quad (2.3.58)$$

which are the real and imaginary part of the dynamic cross correlation function $X(v_1, v_2)$ [188, 189, 191].

2.3.10.2 Synchronous 2D-correlation spectrum

Synchronous 2D-correlation spectra show simultaneous or coincidental changes of two separate spectral intensity variations along the perturbation axis and consist of two kind of peaks. Autopeaks are located along the diagonal axis of the contour map. Those peaks which are always positive form the auto power spectrum. The intensity represents the overall extend of spectrum intensity variation between T_{min} and T_{max} . The spectral region which changes during perturbation develop autopeaks, those which don't show only weak or no autopeaks.

Crosspeaks are located off the diagonal axis and represent spectral changes in intensity at two wavenumbers v_1 and v_2 . A crosspeak suggests a correlation between the peaks at v_1 and v_2 . In contrast to a autopeak, a crosspeak can be positive or negative. A positive crosspeak at $\Phi(v_1/v_2)$ shows that v_1 increases or decreases together with v_2 while a negative crosspeak indicates a change in opposite direction.

2.3.10.3 Asynchronous 2D-correlation spectroscopy

In contrast to synchronous 2D-correlation spectra, the asynchronous spectra consist only of crosspeaks which are located antisymmetric with respect to the diagonal line. The crosspeaks show sequential or successive but not coincidental changes. It develops if the intensity changes are out of phase with each other. This makes it useful to identify overlapping bands because the spectrum can be deconvoluted as long as the spectral changes along the external variable are different.

The crosspeaks can be positive or negative and provide useful information about the sequential order along the perturbation [188].

Figure 2.18: (A) **Synchronous 2D IR spectrum:** The cross peaks indicate the correlation of the peaks A and C and B and D. (B) **Asynchronous 2D IR spectrum:** The cross peaks provide information about the temporal sequence of different components of the spectrum. Changes of peaks B and D occur before A and C [188, 189]. (C) **Phase angle:** Different components can be resolved by asynchronous spectra because of their different response time caused by an external excitation. This phase lag of component A is lower than the phase lag of component B.

2.3.11 Nodas rules

Nodas rules can be used for correlation spectra interpretation.

1. If a crosspeak in the asynchronous spectrum at $\Psi(v_1/v_2)$ is positive, the dipole moment at v_1 changes before v_2 .
2. If a crosspeak in the asynchronous spectrum at $\Psi(v_1/v_2)$ is negative, the dipole moment at v_1 changes after v_2 .
3. If a crosspeak in the asynchronous spectrum at $\Psi(v_1/v_2)$ is negative, the rules 1+2 are reversed.
4. If the crosspeak in the synchronous spectrum at $\Phi(v_1/v_2)$ is negative, the rules 1-3 are reversed.
5. If a crosspeak in the asynchronous spectrum at $\Psi(v_1/v_2) = 0$, the dipolemoment changes simultaneous.
6. If a crosspeak in the synchronous spectrum at $\Phi(v_1/v_2) = 0$, the sequential order can't be determined.

2.3.11.1 Moving window 2D correlation spectroscopy

A further application of 2D correlation spectroscopy is moving window 2D (MW2D) correlation spectroscopy . It correlates the spectral change in the spectrum with the perturbation variable. In contrast to the classic 2D correlation spectra, presented in chapter 2.3.10.2 and 2.3.10.3, the moving window spectra are plotted on a plane between the spectral variable v and the perturbation variable t . A big advantage is the direct visualization of intensity change due to perturbation. However a drawback is the inefficiency in separating overlapping bands.

A spectra intensity data matrix $y(v, t)$ is introduced. The rows provide information about the spectral variable v and the columns provide information about the perturbation variable t . The spectral data is subdivided by a moving window with a windows size $N = 2m + 1$ around the j^{th} spectrum:

$$y_j(v, t_1) = \begin{bmatrix} y(v, t_{j-m}) \\ y(v, t_{j-m+1}) \\ \vdots \\ y(v, t_j) \\ \vdots \\ y(v, t_{j+m}) \end{bmatrix} \quad (2.3.59)$$

where J is the index of the selected window position and j is the index of the individual spectrum within the window. The dynamic spectrum in the j^{th} window is:

$$\overline{y}_j(v) = \frac{1}{2m+1} \sum_{J=j-m}^{j+m} y_j(v, t_J) \quad (2.3.60)$$

and the autocorrelation spectrum in the window is defined by

$$\Omega_{A,j}(v, t_j) = \frac{1}{2m} \sum_{J=j-m}^{j+m} \bar{y}_j^2(v, t_J) \quad (2.3.61)$$

The autocorrelation spectrum $\Omega_{A,j}$ is obtained by moving the window along the perturbation axis from $j = 1 + m$ to $j = N - m$ while the equations 2.3.44, 2.3.60 and 2.3.61 are calculated for every window [192, 193].

2.3.12 Raman spectroscopy

Raman spectroscopy is a further method which is used to analyse the vibrational and rotational modes in a system. It is based on the interaction of photons with matter, called the Raman effect. In contrast to infrared spectroscopy, which requires a change in dipole moment during vibration, Raman spectroscopy requires a change in polarisation. In general, very polar bonds like carbonyl-, carboxyl-groups give a weak band, and apolar bonds (C-C, C-H,...) have a strong signal [155].

A laser beam with wavelength v_s is irradiated on the probe and the photons are scattered inelastically (Raman scattering). The photons interact with the vibrations of the system and energy is transferred, which results in a change of frequency (Raman shift $\Delta\tilde{\nu}$):

$$\Delta\tilde{\nu} = v_s - v_0 = \frac{\Delta E}{h \cdot c} = \frac{E_0 - E_S}{h \cdot c} \quad (2.3.62)$$

The Raman shift can result in an up- ($\Delta\tilde{\nu} > 0$, anti stokes shift) or downshift ($\Delta\tilde{\nu} < 0$, stokes shift) of the energy of the photons.

The scattered light is collected, sent through a monochromator and detected. The highest intensity of the system is generated by elastic scattering ($\Delta\tilde{\nu} = 0$, Rayleigh scattering), which has to be filtered out. Generally, the Raman scattering is much weaker ($10^{-5}\%$ of beam intensity). This is also the major disadvantage compared to infrared spectroscopy because it requires a high concentration of the analyte, a high laser intensity or special prepared surfaces for surface enhanced Raman scattering to receive high intensities.

Raman spectroscopy can be used for the analysis of point group, orientation, composition, doping or temperature of materials. One of the big advantages of Raman spectroscopy is the low intensity of water bands, which makes it very useful for the investigation of biological samples [155]. One special application of Raman spectroscopy is Raman microscopy, which combines a normal optical microscope and a Raman spectrometer. This makes it possible to map the surface and to receive spacial and spectral information .

Figure 2.19: Raman spectra of graphene monolayer (A) without and (B) with defects. In the ideal case of a impeccable graphene monolayer, the Raman spectrum is dominated by the 2D band and two smaller bands (G and 2D'). The introduction of defects causes the forming of a D, D' and D+D' band [194].

2.3.12.1 Raman spectroscopy of Graphene

Raman spectroscopy is a fast and easy method to characterise graphene. The spectrum of a defect free monolayer of graphene is characterized by two prominent bands, namely G-band and 2D(G')-band (figure 2.19a). In consequence of defects, oxidations, number of layers,... the Raman spectrum can change [195, 196]. Instead of two bands, the number and intensity of the bands can vary (figure 2.19b).

Each band can be assigned to different vibrational modes [195, 197–200]

- The **D-band** is situated at about 1350cm^{-1} and is associated with breathing oscillation of a hexagonal carbon ring. In a perfect lattice it is a forbidden vibration and so it is also an indicator for defects in graphene. The intensity decreases with an increasing number of layer of graphene layer, where it's not visible in graphite (2.20A).
- The **G-band** is localised at about 1570cm^{-1} and attributed to the in plane sp^2 -vibrations of the carbon atoms (LO and TO phonon mode) (2.20A).
- The **D'-band** at 1620cm^{-1} and **D+G-** or **D+D'-band** at 2940cm^{-1} are caused by disorder in the graphene lattice (2.20B).
- The **2D or G'-band** is at 2673cm^{-1} and is the overtone of the D-band (2.20B).

Figure 2.20: Vibrational modes of graphene (A) G-band consists of sp^2 C-C stretching modes. (B) The D and 2D band originates from the breathing mode of the carbon rings. The D band requires a defect in the lattice. [194]

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The ratio between the G and 2D band provides information about the number of layers. A monolayer is characterized by a high intensity of the 2D band and a low intensity G-band (about 1/4 of 2D band). The ratio changes drastic with increasing layers. The 2D-band of a bilayer graphene has equal intensity to the G band. The spectrum of a multilayer has a much lower 2D-intensity, than the G band. The shape of the 2D-band can also be used to discriminate between a multilayer of graphene and graphite.

A second ratio of interest is the G/D-ratio. It provides information about the average distance between the defects [195, 197].

2.4 Quartz Crystal Microbalance

The concept of a quartz crystal microbalance (QCM) has been introduced in 1948 by Mason, when he suggested the use of piezoelectric resonators for mass detection on surfaces. In 1959, Sauerbrey published in “Verwendung von Schwingquarzen zur Wägung dünner Schichten und zur Mikrowägung” the linear dependence of the change in resonance frequency on the the deposited mass and so the system came into focus of interest [201]. For long time it was thought that the system can not be used in liquid phase because of the damping of the aqueous bulk phase. So, it was used as thickness monitor in vacuum chambers or in air. Kasemo and coworkers were the first to build a quartz microbalance with dissipation monitoring, which was also able to measure in liquid [202]. This revolutionized the technique.

2.4.1 Principle of QCM-D and Sauerbrey model

Piezoelectric crystals like quartz exhibit the property of induction of a electric field when mechanical stress is applied. Accordingly the crystal oscillates when an electric field is applied. The frequency is dependent of the thickness of the resonator, while resonance occurs when the thickness is a multiple of half the wavelength $t_q = n \cdot \frac{\lambda}{2}$. The resonance frequency can be calculated by:

$$f = n \cdot \frac{v_q}{2 \cdot t_q} = n \cdot f_0 \quad (2.4.1)$$

where v_q is the wave velocity, t_q the resonator thickness and n the overtone number (1, 3, 5,...). Only odd harmonics can be excited electrically because they lead to opposite charges on the edges of the crystal.

The adsorption of mass Δm on the surface leads to a decrease in the resonance frequency Δf .

$$df = \Delta f = -\frac{f}{t_1 \cdot \rho_q} \cdot \Delta m = -n \frac{2 \cdot f_0^2}{c_q \cdot \rho_q} = -n \cdot \frac{1}{C} \cdot m \quad (2.4.2)$$

where C is the mass sensitivity constant ($C=17,7 \text{ ng} \cdot \text{cm}^{-2} \cdot \text{Hz}^{-1}$ at 5 Mhz). Strictly speaking, the Sauerbrey equation can only be applied for rigid and homogeneous adsorbed layers [201]. However, Sauerbrey's equation gives a good approximation for the adsorbed mass on the crystal but the mass is underestimated for thin viscous films in liquid because they are not completely coupled to the vibration of the resonator.

2.4.2 Dissipation

In many cases, the adsorbed or bound molecules are not rigid. Especially in macromolecules like proteins are hydrated and dissipate energy due to viscous or hydrodynamic. In this case, dissipation also has to be taken into account [203–205]. The (energy) dissipation is defined as:

$$D = \frac{1}{Q} = \frac{E_{dissipated}}{2 \cdot \pi \cdot E_{Stored}} \quad (2.4.3)$$

and is a measure of the damping of the resonance oscillation. After switching off the applied voltage, the oscillating crystal still oscillates sinusoidally but decays exponentially (Fig. 2.21).

$$A(t) = A_0 \cdot e^{-\frac{t}{\tau}} \cdot \sin(2\pi ft + \vartheta) \quad (2.4.4)$$

Figure 2.21: Principle of QCM-D: The resonator gets excited by applying a current. After the excitation stops, the crystal vibrations decay exponentially. The change of frequency and dissipation is direct proportional to the amount of the adsorbed mass and the dissipation provides information about the structure. [206].

where A_0 is the amplitude while voltage is applied, τ is the decay time constant and ϑ is the phase angle. A rigid film causes no damping and leads to a slower decay of the oscillation. In viscous films the damping effects the decay time and consequently the decay time is shorter. The decay time constant is related to the dissipation factor:

$$D = \frac{1}{\pi \cdot f \cdot \tau} \quad (2.4.5)$$

In case of protein adsorption on a substrate, the dissipation is influenced by protein-substrate and protein-bulk interactions and changes in the protein layer. Studies have shown that the change of dissipation arises primarily from the protein layer interface such as changes in protein conformation and the amount of water trapped within the adsorbed layer [207].

Chapter 3

Instruments and Methods

3.1 Instrumentation

3.1.1 Infrared spectroscopy

The infrared measurements for characterization of the GFET were done on a Bruker Vertex 70V FT-infrared spectrometer (Bruker, Ettlingen, Germany). The used ATR-crystals were

- Si-single reflection element (custom made): $\Theta=60^\circ$; Korth Kristalle GmbH, Altenholz, Germany; mounted on a home-made ATR-setup described in [173]
- Si-multiple reflection element: 12.5 active internal reflections, $\Theta=45^\circ$; Harrick Scientific, Pleasantville, New York, USA; 50x10x2mm
- ZnSe-multiple reflection element: 12.5 active internal reflections, $\Theta=45^\circ$; Harrick Scientific, Pleasantville, New York, USA; 50x10x2mm

The MIRE was mounted in a Harrick Horizon (Harrick Scientific, Pleasantville, New York, USA) with Horizon Liquid Sampler. Measurements on the MIRE setup were done with temperature control (23°C).

A globar was used as IR-source and a liquid nitrogen cooled MCT-detector as detector. In order to increase the signal to noise ratio, the interferogram was measured double sided and the method to calculate absorbances was power phase correction mode. The spectral resolution was set to 4 cm^{-1} and the mirror velocity was adjusted to 80 kHz.

In order to reduce interfering effects of CO_2 and water-vapor, the chamber was purged with dry carbon-dioxide free air.

Spectra were analyzed using Brukers OPUS 7.2 software and OriginLab Origin 8.5.

3.1.2 QCM-D Measurements

The quartz crystal microbalance measurements were performed on a Q-Sense E4 system (Q-Sense AB, Västra Frölunda, Sweden) with flow module QFM 401. The system allows the simultaneous measurement of frequency and dissipation. The used sensors are:

- 100 nm Gold on Cr (QSX 301)
- 100 nm SiOx on Gold (QSX 303)

The QCM sensors (Q-Sense AB, Sweden) consist of an AT-cut piezoelectric quartz disc (5 MHz) with gold electrodes evaporated on both sides. These electrodes serve as contacts to the measuring unit. The used software for measurements was Q-Soft (Version 4.3) and QTools (Version 3.5) was used for data analysis.

All measurements were done at a controlled temperature of 25°C.

3.1.3 Raman spectroscopy

Raman measurements were done using a Horiba LabRAM HR Raman confocal microscope with a laser with a wavelength of 532 nm (52 mW, 10% filter) used for excitation. The laser spot size was of 520 μm^2 . A silicon wafer was used as substrate. Labview 6.1. was used for measuring and as analysis software.

3.1.4 Scanning electron microscopy

Scanning electron microscopy (SEM) pictures were made on a Zeiss SUPRA 40 Field Emission Scanning electron microscope. The control software was SmartSEM V05.

3.1.5 Contact angle measurements

Static contact angle measurements were performed using a DSA 25E contact angle measuring system (Krüss, Hamburg, Germany). 5 μl Milli-Q-water were dropped on a functionalized silicon waver. The static contact angle (Θ) was determined via drop shape analyser software using circle method.

3.2 Preparation methods

3.2.1 Preparation of silicon oxide surface

A silicon oxide surface was immersed for 1 hour in a 2%-ethanolic APTES (amino-propyl-triethoxy-silane) solution and baked for 1h at 120°C. A aqueous suspension of graphene oxide was incubated for 1h and afterwards washed with water.

The reduction was done with two reducing agents: hydrazine (used for QCM-crystal) and hydroiodic acid (used for silicon wafer). For hydrazine reduction was done in gas phase by evaporating the liquid in a closed container. The reduction with hydroiodic acid was done by placing liquid onto the GO-surface or evaporating HI in a closed chamber with the chips. The GO products were provided by Ciril Reiner-Rozman (GO Larisika, AIT, Austria), Nikolaus Leitner (GO Marcano, AIT, Austria) and University of Bayreuth (GO Feicht, Group Breu).

5 μM pyrene-butyl-succinimide ester (PBSE) was dissolved in tetrahydrofuran (THF) and dropped onto the reduced GO. After evaporation of the solvent, the surface was washed with THF and dried with argon. 10 μM OBP14 was added onto the surface and incubated for 1h. The unspecific bond protein was washed off with PBS buffer.

25 nm Tantal pentoxide were sputtered on a clean SiOx surface using a Univex 450c (Leybold System) sputtering system. A mixture of argon and oxygen (4:1) was pumped into the sputtering chamber with a flow rate of 6sccm and a chamber pressure of 2 μbar . Applying a power of 40W

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on the target holder, a rate of 0.048nm/s was achieved. After the coating, the surface was activated via UV-ozone treatment and afterwards immersed in a 2%-ethanolic APTES solution for 3h.

3.2.2 QCM-measurements

A clean SiO_x-sensor was functionalized with an ethanolic 2% APTES solution and baked for 1h at 120°C. Baseline was recorded with distilled water at a flow speed of 25 μ l/min. When the baseline was stable, 0.1 mg/ml (GO Marcano), 0.5 mg/ml (GO Feicht) was pumped through the chamber using a peristaltic pump (Ismatech SA, Switzerland) with a flow speed of 25 μ l/min. After saturated adsorption was reached, the cell was rinsed with distilled water.

For adhesion studies, the SiO_x crystal was prepared with GO or Ta₂O₅ as described in 3.2.1. The GO was reduced with hydrazine at 105°C overnight. The cell was filled with PBS pH 7.5. After the baseline was stable 30 μ M lysozyme was pumped into the cell and subsequently washed with PBS.

3.2.3 Determination of extinction coefficient

Transmission measurements were done in a Tensor 39 IR-spectrometer (Bruker, Ettlingen, Germany) using CaF₂ transmission cell with a 25 μ m Pb-spacer. For measurements, all parameters were set as for the Vertex 70V. Cell thickness d (μ m) was calculated via the fringe pattern of the empty cell:

$$d = \frac{10000 \cdot n}{2 \cdot \tilde{\nu}_2 - \tilde{\nu}_1} \quad (3.2.1)$$

where n is the number of maxima, $\tilde{\nu}_2$ and $\tilde{\nu}_1$ are denoted to the higher wavenumber and lower wavenumber which include the maxima.

Various PBSE concentrations (in THF) were measured with 32 scans for a spectrum, where the cell, filled with pure THF is used as reference. The integral of the 1745, 1785 and 1815 cm^{-1} was calculated and Lambert Beer's law was applied to obtain the extinction coefficient of the C=O vibrations.

$$\epsilon = \frac{\int \tilde{\nu}_{\text{C=O}} d\tilde{\nu}}{d \cdot c} \quad (3.2.2)$$

3.2.4 Determination of surface concentration

In order to determine the surface concentration, polarised measurements have to be done in parallel (pp) and perpendicular (vp) polarization. Therefore the Si ATR-crystal was prepared as described in chapter 3.2.1 but modifications have to be applied. Due to the water sensitivity of the reactive ester, a buffer measurement (reference measurement for protein quantification) has to be done before the linker is immobilised. The reference measurements (500 scans) for the Linker are done in THF.

The calculation of the surface concentration Γ was done applying Harrick's thin film approximation and the concept described in chapter 2.3.9. An Excel file, provided by Prof. Baurecht (University of Vienna, Austria) was used for computation.

Figure 3.1: Schematic figure of the modulated excitation setup: A buffer solution and a second buffer solution, that contains a ligand a periodically injected to the flow cell. The immobilized OBP changes its structure due to the change of environment. The IR-spectrometer triggers the pumps via a pump controller, which switches the pumps on and off alternating.

3.2.5 Modulated Infrared spectroscopy

The ZnSe-crystal was functionalized overnight using a solution of 5mM DTNTA and 5mM di-thiopropionic acid (DTP) (dissolved in DMSO) (Fig 3.3). After rinsing with water, the surface is immersed in 40mM NiCl_2 in acetate buffer (pH 5.5) followed by washing with Milli-Q-water. Recombinantly produced OBP14 (dissolved in PBS, pH 7.2) with a his-tag engineered on the N-terminus was immobilised on the prepared ZnSe crystal. Temperature was adjusted to 23°C. In the course of protein binding, spectra were taken in 5 minutes intervals with 1100 scans per spectrum. After 4h incubation, the unbound protein was rinsed with PBS buffer.

The setup for modulation measurements is shown in Fig. 3.1. The IR spectrometer triggers the (home made) pump controller which controls two peristaltic pumps (Ismatec SA, Switzerland). The trigger signal is generated by the infrared spectrometer and gets controlled via an oscilloscope. Pump A pumps a buffer solution which contains e.g. a ligand like eugenol. After one half period pump A is switched off and pump B is pumping. Solution B contains the same buffer as A, but no ligand. For pH-modulated measurements solution A contains the lower pH buffer and solution B the higher pH solution buffer. The cell volume was 0.5 ml and the used flow rate was 0.75 ml/min.

The modulation signal alternates periodically between two states as shown in Fig. 3.2. Infrared spectra were recorded successively in the course of the modulation.

Figure 3.2: Principle of time resolved infrared measurement: A modulation signal is periodically changed between two states (eg. buffer with ligand - pure buffer). During this modulation, spectra are measured which show the change of the system due to the modulation signal. Multiple spectra are recorded to observe the kinetics. The band area of the observed bands are calculated and plotted as a function of time. They should show an exponential increase and decrease of the band area.

Figure 3.3: DTNTA and DTP are immobilised on the ZnSe ATR crystal, where the di-sulphur-bonds (red atoms) break and bind covalently to the surface. The NTA-structure of DTNTA (blue atoms) is able to complex Ni or Co. This complex binds His-tag labelled proteins. DTP serves as spacer between the DTNTA molecules to regulate protein density.

The modulation was repeated 100 times at a modulation frequency of 1.7 mHz, where every modulation period was divided into 32 sample point spectra (SPS), where each half period contained 16 SPS. Each SPS was acquired via 64 scans.

Absorbance was calculated via $A = \frac{I}{I_0}$, where the last SPS was defined as I_0 (high pH for pH modulation and buffer for ligand modulation).

Every modulation was taken with and without protein. Difference spectra were acquired by subtracting each absorbance spectrum of the measurement without OBP from the equivalent spectrum with protein. These difference spectra were further used for analysis.

In order to acquire information about the band kinetics, the band area of the bands were calcu-

lated and plotted as a function of time.

Prof. Baurecht (University of Vienna, Austria) provided the OPUS modulation control macro, the PSD-macro and the pump control equipment.

Chapter 4

Results

4.1 Characterisation of the rGO-FET setup

In this chapter, the fabrication of the rGO-FET is characterized layer by layer (Fig.4.1) via vibrational spectroscopy, QCM-D, SEM and contact angle measurements. The gained data is used to optimise the production of the FETs and to control the successful functionalization and immobilization of the components.

Figure 4.1: Layer by layer setup of the GFET. A silicon oxide surface is functionalized with APTES to bind GO. After reduction a PBSE linker adsorbs on the surface and binds the OBP in random orientation

4.1.1 immobilization of APTES

Graphene oxide does not interact with the siliconoxide surface to form a stable layer. Consequently, washing would result in removing the graphene layer. In a first fabrication step, the surface of the silicon wafer was functionalized with a binding layer. APTES interacts with the Si-OH on the surface via H-bonds and heating causes a condensation reaction and the formation of a stable Si-O-Si [208]. The amino-group will be used to bind GO to the surface.

After cleaning, the Si-crystal was immersed in a 2% ethanolic solution of APTES and baked at

120°C for 1h. The spectrum (Fig. 4.2A) shows a increase of C-H stretching vibrations near 2900 and 2800 cm^{-1} and N-H vibrations at 1556 cm^{-1} .

Figure 4.2: (A) APTES was immobilized on a clean Si ATR-crystal. (B) The static contact angle changed from $<10^\circ$ of a silicon substrate (C) to approximately 80° after immobilization.

This indicates the successful functionalization of the Si ATR-element with APTES. The high intensity of the C-H and N-H vibrations suggests the formation of a several APTES-layers rather than a monolayer functionalization [209]. In order to form a monolayer the functionalization process should be done in a water-free environment. However, since the APTES-layer is only used as layer for graphene binding, this has no effect on the functionality of the FET.

Static contact angle measurements before and after APTES functionalization (Fig. 4.2B and C) showed a change in contact angle from $<10^\circ$ to 83° which is in agreement with literature [210]. These measurements indicate a dense or complete coverage of the silicon surface.

4.1.2 Graphene

Lariska GO synthesis is the standard synthesis route used in our group. Several works were published using this GO [35, 37, 149, 211]. However, the resulting quality of rGO-FETs was not sufficient. High variation of resistance and baseline drifts caused problems like difficulties to compare measurements and reproducibility. It was assumed that this is due to the quality of the GO. In order to overcome this problem other GO products were synthesized or received from a collaboration partner and tested on the GFET.

4.1.2.1 Infrared spectroscopy of rGO-FET

In order to get information about the extent of oxidation, the functional group composition and possible contaminants of graphene oxide, infrared spectroscopy was used. A higher degree of oxidation could prevent the formation of aggregates which are otherwise caused by hydrophobic effects and enhance the stability of GO. Therefore the three GO products were dried on the

4.1. CHARACTERISATION OF THE RGO-FET SETUP

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surface of an ATR crystal.

Figure 4.3: (A) Infrared spectra of different GO synthesis products. (B) Enlarged segment of the range 1900-1450 cm^{-1} .

The ATR-infrared spectra of all GO products have one band in the C=C region around 1600 cm^{-1} , one broad band between 1850 and 1650 cm^{-1} (C=O vibrations) and several peaks between 1500 and 900 cm^{-1} 4.3. At higher wavenumbers the spectra show intense hydroxyl vibrations at 3460 cm^{-1} and weaker C-H vibrations at 3000-2800 cm^{-1} .

An interesting feature of the spectra is the different amount of oxidation. The C=O band (1745 cm^{-1}) of Feicht GO and Marciano GO can be attributed to carboxyl groups in the lattice. The 1748 cm^{-1} /1620 cm^{-1} -ratio of the Marciano GO shows the highest content of C=O vibrations in comparison to the other GOs. The Feicht GO has a 1748/1620 cm^{-1} ratio of ≈ 1 . The Larisika GO spectrum contains very weak bands which are assigned to acidic C=O vibrations. This is indicated by a broad, weak shoulder in the region above 1700 cm^{-1} . The higher intensity peak at 1684 cm^{-1} can be attributed to (cyclic) ketones which is also indicated by the C=C vibrations at 1590 cm^{-1} . Very prominent are the high intensity bands of the ester (C-O-C) and hydroxyl (-O-H) vibrations at 1140, 1080 and 1035 cm^{-1} . Additionally there are only weak O-H vibrations at 3200 cm^{-1} .

Table 4.1: Band assignment of different GO vibrations.

Range (cm^{-1})	Vibration	Assignment [155, 212, 213]	Band (cm^{-1})
4000-3000	ν O-H	≈ 3600 -O-H hydroxyde	3640
		3500 – 3400 -O-H acidic	3460
		3600 – 3000 -O-H H-bridge	3220, 3120
		≈ 1830 5-ring lactone	
		≈ 1790 6-ring lactone	
1850-1650	ν C=O	≈ 1780 Lactol	1830
		1790 – 1750 carbonyl	1745
		1700 – 1650 cyclic ketone	
		1700 – 1660 ketone	
1650-1550	aromatic ν C=C	1700 – 1600 cyclic ethers	1684
		1600 – 1585 aromatic C=C	1620
		1600 – 1550 cyclic ketone	1596
1400-1000	δ C-O		1365
		1500 – 1250 C-OH	1220
		1150 – 1070 C-O-C	1140
		1350 – 1000 C=C-H	1080
			1035
		1320 – 1280 + 900 – 800	960
	δ C-O epoxides		880

The direct assignment of the bands to vibrations proved to be difficult [214]. The bands are very broad and according to Fuente et al., the C=O bands can shift to lower wavenumbers up to $40 - 90 \text{ cm}^{-1}$ due to inter- and intramolecular H-bridges [212]. Graphene-oxide contains many different defects (see Fig. 4.4) which are present in various amounts. This causes a overlapping and broadening of the bands, as they consist of many different vibrations which can not be deconvoluted. Table 4.1 provides an overview of different vibrations which could be assigned to found bands.

The IR-spectra reveal, that the Larisika synthesis results in the lowest oxidised product as there are only weak carboxyl C=O vibrations and there is also no strong band at 3460 cm^{-1} . Additionally, there are strong C=O carbonyl vibrations and C-O vibrations in the lower wavenumber range. The most intense peaks are found in the fingerprint region. Bands in this region indicate a high amount of ether and epoxide residues. The highest oxidized product is produced by the Marcarno synthesis. This is indicated by the highest ratio of the C=O and O-H vibrations to C=C vibrations ($\approx 2 : 1$). The Feicht GO showed low C-H vibrations around 2900 cm^{-1} which can be attributed to the presence of 1-dodecylamine.

The spectra of Feicht GO and Marcarno GO have high similarities to graphene IR-spectra found in literature [131, 141, 142, 215, 216]. Many bands can be assigned to different components e.g. lactones, hydroxyl or caboxyl groups. The Larisika GO shows a complete different spectrum. Its composition seems to be completely different from the other two products. Its defects seem to be primarily due to cyclic esters and epoxides.

Figure 4.4: Different surface oxides of a polycyclic aromatic system (like graphene oxide) according to Fuente et al. [212].

After the analysis of GO, the products were reduced on the crystal with hydrazine and hydiodic acid. The spectra of the reduced graphene oxide showed very strange properties and in some cases the crystal was damaged. Consequently infrared spectroscopy could not be used for further examination of rGO.

4.1.2.2 Raman spectroscopy of rGO-FET

Raman spectroscopy is one of the key methods to characterize graphene. It is a fast and easy method to provide information about the structure of graphene and is able to discriminate between single, double and multilayer graphene as well as graphite. In order to conduct this experiment, GO was immobilized on Si-wafer and spectra taken with a Raman-microscope.

Figure 4.5: (A) Raman spectra of graphene oxide and reduced graphene oxide (with hydrazine), (B) enlarged segment at the G-band and (C) enlarged range of the 2D and D+G band.

In a first experiment, GO and rGO were compared. The Raman spectrum (Fig. 4.5) of GO

shows two prominent bands at 1342cm^{-1} (D-Band) and 1581cm^{-1} (G-Band) and three less intense bands at 2680cm^{-1} (2D Band), 2930cm^{-1} (D+G Band) and 3230cm^{-1} (2D' Band). After reduction, the G and 2D bands are shifted to slightly higher wavenumbers. The D/G ratio-change of GO due to reduction is widely used to verify reduction. In Fig. 4.5 the ratio increased from 0.95 to 2.04 after the reduction which indicates successful reduction. However, this ratio has a high batch to batch variation, so the change is often not as drastic.

Table 4.2: Experimental and literature Raman bands of graphene oxide and reduced graphene oxide after hydrazine reduction

Band	Graphene oxide		Reduced graphene oxide	
	Exp. cm^{-1}	Lit. cm^{-1}	Exp. cm^{-1}	Lit. cm^{-1}
D	1342	1341 [39]	1338	1341 [39]
G	1581	1596 [39]	1573	1595 [39]
D'	1607	1620 [200]	1608	-
2D	2680	2770 [217]	2671	2726 [218]
D+G	2930	2950 [217]	2927	2960 [217]
2D'	3230	-	3215	3220 [217]

In contrast to the IR-spectra, there are only small differences visible in the Raman spectra of the different GO synthesis products (Fig. 4.6). GO Feicht seems to have a G band shift to higher wavenumbers of up to 50cm^{-1} compared to the other GO products. This is due to the high intensity of the D' band as shown in Fig.4.7D All show characteristic bands of graphene however none is a monolayer. The preparation results in a multilayer of graphene with 5 to 10 layers. This is indicated by the low 2D/G-ratio ($\ll 1$) and the shape of the 2D-band, which is a smooth single peak [196, 198].

Figure 4.6: (A) Raman spectra of graphene oxide, produced via different synthesis routes (B) enlarged segment at the G-band and D-band and (C) enlarged range of the 2D and D+G band.

The reduction is the critical steps in graphene production. Graphene oxide is a very good in-

Figure 4.7: (A) The ratio I_D/I_G as a function of average distance between defects in a graphene lattice for a 532nm laser (adapted from [219]). Fitting of the Raman spectrum of the (B) Larisika GO, (C) Feicht GO and (D) Marciano GO enables to find the FWHM of the D and G band and the different intensities of the D'-band for the GO products.

insulator as the π -network, which is responsible for the good conducting properties of graphene, is destroyed by esters, hydroxyl or carboxyl groups. Hydrazine is a very common reductant to remove those defects from GO, although the mechanism is still unclear. One mechanism proposes highest reduction efficiency for epoxides but claims to be inefficient against carboxyl- and carbonyl groups [135]. Other reductants, which are commonly used in organic chemistry, like $LiAlH_4$ or $NaBH_4$ react with water and are consequently rarely used [136, 139]. The reduction with hydrazine shows the significant increase of D/G-ratio which indicates a increase of average distance between defects. This can be attributed to the removal of defects in the lattice.

The D/G-ratio differs between the synthesis products. The synthesis product of Larisika shows the highest D/G ratio of approximately 1.9, while GO Feicht has a ratio of 1.7 and Marciano GO 1.75.

In order to get information about the average distance between defects, the FWHM of the D and G band has to be obtained via curve fitting of a Lorentzian function. Cancado et al. reported a L_D -dependency of the FWHM of the G (Γ_G) and D (Γ_D) band (Fig. 4.7A). In the first stage the Γ_G and Γ_D is between 20 and 10 cm^{-1} and increases exponentially in stage 2 [220].

The FWHM of the bands are presented in Table 4.3 and are all above 20 cm^{-1} which means that all are in stage 2 and the distance between the defects is below 3.2nm (Fig. 4.7).

Consequently this means, the GO Larisika has the lowest defect density, but the batch to batch

Table 4.3: I_D/I_G -ratio, FWHM of the D and G band and average distance between defects of different GO products.

GO product	I_D/I_G	Γ_D (cm^{-1})	Γ_G (cm^{-1})	L_D (nm)
Larisika	1.9	62	61	2
Feicht	1.7	66	56	1.7
Marcano	1.75	128	67	1.75
GO	0.95	68	58	1.5
rGO	2.04	62	50	2.2

variation is very high. Some batches showed a very high defect density as shown in Fig. 4.5. Marcano GO and Feicht GO have a similar and significant higher defect density.

The change of defect density due to reduction of GO was also examined. Raman spectra of GO before and after the reduction were recorded. Remarkably, the D/G ratio of the Raman spectra of GO (0.95) and rGO (2.04) change significantly. According to literature, the D/G ratio should increase after reduction [200]. In order to acquire information about the reduction efficiency, the method, used above was applied. The average distance between defects increased by 47% after reduction. This indicates that the graphene is still oxidised but many defects caused by oxidation are removed. However, structural defects which are not caused by heteroatoms can not be removed with this method and contribute to this defect density as well as damages in the GO lattice.

4.1.2.3 Contact angle measurements

Beside the choice of a good GO product, the efficient reduction of graphene oxide is of high importance for the consistency of a GFET batch. Hydrazine was used till now but beside the general issues about hydrazine due to its toxicity and reactivity it did not show consistent results. Hydriodic acid (HI) proved to be a very effective graphene oxide reductant in literature [132, 221]. Previous experiments with HI showed lower and more consistent resistances (verbal communication with Nikolaus Leitner and Max Kosok). However they applied HI directly on the surface. However the dropcasting of HI solution onto the graphene uses high amounts of the reductant. Furthermore, this reduction causes contamination of the surface which has to be cleaned before further preparation. This is done with intense rinsing to remove HI residues and incubation of the chip for 1h at 180°C [211] Another approach would be to use HI vapor for reduction similar to the hydrazine method.

Static contact angle measurement were performed to compare the hydrophilicity, and consequently the oxidation of the samples and furthermore the efficiency of different reduction attempts. Distilled water was dropped on a prepared Si-wafer and measurements were done after 5 seconds. The contact angle was calculated via circle method.

Figure 4.8: Static contact angle of GO products before and after reduction with HI gas. A significant increase of hydrophobicity can be observed.

The immobilization of GO on the surface caused a reduction of the contact angle from 83° (APTES) to 36° - 47° due to the high content of polar groups on the GO. Feicht GO showed the highest contact angle due to the presence of remaining dodecylamine. After reduction, the contact angle increased to 81-92° which indicates a loss of hydrophilic groups on the surface.

Table 4.4: Water contact angle measurements of GO and rGO using different reduction techniques.

Substrate	Contact angle Θ_w	Literature
GO Feicht	$47^\circ \pm 2.5$	30-60° [222-224]
GO Marcano	$37^\circ \pm 2$	
GO Larisika	$36^\circ \pm 1.5$	
rGO Feicht (vapor HI)	$88^\circ \pm 0.5$	95-100° [225, 226], 80-95° [227, 228]
rGO Feicht (liquid HI)	$98^\circ \pm 4$	
rGO Feicht (hydrazine)	$81^\circ \pm 2$	
rGO Marcano (vapor HI)	$90^\circ \pm 4$	
rGO Larisika (vapor HI)	$92^\circ \pm 2$	

The reduction was expected to cause a massive increase in contact angle due to a very hydrophobic rGO surface, which was reported to be up to 130° (this value is still under discussion [225]). The relatively low contact angle of rGO indicates that there are still remaining hydroxyl- and carboxyl groups on the rGO film. Those are believed to remain at the sheet edges [227]).

The three different GO products showed no significant difference in contact angle after reduction with HI vapor. For comparison of the reduction methods only Feicht GO was used. This experiment results that the reduction with HI in liquid phase shows the most efficient removal of hydrophilic residues. The lowest contact angle was obtained via hydrazine reduction. Lower contact angles are assumed to correlate with higher electrical variable resistance and lower consistency, while higher contact angles correlate with low resistance of the FET. These results are in accordance with resistance measurements of Reiner-Rozman [211]. The reduction via HI vapor

can be used as alternative for hydrazine since it has better reduction properties. Taking into account environmental issues, storage and handling HI is way superior to hydrazine. However, the resistance of a GFET after HI vapor reduction is higher ($100\text{-}800\Omega$) than after reduction using dropcasted HI ($\leq 200\Omega$). Since the resistance should be below 1000Ω to ensure that 99% of the current is conducted via graphene (and not over the buffer solution), the reduction via HI vapor is sufficient and has advantages compared to the dropcasting method.

Figure 4.9: Contact angle of Feicht GO after reduction with hydrazine vapor (A), HI vapor (B) and HI dropcasted onto the wafers (C).

4.1.2.4 GO binding on Silicon oxide

Graphene oxide interacts with the APTES binding layer via a reaction of the epoxy and carbonyl groups in the GO lattice and the amine groups on the surface [229, 230]. In order to immobilize GO on the surface, the dispersion was dropcasted or dip-coated and incubated over 1h [37, 39, 149]. However, this incubation time was never validated or optimized, which led to high variation in the surface coverage.

Information about the binding kinetics and coverage of GO is very important. High surface coverage is needed to have many connections between the two electrodes (source and drain) and increase the sensitivity of the system. Too long exposure to the graphene oxide solution might cause the aggregation and formation of multilayers. This would decrease the conductivity and is not favorable.

QCM-D was used to determine the binding of the graphene products and SEM provides information about the effective coverage and quality of the graphene layer.

Figure 4.10: SEM pictures of the three used GO products: (A) Larisika, (B) Marcano and (C) Feicht GO

The SEM pictures in Fig. 4.10 show the shape and size of the different GO products. The Larisika GO shows a highly variable size, depending by the batch, where it is dominated by many small sheets ($< 10\mu\text{m}$). Furthermore, it shows a high content of graphite aggregates which are immobilized on the surface. The Marcano GO contains a high content of overlapping graphene aggregates, shown as dark grey sheets. However the size is comparably high and some graphene sheets have a diameter $> 50\mu\text{m}$. The Feicht GO shows a high content of graphene flakes with few layers. The size varies but lies between 10 and $60\mu\text{m}$. The low number of aggregates and high amount of large flakes could be attributed to the 1-dodecylamine which could prevent sheet aggregation. This could also be the reason for high stability of the solution.

4.1.2.4.1 GO Larisika et al.

Due to the unknown GO-concentration and the contamination with larger graphite flakes, only SEM-pictures were taken to study the coverage. Two batches were tested to control reproducibility under different batches.

Figure 4.11: SEM pictures of one batch graphene oxide bound on a APTES functionalised silicon oxide surface taken at different incubation times (5, 30 and 60min.).

Batch I: The SEM pictures show the slow increase of GO coverage. After one hour, the surface is not covered completely (Fig. 4.11). Due to the insufficient coverage, the FET often had a high electrical resistance $\geq 1M\Omega$ (communication with Reiner-Rozman).

Figure 4.12: SEM pictures of a second batch graphene oxide immobilised on a silicon wafer at incubation times of 5, 30 and 60 minutes.

Batch II: The second batch shows a different picture. Within a few minutes the surface has a coverage of approximately 50%. Increasing the incubation time does not change the surface coverage significantly (Fig. 4.12).

The product of the Larisika synthesis showed a low reproducibility, high contamination with graphite flakes and low coverage. The two tested batches show very drastic the disadvantage of this product.

The Larisika GO product also has a low stability, which is estimated to be 2-4 weeks and a optimal incubation time has to be found for every batch. The low reproducibility among the synthesis batches causes a high variability of the GO quality, surface coverage and consequently resistance of the rGO-FET.

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4.1.2.4.2 GO Feicht et al.

Graphene oxide binds rapidly on the surface and reaches almost full coverage after 5 minutes (Fig. 4.13). Further incubation leads to a higher coverage but also to a increased formation of multilayer of GO-sheets. The high dissipation ($\Delta D \approx 150$) and spreading apart of the overtones indicate the high content of water bound to the GO flakes and between the GO sheets and the silicon surface. The frequency shifts up to $\Delta F \approx 200\text{Hz}$.

The adsorption of Feicht GO on the silicon surface showed high reproducibility. In addition, the fast adsorption makes it a good product for the rGO-FET fabrication.

Figure 4.13: QCM measurement of graphene oxide (Feicht et al.) on a APTES functionalised silicon oxide surface and SEM pictures taken at different points of time (30 sec., 1min., 5 min. and 30 min). After 5 minutes, the kinetics reaches its plateau.

4.1.2.4.3 GO Marcano et al.

QCM measurements show that graphene oxide binds on the silicon surface and does not saturate within two hours (Fig. 4.14). Further incubation time was not investigated because longer incubation times were not practical for the fabrication. The frequency changes by 65Hz. Dissipation changes drastic, which indicates a non uniform surface and high water content, bound to the GO. The SEM pictures exhibit the low surface coverage and a low stacking of GO-flakes.

Marcano GO shows a discrepancy between the SEM pictures and the QCM data. According to QCM data, saturation is not reached within two hours. However, SEM pictures don't show significant changes of the surface concentration between 30 and 60 minutes. The reason for this difference is not known but shows the poor reproducibility of the GO.

Figure 4.14: QCM measurement of graphene oxide (Marcano et al.) on a APTES functionalized silicon wafer and SEM pictures taken at different points of time (30 sec., 1min., 5 min. and 30 min).

Comparing all three products, the GO Feicht shows the highest reproducibility and fastest binding kinetics. The properties of the product are favourable for a commercial product. Marcano GO had a lower reproducibility and the binding takes longer and the surface coverage is not as good as the Feicht GO. The poorest product was the Larisika GO as it had poor reproducibility and unknown concentration and binding kinetics.

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4.1.3 Pyrene-butyl-succinimide-ester

Figure 4.15: (A) Kinetics of the 1815, 1785 and 1745 cm⁻¹ bands of C=O vibration of the PBSE linker during hydrolysis. The dashed line leads the eye. The dotted line indicates the half life. (B) Hydrolysis of the reactive ester in PBS buffer pH 7.2. followed via infrared spectroscopy. Spectra are taken in 5 minute intervals. (C) Band change of PBSE after 250 minutes hydrolysis (black line) in comparison with a dry PBSE on GO (blue line).

Pyrene-butyl-succinimide-ester (PBSE) is used as linker molecule to bind proteins. It contains a pyrene group which interacts with the graphene surface via $\pi - \pi$ -interactions [231, 232]. This interaction immobilizes the PBSE on the graphene-surface. The reactive group is a N-hydroxysuccinimide-ester (NHS) which is able to react with primary amines (eg. lysine in proteins) and forms an amide bond. The NHS is very water sensitive as it hydrolyses with formation of a carboxyl group. This limits its usability. The immobilisation of the linker on the surface and binding of the protein to the linker were examined. In a first experiment, the immobilization of PBSE was controlled. Subsequent examinations were performed to study the hydrolysis with and without protein. This provides evidence whether the protein binds to the linker or not.

PBSE was dissolved in THF and dropcasted on the GO surface. The surface was rinsed with THF and dried with argon. Infrared spectra show C-H vibrations between 2800 and 3000 cm⁻¹ as the dominant bands of the spectrum (Fig. 4.15C). The bands at 1815 and 1785 can be attributed to the symmetric and asymmetric stretching C=O vibrations of the succinimidyl residue

and 1745 cm^{-1} is assigned to the C=O vibrations of the reactive ester [233–236]. The bands at 1668 and 1644 cm^{-1} are assigned to C=C aromatic vibrations of the pyrene anchor and 1460 , 1445 and 1434 cm^{-1} can be referred to CH_2 bending vibrations [237].

4.1.3.1 PBSE hydrolysis

The immobilization of the PBSE or Pyrene-NTA anchor is used in several publications to non-covalently bind molecules or proteins on graphene or carbon nanotubes [238–244]. The major advantage, compared to covalently bound linker molecules, is that $\pi - \pi$ stacked linkers have almost no effect on the electrical properties of graphene [238, 239]. The introduction of heteroatoms like oxygen into the graphene lattice would increase the resistance and so affect the functionality of the FET. Another major advantage of using a reactive ester to bind proteins in a random orientation is that protein modifications as his tag or FLAG tag are not required for binding.

NHS-esters are known to react with water, where the half life is dependent on pH and temperature of the buffer solution. Additionally the weak interaction between linker and surface raises the question about the stability in water. In order to observe the hydrolysis of the PBSE reactive ester during incubation in PBS buffer (pH 7.4, room temperature), PBSE was immobilized on a GO surface and immersed in buffer, while spectra were recorded in 5 minute intervals.

Table 4.5: Band assignment of PBSE hydrolysis

Band (cm^{-1})	Hydrolysis	Vibration
2922	↓	$v_{as}\text{ CH}_2$
2852	↓	$v_s\text{ CH}_2$
1812	↓	$v\text{ C=O of NHS}$
1783	↓	$v_s\text{ C=O of NHS}$
1738	↓	$v_{as}\text{ C=O of ester}$
1437	↑	$\delta\text{ O-H of carboxyl group}$
1285	↑	$v\text{ C-O of carboxyl group}$
1210	↓	$v_{as}\text{ C-N-C of succinimide}$

The hydrolysis shows a decrease of C-H vibrations (2822 and 2852 cm^{-1}), C=O (1812 , 1783 and 1738 cm^{-1}) vibrations and C-N vibrations (1210 cm^{-1}) (Fig. 4.15B,C). All bands are assigned to the succinimide residue of the reactive ester. The hydrolysis causes the dissociation of hydroxy-succinimide and formation of a carboxyl group. This formation can be observed by the increase of the O-H band at 1437 cm^{-1} and C-O band at 1285 cm^{-1} .

The bands of the reactive ester reach their maximum intensity after approximately 250 min which indicates the complete hydrolysis of the linker. As shown in Fig. 4.15A, the linker has a half live of approximately 50 minutes at pH 7.4 and room temperature. The complete hydrolysis is measured after 250min which is in the range of other hydrolysis studies of N-hydroxy-succinimide-reactive esters (NHS-ester) [236]. The small rising band at 1630 cm^{-1} is water which replaces the removed succinimide [245].

The spectrum shows no decrease of C=C vibrations which would be an indication for desorption

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of the pyrene anchor. Consequently it can be assumed that the linker stays on the GO surface in water.

4.1.3.2 OBP14 binding

The successful immobilization of the protein is of major importance for a working sensor. OBP14 should bind to the linker which immobilizes it and keeps it from the hydrophobic surface. In order to have an optimal working sensor, a high coverage of the surface is required. Additionally it should be ensured that the linker with bound protein is still stable on the surface.

10 μM OBP14 in PBS-buffer (pH 7.4) was dropcasted and incubated on the PBSE functionalised GO surface. The increase of the amide I and II bands show the immobilization of the protein on the surface (Fig. 4.16). The reactive ester reacts either with water (hydrolysis) or with one of the 15 NH_2 -groups of lysine (aminolysis). Cleavage of the active ester group leads to a decrease of $\text{C}=\text{O}$ bands at 1738, 1783 and 1812 cm^{-1} . Simultaneously an increase of the amide I and amide II band can be observed. The maximum intensity of the amide bands and $\text{C}=\text{O}_{\text{Linker}}$ -band is reached after approximately 35-40 minutes, which indicates the complete binding of the protein on the surface.

Figure 4.16: (A) Infrared spectrum of OBP14 binding on PBSE linker in PBS buffer pH 7.2. (B) Kinetics of Amide I and Amide II band which are assigned to the protein (increasing) and 1738 cm^{-1} band which is assigned to the $\text{C}=\text{O}$ vibration of the PBSE linker (decreasing) [149].

The addition of 10 μM protein causes a drastic decrease of half life of the reactive ester. Since the protein bands (amide I and amide II) reach their maximum simultaneously with the $\text{C}=\text{O}$ vibration (1738 cm^{-1}) of the reactive ester, it can be assumed that the hydrolysis is not interfering with protein binding. Hydrolysis only plays a minor role as the reaction of the linker with the NH_2 -groups of the protein is much faster than the hydrolysis. The half life of the reactive ester was calculated to be 5 minutes. This is according to literature, where Lim et al. reported a significant decrease of NHS-ester half life after addition of ethylamine as a primary amine source [234].

Protein binding also shows no decreasing of bands at 1668 and 1644 cm^{-1} , which are attributed

to aromatic C=C vibrations (Fig. 4.16). This indicates that the pyrene anchor is also stable on GO when bound to a protein. Consequently, it can be assumed that the protein binds on the PBSE linker in significant amounts.

4.1.4 Tantalpentoxide as passivation layer

A major problem is the unspecific adsorption of organic material on the graphene surface. This causes a change in charge density on the graphene surface hence a false positive signal.

In order to reduce these interactions, different passivation methods were tested. In his work, Reiner-Rozman approached the problem with three chemical passivation layers and one metaloxide layer [211]. In his FET-measurements, a sample solution was pumped through a measuring chamber containing a passivated rGO-FET. The sample solution was a filtrate (pore size $0.45\ \mu\text{m}$) of blended corn in PBS and 1% Tween 20. 1,4-n-butylbenzene, fluorobenzene and polyethylene glycol PEG-2000 were incubated on the rGO-FET before measuring. In case of the metaloxide layer, a 20nm tantal pentoxide layer was sputtered on the reduced GO. The usage of 1,4-n-butylbenzene or fluorobenzene still showed strong unspecific signals in rGO-FET measurements. The widely used passivation molecule polyethylene glycol (PEG) showed better results but still interferences of the signal with the bulk solution. Additionally, the problem with chemical passivation layers is how to ensure that the layer stays stable on the rGO. The functionalization of rGO would reduce the properties and increase the resistance. The adsorption might be working with benzene derivatives but PEG might not adsorb stable on the surface. Tantal pentoxide (Ta_2O_5) was reported to show the best passivation properties. Ta_2O_5 is a metaloxide which is sputtered on the rGO surface to create a smooth and equally covered surface.

The passivation progress has also a drawback. The immobilization of the protein can not be granted. In case of the benzene derivatives (1,4-n-butylbenzene, fluorobenzene) a pyrene-anchor might still work due to $\pi - \pi$ -interactions between the benzene and pyrene-ringsystem. However, for PEG and tantal pentoxide a new approach is needed. Therefore silanes were used for tantal pentoxide. 3-Glycidyloxypropyl-trimethoxysilane (GPTMS, silane with epoxy-group), the linker molecule with the best properties in FET measurements was used instead of PBSE.

Since the chemical passivation didn't show a sufficient reduction of unspecific signals only the Ta_2O_5 was used for further investigation. The aim of these experiments was to test if the tantal pentoxide layer shows any passivating properties and examine the successful immobilization of GPTMS on the Ta_2O_5 layer.

In order to control the the binding of silanes on Ta_2O_5 , a silicon wafer was coated with 20nm Ta_2O_5 . The surface was cleaned with ozone to remove contaminants for 10 minutes and immersed in a 2%-ethanolic solution of APTES. APTES was used instead of GPTMS as it had a known contact angle and a stable residue. After 90 and 180 min. incubation time, the contact angle was determined.

The treatment with ozone shows significant effect due to the removal of organic contaminants adsorbed on the surface during storage. The introduction of $-\text{OH}$ groups on the surface makes it accessible for silanes like APTES, which bind to metalhydroxides. After APTES immobilization the contact angle rose to 37° after 90 minutes and 52° after 180 minutes indicating a binding on the surface. The contact angle measurements show a significant change of contact angle after APTES functionalization. This shows, that the reaction takes longer than for siliconoxide but the functionalization of Ta_2O_5 with silanes is feasible. However, the interaction between the silane and the surface is based on weak hydrogen-bonds. In order to form a stable covalent bond, the chip should be backed for 1h at 100°C . This was not done to prevent the degradation of the epoxide. Consequently, it can not be ensured that the protein is kept immobilized on the surface over a longer period of time.

Figure 4.17: Contact angle measurements of Ta₂O₅-coated Si wafer.

Table 4.6: Water contact angle measurements of the tantal pentoxide coated Si wafer

Substrate	Contact angle Θ_w	Literature
Ta ₂ O ₅ untreated	$69^\circ \pm 0.5$	-
Ta ₂ O ₅ ozone treated	$< 10^\circ$	“very hydrophilic” [246]
Ta ₂ O ₅ APTES after 90min	$37^\circ \pm 0.5$	$83.2^\circ \pm 2$ on silicon [210]
Ta ₂ O ₅ APTES after 180min	$52^\circ \pm 2$	

The FET experiments showed a significant improvement of signals and the cause of these changes was explained by a decrease in nonspecific adsorption. In order to verify the passivating properties of Ta₂O₅, a QCM-experiment was conducted compare the passivation layer with GO, rGO and PEG on rGO. Therefore GO (Feicht et al.), rGO (reduced with hydrazine) and Ta₂O₅ was immobilized on QCM SiOx crystals. One rGO functionalized crystal was additionally coated with PEG2000 by incubating in 10% aqueous solution for 4h. 30 μ M lysozyme PBS solution was pumped into the cells and washed with PBS.

In all four cases high amounts of protein are adsorbed (Fig. 4.18. The tantal pentoxide surface showed the highest frequency shift $\Delta F \approx 22$ Hz. GO and rGO, coated with PEG2000, showed comparable results with a lower adsoption of $\Delta F \approx 13$ Hz. This might be due to insufficient coverage of the surface. The lowest lysozyme adsoption was achieved by the blank rGO surface $\Delta F \approx 6$ Hz. The dissipation was in all cases equal low between 0.4 and $1.2 \cdot 10^{-6}$.

The QCM measurements showed a significant difference between the affinity of lysozyme and the substrates. rGO showed to have the lowest adsoption of proteins compared to all other surfaces. This phenomenon is surprising because PEG is a standard anti fouling molecule but showed comparable results as GO. However, the usage of Ta₂O₅ increased the adsorption even to approximately 300% of the rGO substrate.

In his work, Reiner-Rozman shows comparable measurements with QCM-D and claims a reduction of nonspecific adsorption due to the passivation layer [211]. However he compares the unreduced GO-layer with Ta₂O₅. Since the FET uses rGO, the measurements are not comparable. Additionally, there is a experimental problem. The corn extract is a complex medium with variable particle sizes and composition. The low reproducibility between batches showed high variations which made a comparison almost impossible. To overcome this problem a standardized protein solution was used in these experiments. This ensured that all experiments are done under comparable and reproducible conditions.

Taking these factors into account, it can be concluded that Ta₂O₅ has no passivating effect. The change of signal might be due to the insulating effect of the layer.

4.1. CHARACTERISATION OF THE RGO-FET SETUP

Figure 4.18: Affinity of 30 μM lysozyme on (A) 25 μ m Tantal pentoxide, (B) GO, (C) rGO and (D) rGO coated with PEG 2000.

4.2 Determination of surface concentration of PBSE and OBP

4.2.1 Surface concentration of PBSE

The quantification of PBSE linker and OBP14 provides important information about the surface composition of the rGO-FET. OBP14 is bound via the reactive ester in random configuration to ensure the functionality of the protein. Nonspecific adsorption on the hydrophobic surface potentially causes massive conformational changes due to hydrophobic effects. This could lead to loss of functionality of the protein and consequently to failure of the sensor.

An important question is whether a monolayer of linker and protein is formed. Quantification of the amount of PBSE linker on the surface yields information of whether the linker can be adsorbed in sufficient amount on the rGO surface. The PBSE linker is also able to stack via $\pi - \pi$ -interaction one over the other. Multilayer of PBSE could result in low sensitivity of the device as it builds up a more and more graphite like structure. The quantification enables the validation, if the preparation is efficient to prevent PBSE-stacking.

Figure 4.19: Determination of molar extinction coefficient: (A) Transmission spectra of C=O vibrations of PBSE at various concentrations. (B) Parallel and perpendicular polarized ATR-spectra of PBSE on GO in THF.

Table 4.7: Calculated molar extinction coefficient and surface concentration of the reactive ester bands of PBSE.

Band	ϵ (l mol ⁻¹ cm ⁻¹)	Γ (pM/cm ⁻²)	Γ (ng/cm ⁻²)
1738	$2.01 \pm 0.32 \cdot 10^6$	348 ± 108	133 ± 42
1783	$1.86 \pm 0.28 \cdot 10^5$	545 ± 85	210 ± 32
1812	$1.05 \pm 0.17 \cdot 10^5$	396 ± 69	150 ± 26

The molar extinction coefficient of the C=O bands of the reactive ester at 1738, 1783 and 1812 cm⁻¹ was determined by measuring various PBSE concentrations (dissolved in THF) in trans-

mission cells. Thickness of the cells was calculated via the fringe pattern of the empty cell ($d = 26.6\mu\text{m}$). Integral band area was determined and the coefficients were calculated via Lambert-Beers law. Several concentrations were measured multiple times and the extinction coefficient of each band was averaged respectively. The averaged extinction coefficient was calculated as shown in Table 4.7

In order to obtain the surface concentration, the area of C=O bands of the measured ATR spectra (Fig. 4.19B) were calculated. The parameters used for the quantification are collected in table 4.9.

The calculated theoretical surface coverage assuming that only the pyrene anchor interacts with the graphene was determined to be $220\text{ pM}/\text{cm}^2$ or $86\text{ ng}/\text{cm}^2$ (assumed theoretical area of pyrene: 0.75 nm^2). This is significantly lower than the concentration determined by ATR-measurements ($330\text{--}550\text{ pM}/\text{cm}^2$ or $130\text{--}210\text{ ng}/\text{cm}^2$).

The quantification of the PBSE-linker showed a more than twice as high concentration compared to calculated maximum concentration. However assuming the space needed for the binding of the PBSE is similar with the one for dithio-propionon-succinimidyl-ester (also a NHS-ester) the results are very similar to literature values ($520\text{ pM}/\text{cm}^2$) [234].

The calculated maximum surface concentration assumes a optimal monolayer where all pyrene are aligned perfectly on the surface. Therefore the rGO has to cover the whole surface equally. In reality, this is not the case as shown in chapter 4.1.2.4. The surface coverage of GO varies, depending on the used GO and incubation time. However PBSE also covalently binds to APTES as it contains also free NH_2 groups. Also those APTES-bound-PBSE molecules are taken into account. A hint for this is the broadening of the 1738 cm^{-1} band to lower wavenumbers as the bond PBSE has a C=O vibration at 1735 cm^{-1} . As the carboxyl groups can bind the APTES in a much denser way than via the pyrene-anchor, this contributes to the high surface concentration of PBSE.

In order to make concentration measurements, the setup had to be simplified. The reduction of GO caused damage to the ATR-crystal, which made it impossible to make IR measurements with rGO. Additionally the high hydrophobicity of the surface caused a change in water-band intensity as less water covers the surface. This made it very difficult to find the bands of interest. So GO was used instead to overcome the problem as PBSE was already used successfully to functionalize GO in a sensor system of Song et al. [247]. Those simplifications make the result even more interesting as it was not expected that such a high amount of linker would stable adsorb on a graphene oxide surface. This can be explained by the incomplete oxidation of GO, which predominantly takes place at the edges.

The usage of ATR-IR-spectroscopy is a good tool to verify the adsorption due to its high surface sensitivity. Nevertheless it was not always possible to ensure the binding. After washing of the linker with THF, the surface was dried and the C=O bands were visible in the IR-spectra. For quantification, the measurements were taken in THF as bulk solution. In only a few of several attempts to quantify the surface concentration, the signal of the linker (measured in THF as bulk) was strong enough to be quantified. This indicates that the THF causes a damping in signal strength. A reason for this damping could be a high solvent sensitivity of the C=O bands due to a loss intermolecular H-bridges in solution [154, 248].

Figure 4.20: Dichroic spectrum of PBSE in (A) air and (B) THF. The negative vibrational bands indicate a orientation parallel to the surface.

Table 4.8: Orientation of PBSE vibrations in THF.

	C=O 1738 cm ⁻¹	C=C 1668 cm ⁻¹
R	0.866 ± 0.487	1.605 ± 0.756
cos(Θ)	0.041 ± 0.018	0.376 ± 0.328
Θ	78.6 ± 2.7	61.4 ± 18.7
S	-0.439 ± 0.027	-0.072 ± 0.383

In addition to the surface concentration, the dichroic ratio was used to gain information about the orientation of the C=C aromatic and C=O ester vibrations. Table 4.8 shows the order parameter *S* and the average angle of the vibrations in THF. The C=O vibration of the ester shows an average orientation of $78.6 \pm 2.7^\circ$ angle with respect to the *z*-axis. It can be assumed that the vibration is oriented almost parallel to the surface. This is according to the expectation that the ester groups would align into the bulk solution to overcome steric hindrance.

The average C=C vibration of the pyrene anchor is oriented at an angle of $61.4 \pm 18.7^\circ$. If the surface is defect free and flat, the vibrations should be oriented parallel to the surface as it adsorbs on the graphene. However, the Si surface is not smooth and the adsorbed graphene causes the surface to become rougher. Consequently an angle of 90° would be unlikely. The angle of 62° is also very low. However, baseline drifts made it difficult to choose the optimal integration range. Two measurements were used for the calculations with an C=C vibration angle of 80.1° and 42.7° respectively which also caused the high standard deviation of the values. Additionally the orientation of APTES-bound-PBSE has to be taken into account as it also contributes to this result. The pyrene anchor will be oriented in a random way which causes the average orientation to be lower. Consequently it is assumed that in one measurement the average C=C vibrations of the pyrene anchor are oriented parallel to the surface and less PBSE is bound to the APTES surface. In the other measurements it is assumed that more PBSE is bound to the surface.

Dichroic spectra *D** show, that in air, as well as in THF C-H, C=O (of the ester) and C=C aromatic vibrations of the pyrene anchor are oriented parallel to the surface (Fig. 4.20). There-

Table 4.9: Parameters used for protein quantification.

Parameter	Symbol	Value
Angle of incidence	Θ	45°
Number of active internal reflections	n	12.5
Refractive index of Si MIRE	n_1	3.5
Refractive index of ZnSe MIRE	n_1	2.4
Refractive index of protein layer	n_2	1.45
Refractive index of aqueous bulk layer	n_{3,H_2O}	1.32
Refractive index of C=O PBSE ester	$n_{2,C=O}$	1.45
Refractive index of THF bulk layer	$n_{3,THF}$	1.4 [114]
Integrated molar absorption coefficient of amide II band	$\int \epsilon_{amideII} d\tilde{\nu}$	8.25*10 ⁶
Integration range		OBP14 1590-1500 cm ⁻¹ , BSA: 1600-1500 cm ⁻¹ PBSE: C=O 1770-1710 cm ⁻¹ PBSE: C=C 1680-1660 cm ⁻¹
Number of equal functional groups	N	OBP14 124, BSA: 582 C=O ester: 1
Molecular weight	m	OBP14 16.201Da, BSA: 66.400Da PBSE: 385Da

fore, the spectrum was calculated according to eq. 2.3.37. The isotropic dichroic ratio R_{iso} was calculated to be 1.57. This supports the orientation calculation since it is not as effected by baseline drifts and the chosen integration range of the bands.

4.2.2 Surface concentration of OBP14 on PBSE

The polarized ATR-spectra are dominated by three bands in the amide region. Beside the most prominent amide I and II bands, a less intense band is observed in the spectra. The band at 1735 cm^{-1} is attributed to C=O vibrations of free carboxyl groups [237] caused by the hydrolysis of PBSE and the APTES-bond-PBSE. Determination of surface concentration was done using the amide II band because of interference of the amide I band (1650 cm^{-1}) with H₂O bending vibrations at $\approx 1640\text{ cm}^{-1}$.

Figure 4.21: Polarized ATR-spectra of protein and PBSE on GO: (A) pp and vp spectra of PBSE and BSA. (B) pp and vp IR-spectra of OBP14.

The quantification of proteins adsorbed and bound to different fabrication stages of the rGO-FET provides insights into the surface coverage. BSA was used as a standard protein to compare it with literature values. It can be shown that the protein concentration is well in the range of literature which uses the same concentration determination technique but different substrate or protein (with comparable molecular weight, solubility and size). In order to obtain the percentage of coverage, the theoretical maximum concentration of the proteins had to be calculated. This was determined to be $\Gamma = 3.89\text{ pM/cm}^2$ for BSA and $\Gamma = 16.6\text{ pM/cm}^2$ for OBP14. The dimensions of BSA are $14 \times 4 \times 4\text{ nm}$ [249] and of OBP14 were estimated to be $3.8 \times 3.2 \times 3.2\text{ nm}$ (via Chimera software). An average molecular area of 43 nm^2 and 11.6 nm^2 were calculated. The concentration of OBP14 on PBSE linker was obtained to be $\Gamma = 9.68 \pm 3.03\text{ pM/cm}^2$ which is equivalent to $\Gamma = 157 \pm 50\text{ ng/cm}^2$ which is a surface coverage of approximately 60%. BSA incubation also resulted in a surface concentration of $\Gamma = 2.21 \pm 0.31\text{ pM/cm}^2$ or $\Gamma = 147 \pm 30\text{ ng/cm}^2$. BSA had a high affinity to the GO ($\Gamma = 3.51\text{ pM/cm}^2$) compared to OBP14 ($\Gamma = 9.77 \pm 0.57\text{ pM/cm}^2$), though it is known that BSA has a high tendency to stick on surfaces [250].

Comparing the unspecific adsorption of OBP14 on GO with binding on PBSE, there is almost no quantitative difference, which makes it difficult to determine if the protein binds unspecific on the surface or binds to the PBSE-linker. In conclusion, a specific binding to the PBSE can not be demonstrated.

4.2.3 Surface concentration of OBP14 on DTNTA

OBP14 is immobilized on a Ni-NTA-functionalized ZnSe surface via his-tag. The binding of the protein takes about 180 minutes (Fig. 4.22A). The maximum intensity of the amide I band is at 1653 and of the amide II band at 1549 cm^{-1} . The surface concentration calculated from band area of amide II band of measurements with parallel and perpendicular polarized light (Fig. 4.22B). The calculated mean surface concentration of OBP14 was $\Gamma = 5.24 \pm 0.31 \text{ pM cm}^2$ which corresponds with $\Gamma = 84.9 \pm 5.1 \text{ ng/cm}^2$ with a molecule molecular area of $3227 \pm 440 \text{ \AA}^2$.

Figure 4.22: (A) Infrared spectra of OBP14 binding on the DTNTA functionalized ZnSe-surface. Spectra were taken in 5 minutes interval. Protein binding causes an increase of amide I and amide II band and reaches its maximum after approximately 70 minutes. (B) Parallel (pp) and perpendicular (vp) polarized infrared spectra of the amide I and amide II region.

The surface coverage of the protein on DTNTA is about 32% which is significantly lower than the immobilization on graphene (Table 5.1, approx. 60%). This caused by the usage of dithio-propionic acid (DTP) as spacer molecule. The usage of a spacer has shown to be beneficial for the function of immobilized proteins [51, 176]. The space per molecule provides enough room for conformational change without steric hindrance and prevents denaturation. Obviously, this difference could also be due to nonspecific adsorption on graphene. Another reason is the low binding efficiency of a hexa-histidine-tag. Pinkerneil et al. reported a 4 fold higher binding efficiency with a deca-histidine-tag compared with a hexa-histidine-tag [251].

Additionally the dichroic ratio was calculated for amide I and amide II band. This provides information about the orientation of the amide transition moment and has been used to study the orientation of α -helices [185, 252, 253] or β -sheets in proteins [254]. For the immobilised OBP14, the obtained average dipole moment orientation of the N-H vibrations in the amide II band is $55.5^\circ \pm 2.1^\circ$ and the average orientation (assuming an angle of 70° between the N-H and the helix orientation) α -helices is $58.4^\circ \pm 2.6^\circ$ (Table 4.10). It can be assumed that the helices in the protein are not oriented, which in agreement with X-ray diffraction studies.

The amide I band would have been a more suitable band for orientation measurement as it consists to 80% of C=O protein-backbone vibrations. This makes it more useful compared to

Table 4.10: Orientation of Amide II vibrations and α -helix (via amide II band).

	Amide II	α -helix orientation
R	1.155 ± 0.086	-
$\cos(\Theta)$	0.320 ± 0.033	-
Θ	55.5 ± 2.1	58.8 ± 2.6
S	-0.019 ± 0.049	0.181 ± 0.03

the amide II band which is less pure and consists to 60% of N-H vibrations. As consequence, the angle of the vibration and the angle of the helix orientation is a rough estimation. The amide I band can not be used as it is covered by water bending vibrations. Additionally to these disadvantages of the amide II band the amide II band overlaps with the COO^- vibrations of carboxyl amino acids. Goormaghtigh also points out to separate bands of secondary structures if the protein does not only consist of α -helical structure [177]. In this case it is not possible as the exact bands of random coil structure is not known and partly overlap with helical structure. However, in many publications this is not discussed.

4.3 Modulated IR spectroscopy of OBP14

Infrared spectroscopy is widely used to investigate the structural changes of proteins due to an external stimulus. However, the majority of published articles are static measurements. The protein is dissolved in buffer with different pH and injected either into a transmission cell [255] or adsorbed on a ATR crystal [256–258]. More sophisticated measurements were done to examine the change of secondary structure due to ligand binding [259, 260] in D₂O-buffer. However transmission experiments require high protein amounts ($\geq 1\text{mg/ml}$) and these experiments cannot be done by changing the conditions in vitro but by exchanging the protein solution. Static ATR-experiments require less material but provides no kinetic information.

In order to measure the conformational changes as well as the kinetics of the ligand-receptor-system modulated infrared spectroscopy was applied. A major drawback of infrared spectroscopy is the overlapping of the amide I band and the absorption of water bending vibrations. This is especially a problem when a ligand is modulated. Spectral changes, caused by structural changes of the protein interfere with the absorption of H₂O-bending vibrations, which decreases with the increase of ligand concentration and ligand-bands, which are located in the amide I region (Fig. 4.37).

To overcome the water absorbance problem, deuterium oxide is used in to determine the secondary structure of OBP14. This shifts the D-O bending vibration band to lower wavenumbers [256, 259, 260]. The so called amide I' band is used for structural analysis. However the usage of deuterated water can cause structural changes in the protein [261] like denaturation [262], change of function [263] and altering of salt bridges [153]. Therefore the modulated measurements were carried out in H₂O-buffer.

4.3.1 Secondary structure of OBP14

In a first experiment the secondary structures of the protein are examined. FT-IR spectroscopy has been used for the analysis of secondary structure of several proteins [158, 180, 264, 265]. One big advantage is the fast and easy application compared to X-ray crystallography and the high information content though it is often used in combination with circular dichroism spectroscopy [266, 267].

The infrared spectrum of OBP14 was taken in deuterated water (150mM NaCl, pD 6.5) to remove the interfering water band from the amide I band. This offers the examination of the secondary structure of the protein. Therefore the second derivative of the spectrum was taken and smoothed via Savitzky-Golay function (factor = 13) to obtain the position of the bands. The gained parameters are used to fit Gaussian peaks on the spectrum, where the full width-at-half-maximum (FWHM) boundary conditions were set to a range of 10 to 50 cm⁻¹.

Figure 4.23: Infrared spectrum of OBP14 in D₂O (black line) and second derivative spectrum (grey line). The band fits (green lines) base on the peak positions gained by the second derivative analysis. The red line represents the sum of all fitted bands.

Second derivative analysis (grey line) and fitted peaks (green lines) is shown in Fig. 4.23. There are three main peaks in the amide I band at 1647 (α -helix, 74%), 1670 (loop, 11.5%) and 1633 cm⁻¹ (random structure, 14.5%) and one very weak peak at 1604 cm⁻¹. The latter is assigned to histidine ring vibrations and two other bands at 1575 and 1564 cm⁻¹ are attributed to carboxyl groups $v_{as}(COO^-)$ of aspartate and glutamate [268].

Fig. 4.23 has similar characteristics as the IR-spectra obtained by Schwaighofer et al. [109]. As shown in the spectra protein structure is dominated by α -helical structure as known from X-ray crystallography. The three main bands have comparable ratio to literature but still show a significant difference. The difference in area could be due to the inaccuracy of fitting as described by Baldassarre [269]. There he points out that fitting of a spectrum with band positions, known from second derivative analysis can bring up many results with good quality but does not necessarily reflect the real structure of the protein.

All peaks show very uncommon full-width-at-half-maximum values between 20 and 40 cm⁻¹, which are comparably high in contrast to other IR secondary structure studies (10-20 cm⁻¹) [164, 270]. However, these properties were also reported by Byler and Susi 1986 in FTIR measurement on casein (1644 cm⁻¹ and FWHM=32 cm⁻¹) [159]. This study suggests the overlapping bands which can not be deconvoluted by this analysis caused the broadening of this band. In OBP14, it could be assumed that each helix-band contributes to the big peak at 1649 cm⁻¹ and consequently causes the high FWHM-value of the band.

An other characteristic is the finding of two bands in the β -sheet range. Schwaighofer et. al suggested a assignment of these bands to β -sheets although the OBP14 protein consists only of loops, helical and random/disordered structures [109]. This shows the limitations of FT-IR secondary structure analysis, which seems to find also secondary structures although the protein does not contain these structures according to X-ray data [270].

Hemoglobin and myoglobin, which are also high content α -helical proteins [271], are reported to show bands between 1671–1675 and 1627–1638 cm⁻¹ which are typical ranges for β -sheets. The former is associated with the 2-6 amino acid long loops, which connect the helices and the latter with “less well defined chain segments” [159]. A reason for this down shift could be a higher

Table 4.11: Band positions and assignment of the components of OBP14 amide I band in D₂O. Gaussian curve fit was used to get the relative absorption of each band.

experimental			literature [109]		
$v \text{ (cm}^{-1}\text{)}$	Assignment	%	$v \text{ (cm}^{-1}\text{)}$	Assignment	%
1670	loops	11.5	1672	turns	4.8
1647	α -helix	74	1648	α -helix	76.3
1633	random	14.5	1628	β -sheet	18.9
1604	<i>His</i> (C-C, 1600) [268]	-	-	-	-
1575	<i>Asp</i> (v_{as} COO ⁻ , 1584) [268]	-	-	-	-
1566	<i>Glu</i> (v_{as} COO ⁻ , 1567) [268]	-	-	-	-

strength of H-bridges for these structures. Consequently, the peak at 1669 cm⁻¹ is assigned as loop and turns between helices and are caused by a strong interaction of C=O vibrations. The lower wavenumber peak at 1633 cm⁻¹ is attributed to random structures although it is located in the β -sheet range. In comparison with the assignment of Schwaighofer, this assignment is closer to the results of X-ray diffraction studies [62].

4.3.2 pH modulation of OBP14

4.3.2.1 Modulation between pH 6.5 and 4.5

Odorant binding proteins are localized in the sensillum lymph which has a pH of 6.5. It is assumed that the protein undergoes a structural change and consequently changes the affinity to ligands as it approaches the cell membrane as the pH changes from 6.5 to 4.5. In order to observe the conformational changes, the protein is modulated between pH 6.5 and pH 4.5. This experiment provides important information. It serves as a test that the setup is working. Even without conformational rearrangement a change in carboxyl group bands is expected due to protonation and deprotonation. Additionally, this experiment could prove that the protein is stable over the course of a modulation experiment.

Figure 4.24: Infrared spectra of pH modulation of OBP14: pH-modulation without OBP14 (A) and with OBP14 (B) bound on the surface. The time interval between spectra is 19 sek. (C) Difference spectra (B-A) of the modulated spectra and (D) IR spectra at pH 4.5 without and with OBP14 and the difference spectrum of both spectra.

The measurements were first done without OBP14 to get a reference and measure the reaction

of the DTNTA and DTP caused by the modulation. The decrease of pH causes a protonation of carboxyl groups. This protonation is observed in Fig. 4.24A, which shows two negative bands at 1400 and 1550 cm^{-1} (COO^-) and one weak positive band at 1720 cm^{-1} (COOH). Additionally a band at 1659 cm^{-1} is decreasing with increasing pH, which is assigned to the secondary amine of the DTNTA [272, 273]. The spectra with OBP14 (in Fig. 4.24B) show a similar pattern but a positive band at 1654 cm^{-1} can be clearly observed. In the difference spectra (Fig. 4.24C and D) this band is even more prominent. Three other bands at 1720, 1568 and 1400 cm^{-1} can be assigned to protonation of carboxyl groups of the protein. Additionally one decreasing band can be found in the amide I region at 1643 cm^{-1} which is attributed to random structure (Table 4.12).

Table 4.12: Band assignment of pH modulation of OBP14 between pH 6.5 and 4.5.

Band	Tendency	Assignment	Literature
1720	↑	COOH	C=O Glu (1716), Asp (1712) [153]
1672	↓	amide of DTNTA	1659 [273]
1654	↑	α -helix	1645-1660 [153]
1643	↓	random structure	1640-1645 [154]
1597	↓	COO^-	v_{as} Asp [153]
1568	↓	COO^-	v_{as} Glu [161]
1400	↓	COO^-	v_s C=O (1400) [155]
1320	↓	COO^-	v_s C=O [155]
1225	↑	δ O-H	δ O-H (COOH) [155], δ O-H, v C-O Asp, Glu [165]
1060	↑	v C-OH	v C-OH (COOH) [155]

In the lower wavenumber range the bands of the C-O and O-H vibrations at 1060 and 1225 cm^{-1} can be found.

4.3.2.2 Modulation steps between pH 6.5 and 5.5 and pH 5.5 and 4.5

The pH modulation was extended to obtain information about the major structural changes induced by a change in pH. Therefore the pH modulation was modulated between the isoelectric point (approximately pH 5.45 [107]) and one pH-value above and below.

Figure 4.25: Differencespectra of different pH modulations of OBP14. (A, B) Differencespectra of modulation between pH 6.5 and 5.5. (C, D) Differencespectra of modulation between pH 5.5 and 4.5.

Fig. 4.25A/B displays the difference spectra of the pH modulation of OBP14 between pH 6.5 and 5.5 which have a negative band at 1639 cm^{-1} . This can be assigned to random coil structure. In comparison Fig. 4.25C/D (modulation between pH 5.5 and 4.5) show a prominent band at 1659 cm^{-1} that is attributed to a combination of α -helical and loop structure.

4.3.2.3 Modulation of PBP1 between pH 6.5 and 4.5

PBP1 of *Bombyx mori* is known to change the conformation induced by a change of pH [274]. In order to compare the IR spectra of OBP14, pH modulation was performed on PBP1 between pH 6.5 and 4.5.

Figure 4.26: Infrared spectra of PBP1 pH modulation between pH 6.5 and 4.5. (A) Modulated spectra of pH modulation without PBP1. (B) Modulated spectra of pH modulation with PBP1. (C) Differencespectra of (B) - (A). (D) Structural change during pH decrease.

Fig. 4.26A and Fig. 4.26B show the pH-modulated spectra without and with PBP1. There are significant structural changes, which are better visible in the difference spectra (Fig. 4.26C). The spectra are dominated by the protonation of COO^- groups. Additionally, there is one small positive band at 1658 cm^{-1} which can be attributed to α -helical/loop structure and a more intense negative band 1642 cm^{-1} which can be assigned to random coil structure. Fig. 4.26D shows the progression of conformational change. The α -helical structure increases with the starting of pH change (black line; 19s after injection), while the negative band at 1642 cm^{-1} is much more dominant but changes in time with the modulation (cyan to dark blue spectra).

4.3.2.4 2D-correlation spectroscopy and PSD

In order to deconvolute the spectra and get more information about the change of the different components 2D-correlation spectroscopy was applied.

Figure 4.27: Synchronous (A,C) and asynchronous (B,D) 2D-correlation spectra of OBP14 (A,B) and PBP1 (C,D) modulated between pH 4.5 and 6.5. Moving window 2D correlation spectra of pH modulation of (E) OBP14 and (F) PBP1. Beside the significant change in carboxylic groups due to protonation and deprotonation, the change α -helical structure of OBP14 and the random coil region of PBP1 correlates with the change of pH.

2D correlation spectra were used to get a deeper insight into the structural changes of the protein and find bands, which are not observable in 1D-IR-spectroscopy due to overlapping or low signal-to-noise ratio. Fig. 4.27 shows the synchronous and asynchronous spectra of OBP14 and PBP1 and additionally MW2D-correlation spectra in contour maps. The maps are dominated by the protonation/deprotonation process of the carboxyl groups (1710 and 1565 cm^{-1}). 2D-correlation spectroscopy shows its strengths in the asynchronous spectra, which enables the deconvolution of the big band into the smaller bands of the carboxylic groups of the protein 4.27B,D. The region of special interest is the amide I region (Fig. 4.29). There, significant differences can be seen not only in the asynchronous spectra, but also in the synchronous spectra. OBP14 shows high correlation of protonation of carboxyl groups with the peak at 1657 cm^{-1} while in case of PBP1, it shows high correlation with 1637 cm^{-1} . This indicates the strong coupling between these components. The asynchronous spectra provide more information about the composition of the amide I band.

In the asynchronous spectrum of PBP1, two cross peaks are observed at $\Psi(1662/1638)$ and $\Psi(1655/1638\text{ cm}^{-1})$. This indicates a change in the disordered, loop and helical structure. Applying this information with the spectra, a change from random structure to loops and helix is assumed, which is also reported in literature [104, 274].

OBP14 only shows one synchronous autopeak at $\Phi(1657/1657)\text{ cm}^{-1}$ which implies that the predominant changes are in this spectral range. The asynchronous spectrum is more complex than the one of PBP1 with crosspeaks at $\Psi(1673/1662)$, $\Psi(1673/1652)$, $\Psi(1662/1652)$, $\Psi(1652/1620)$, $\Psi(1662/1645)$, $\Psi(1662/1635)$ and $\Psi(1662/1620)\text{ cm}^{-1}$ (Fig. 4.29). This implies that the structural change is more complex than the one of PBP1 (Table 4.13).

Table 4.13: Band positions and assignment of asynchronous spectra of OBP14-pH-modulation

asyn. ν (cm^{-1})	Assignment
1673	loop structure / Gln ν C=O (1672) [275]
1662	loop structure
1652	α -helix
1645	α -helical/unordered structure
1635	unordered / δ_{asym} NH_3^+ Lys (1637) [276], δ_{as} NH_3^+ Lys (1629) [276])
1620	unordered / δ NH_2 Asn (1622)
1519	δ_s NH_3^+ Lys (1522) [276]

The 2D-moving window spectra of OBP14 and PBP1 (Fig. 4.27E,F) illustrate the main structural changes of the proteins during pH modulation. Fig. 4.13E shows that significant change of secondary structure occurs in the range of 1670 - 1649 cm^{-1} which is assigned to loops and helical structure. In contrast, the changes of PBP1 are primarily in the range of 1647 - 1627 cm^{-1} , which is assigned to helical and disordered structure. In both cases the changes occur synchronous with some delay to the periodic excitation and are reversible. This can also be observed in the band kinetics, illustrated in Fig. 4.28.

Figure 4.28: Kinetics of bands of (A) OBP14 and (B) PBP1 as a function of time during pH modulation between pH 6.5 (first half period) and 4.5 (second half period). The structural changes for both proteins occur simultaneous with the protonation/deprotonation of the carboxylic acids.

Figure 4.29: Synchronous (A,C) and asynchronous (B,D) 2D-correlation spectra (amide I region) of OBP14 (A,B) and PBP1 (C,D) modulated between pH 4.5 and 6.5.

4.3. MODULATED IR SPECTROSCOPY OF OBP14

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Figure 4.30: Phase-resolved difference spectra (0°) of pH-modulation experiments with OBP14 and PBP1. The reduced noise uncovers the spectral changes in the α -helical and random structure (the OBP14 spectra were scaled to the COOH band of PBP1).

Figure 4.31: Band-fit (red) of phase-resolved difference spectra (0° , black) of pH modulation-experiments (pH 6.5-4.5). (A,C) show the structural changes of OBP14 and (B,D) the changes of PBP1. The peak positions were gained from 2D-correlation data. Green peaks represent the fitted peaks, where α -helical structure is shaded red, random structure blue and loop structure is shaded violet.

In order to get a better signal-to-noise ratio, phase sensitive detection (PSD) was applied. Fig. 4.30 shows the phase resolve difference spectra of pH-modulation. The quality of the spectra increased drastically after PSD-treatment. In all spectra, the increase of helical structure can be seen easily in the amide I region. Additionally, the decrease of the disordered structure is also visible. The phase-resolved difference spectra were used band for fitting (Fig. 4.31) with Gaussian curve. Therefore, band positions, gained from 2D-correlation analysis were applied and the FWHM limited to a range between $10\text{--}50\text{ cm}^{-1}$. It clearly shows the structural changes of OBP14 due to pH-change which is dominated by 3 bands (1662 , 1652 and 1645 cm^{-1}) while the other bands play a minor role. The change in the 1652 and 1645 cm^{-1} bands are very strong for OBP14, while the change in the 1655 cm^{-1} band (helix) in PBP1 is comparably low. This is surprising since a very intense change is expected due to the massive structural change observed in X-ray diffraction studies.

4.3.2.5 QCM-measurement

The change in pH to lower values is commonly used to remove his-tag bound proteins from the Ni-NTA. In order to control the stability of OBP14 on the surface, a QCM experiment was performed in which the pH was changed from pH 7.5 to pH 5 and further to pH 3.5. A second QCM experiment with $5\text{ }\mu\text{M}$ lysozyme without his-tag was performed to get comparative adsorption data for a different protein species.

Figure 4.32: QCM measurement of (A) specifically bound OBP14 and (B) nonspecifically adsorbed lysozyme stability at different pH on a Ni-NTA functionalized gold surface. The pH-change shows only little effect on the protein concentration on the surface.

Fig. 4.32 shows two features: Firstly, it confirms the binding kinetics of OBP14 on the Ni-NTA surface. The nonspecific binding takes about 4-5 hours to reach a plateau to complete the binding. It takes significantly longer than in the IR measurements. This can be due a different design of the measuring cell which is larger for IR than for QCM-D measurements. The nonspecific binding of lysozyme takes place within minutes and the ΔHz is about $\frac{1}{3}$ of the specific binding of OBP14. Secondly, OBP14 is stable on the surface, even when the pH is lowered to 3.5. The mass loss after pH 3.5 is about 10% of the value of pH 7.5. This can also be observed in the

lysozyme measurement.

4.3.2.6 OBP14 vs. PBP1

The pH modulated measurements of OBP14 (between pH 6.5 and 4.5) showed a increase of the α -helical structure at 1654 cm^{-1} . This increase can be traced back to four possible effects:

- change of disordered to helical structures in the protein
- change in the molar extinction coefficient ϵ due to the change of pH [277]
- reorientation of helical structure (reorientation of the dipole moment from y plane into x-z plane)
- weakening or stretching of H-bonds

There is no X-ray crystallographic data for OBP14 at pH 4.5 available, but olfactory proteins show a change in ligand affinity due to change of pH. X-ray diffraction analysis of PBP1 revealed massive conformational changes caused by change of pH [88, 104].

Figure 4.33: PBP1 in B-form (violet) and A-form (gold). The A-form is formed at low pH and shows no binding of the ligand. As the pH increases above pH 5.5 the B form is formed which can bind a ligand. The pH change causes a drastic change in the conformation. As the pH increases the N-terminal α -helix (blue) unwinds and forms an unordered chain. As consequence, the C-terminal disordered structure (green) forms an α -helix which is placed into the binding pocket. This mechanism is used to eject the ligand to the receptor. In addition to the structural change, some amino acids (Phe12, Phe 118 and Met61, cyan) which are forming the pocket are rearranged so the affinity to the ligand (red) is reduced.

One interesting aspect of the pH-modulation of PBP1 (Fig. 4.26) is the significant change is the decrease of disordered structure and small increase of α -helical structure (1658 cm^{-1}). According to literature, in which the formation of a C-terminal helix (4.33 green chain) was observed, a

Figure 4.34: OBP14 is stabilized by 2 disulfide bridges and several salt bridges. These salt bridges consist of a positive charged lysine or arginine and a negative charged glutamate and aspartate. At pH 6.5. the latter are deprotonated and form a bond. As the pH decreases, more and more acidic groups are protonated and the salt bridges are destroyed or replaced by weaker H-bonds. This causes more freedom of the mobility of the helices and further decreases the affinity.

drastic increase of a α -helix-band would be expected. However, it is only a weak signal [278][274]. Fig. 4.26D shows the increase of α -helical structure during the first spectra but at the end of the half period, it drops almost to zero. This indicates that at lower pH-values, the extinction coefficient of the disordered structure (negative band at 1642 cm^{-1}) is higher than the one of the α -helical structure which causes the band to virtually disappear due to overlapping. An additional reason for the low intensity of the helical band could be the simultaneous unwinding of the N-terminal helix (Fig 4.33 blue chain). This negative band overlaps with the increasing helix-band.

OBP14 is very stable at physiologic pH. This is due to two disulfide bonds (Cys17 - Cys49 and Cys88 - Cys106) and seven salt bridges which connect different helical structures of the protein (Table 4.14, Fig. 4.34). These salt bridges are formed at physiological pH by positively charged amino acids like lysine, histidine and arginine and negatively charged amino acids like glutamic acid and aspartic acid. A drop of pH causes the protonation of the acid groups and breaks the salt bridges. As consequence, the helical structures gain more freedom in movement. The binding pocket opens and reduces the affinity to ligands. A similar effect was observed with antenna specific protein 1 (ASP1) of *Apis mellifera* [63]. However, in contrast to PBP1 of *Bombyx mori* the protonation of acidic groups caused only a minor conformational change in terms of small reorientation. A reason for the very small change can be seen in X-ray crystallography data. It shows that there is no salt bridge in the protein which connect the helices. The protonation of Asp35 causes the small change which leads to the opening of binding pocket by removing the

Table 4.14: Salt-bridges and H-bonds of OPB14 which are affected by pH change.

Amino acids	connecting structures
Glu4 - Lys7	intra-helical Helix1
Glu13 - Arg9	Helix1 - Helix3
Glu33 - Lys114	Helix2 - Helix7
Asp40 - Lys27	Helix3 - Helix2
Glu48 - Lys52	Helix3 - Loop
Asp87 - Lys109	Helix5 - Helix6
Glu93 - Lys102	Helix5 - Helix6

Figure 4.35: ASP1 is a odorand binding protein, found in the antenna of honeybees (blue pH 4, gold pH 7). A decrease in pH causes a small conformational change which increases its affinity to the ligand. The protonation of ASP35 (red), which forms a H-bond with Val118 (blue), causes the binding pocket to be opened which is otherwise covered by the C-terminus (blue chain). The deprotonation causes a repellant effect between C=O of Val118 and COO^- of Asp35 and closes the binding pocket.

C-terminal chain (Fig. 4.35).

OBP14 (unlike PBP1 or ASP1) does not have a long C- or N-terminal disordered peptide-chain which can form a helix. However, the seventh helix at the C-terminus plays a major role in this mechanism as it covers the binding pocket [62]. As the protein approaches the membrane the pH drops to pH 4.5. The resulting protonation of Glu33, which forms a salt bridge with Lys114 at pH 6.5, destabilizes the compact form and leads to more mobility for the seventh helix (Fig. 4.36). This provides enough space for ligands to enter the binding pocket. The increase of the bands at 1662 and 1652 cm^{-1} is assigned to an increase in H-bond length which causes an upshift of amide bands [279]. As the spectral change is very strong, it is assumed that the disordered structure between helix 6 and 7 becomes more helical, which contributes to the spectral change. As the protein diffuses into the buck solution the protein closes again and the ligand is caged in the protein. As the protein reaches the receptor, the pH decreases again, the protein opens and the ligand is released.

Figure 4.36: Cross sections of OBP14 with (cyan) and without (orange) bound eugenol. Both structures show no channel which can be used by the ligand (magenta) to enter or exit the binding pocket. However a decrease of the pH protonates the Glu33 (red) and causes the break of the tight salt bridge with Lys114 (blue) at helix 7. This leads to a structural change as the helix 7 (orange) can move and causes the binding pocket to open for bulk solution. The connecting disordered structure (black) undergoes conformational change to a more helical form.

Additionally, the pH-modulated measurements of OBP14 below and above the isoelectric point were done. Fig. 4.25B shows only a decrease in random structure while the α -helical structure stays constant during the modulation of pH 6.5. to 5.5. In comparison Fig. 4.25D shows a increase in α -helical structure, while there is almost no change in random structure. This effect seems contradictory because the increase of one secondary structure causes the decrease of another secondary structure. PSD spectra (Fig. 4.30) show that there is a change in both band ranges. Baseline shifts and poor signal-to-noise ratio could be a reason for this. As PSD takes all measured spectra into account it can be assumed that there is a decrease of a band near 1635 and an increase at approximately 1645 cm^{-1} . These results are prove that the pH drop to 5.5 already causes the destabilisation of the salt bridge and conformational change but a further drop below 5.5 results in more proteins changing to an open form.

4.3.3 Ligand modulation of OBP14

In order to observe the conformational changes of OBP14 due to binding of a ligand, ligand modulation is applied. Three ligands with different structure and dissociation constants were used for analysis. In comparison to commonly used techniques like using fluorescent or radio-labeled dyes the method is label free. This eliminates the interfering effect of a fluorescent dye or the problems with radioactive material. Additionally, the kinetic information is collected simultaneously with the information about structural change in the protein.

A 100 μ M solution of odorants in PBS pH 6.5 is modulated and spectra taken during binding and rinsing of the ligand.

4.3.3.1 Ligands

Figure 4.37: IR spectra of OBP14 ligands used for modulation.

In order to enable the discrimination between protein bands and ligand bands spectra were taken of the pure odorants. Those spectra show bands of every ligand in the amide I band region. Eugenol exhibits two bands at 1638 and 1609 cm^{-1} which can be assigned to C=C vibrations of the double bond. Additionally the most prominent band is the aromatic C=C vibration at 1515 cm^{-1} . This is also the most intense band for methyl eugenol with bands at 1638, 1609 and 1590 (C=C double bond) in the amide I region. The geraniol spectrum is dominated by three bands beside the C-H bands between 3000 and 2800 cm^{-1} at 1669 (ν C=C), 1443 ($\delta_{as}(\text{CH})$) and 1379 cm^{-1} ($\delta_s(\text{CH})$).

Figure 4.38: Modulated IR spectra of eugenol (A,B), methyl eugenol (C,D) and geraniol (E,F) without (A,C,E) and with OBP14 (B,D,F). In the first half period (green spectra) the odorant is injected into the flow cell with 0.75ml/min and binds to the odorant binding protein. In the second half period the ligand is washed off by pure buffer (cyan spectra).

The eugenol and methyl eugenol modulated measurements (Fig. 4.38) are dominated by the

increase in the characteristic bands of the ligand. Additionally, a change in the C=O band region (approx. 1700 cm^{-1}) can be observed. Geraniol modulation shows only the decrease of water at 1637 cm^{-1} .

4.3.3.2 Comparison of modulated spectra of OBP14

The difference spectra showed a very low signal-to-noise ratio which is due to the small spectral change of $20\text{--}40\mu\text{AU}$. In order to get more information about the spectral changes, the difference spectra of the bound ligand were smoothed via Savitzky-Golay function (Fig. 4.39).

Figure 4.39: Difference spectra of bound (A) eugenol, (B) methyl eugenol and (C) geraniol to OBP14. The blue spectrum represents the rinsed OBP14 and the black spectrum represents the untreated spectrum of bound ligand. In order to have a better overview of the band the ligand bond spectrum was smoothed (red line).

When comparing the difference spectra a significant change in the amide I region can be observed. The band-maximum is located between 1645 and 1631 cm^{-1} , depending on the ligand. These bands at 1645 (eugenol) and 1640 (methyl eugenol) and 1631 cm^{-1} (geraniol) are assigned to a change of random structure. Two bands can be observed in the geraniol and methyl eugenol measurement. Both bands are not in the amide I range but around 1710 and 1565 cm^{-1} which are typically assigned to COOH and COO^- vibrations. The origin of the shoulders is not known but it might be caused by water-incompensation. Although the spectral quality was sufficient to observe structural changes, it was not enough to provide reasonable information about the binding kinetics.

Figure 4.40: Smoothed IR difference spectra of modulated measurements of (A) OBP14 with eugenol, methyl eugenol and geraniol in comparison to a water spectrum. (B) Amide I region of modulated spectra.

The binding of a ligand causes a small conformational change in the protein. Fig. 4.41A shows the small structural changes of the odorant binding pocket. However the changes are very small and since there is no major change it is unlikely to resolve this change with infrared spectroscopy. A bigger change is observed with the reorientation of the loops between α -helix 3-4 and 5-6 shown in Fig. 4.41B. This change is caused by sterical hindrance due to ligand binding.

The analysis of the modulated measurements proved to be very difficult. A major problem was the small change in absorbance due to the minor structural changes. Also the calculation of difference spectra was very difficult. The protein on the surface replaced some of the water which lead to a difference in the spectra for the water absorption. To overcome this problem, those bands were subtracted with the reference measurements, done without OBP14. As there is no objective way to effectively subtract all water of the spectrum, the band at 1515 cm^{-1} was used for eugenol and methyl eugenol as reference as well as the H-O stretching vibration at 3200 cm^{-1} .

Fig. 4.40 indicates the change in the amide I region. The amount of change is very low and band shape could also be caused by incorrect spectra subtraction. In order to see the difference between the spectra and pure buffer, the spectrum of water was added. Although the position of the bands are quite close to the water bending vibrations the position is mostly slightly but significant shifted and they differ also in the FWHM. Additionally it was not possible to completely remove the band via subtraction of the reference spectrum. This is shown for eugenol-modulation of PBP1 which is not binding the odorant (Fig. 4.42). The difference spectra showed no spectral change.

A further indication of the change to be due to a change in protein structure is the change of ratio between positive ligand band 1515 cm^{-1} and the negative water band, which is clearly seen at the methyl eugenol measurements in Fig. 4.38C and D. The ratio changes from 2.7 for the measurements without protein to 4.2 for the measurements with protein. If there is no observable conformational change, the ratio should be constant. So it can be assumed that the band is due to structural changes or the binding of the ligand. The different maxima of the bands for the ligands suggest that different ligands cause a slightly different change in conformation.

Figure 4.41: Structural changes of the (A) binding pocket and (B) changes of amino acids 54-64 and 89-96 of OBP14 with (cyan) and without eugenol (yellow). The binding of eugenol causes a decrease of the binding pocket volume from $415\text{\AA}^3$ to $360\text{\AA}^3$. This volume change causes a small change of orientation of helix 3, 4 and 5 and their connecting loops. This orientation change could consequently be measured by an increase or decrease of bands assigned to helical structure.

Figure 4.42: (A) Absorbance spectra of eugenol modulation with and without PBP1. The black spectrum shows the last eugenol spectrum without protein. The red spectrum shows the last eugenol spectrum with protein. A significant change in intensity of the bands is visible due to the displacement of water/ligand by the protein bound to the surface. (B) Difference absorbance spectra of eugenol-modulated measurements of PBP1. Eugenol is not a ligand of PBP1 and does not show a signal due to modulation.

Figure 4.43: Phase resolved difference spectra of modulated measurements of OBP14 with eugenol, geraniol and methyl eugenol. It provides a better insight in the structural changes than difference spectra. For all proteins, intense changes are found in the range $1648\text{--}1654\text{ cm}^{-1}$ and for eugenol and geraniol smaller changes at 1625 cm^{-1}

The low signal-to-noise ratio allows only a rough estimation of the structural changes. All ligands show positive changes in the α -helical and unstructured region, although there are small differences in position and also intensity. These small differences in structural changes of different ligands could also contribute to the different binding kinetics measured by the rGO-FET.

As a consequence of the low signal-to-noise ratio, 2D-correlation spectroscopy could not be applied. However, phase resolved difference spectra (Fig. 4.43) showed to increase this ratio and give a better insight into the changes of the protein. For all ligands, a strong band is visible at $1648\text{--}1653\text{ cm}^{-1}$ with a shoulder at 1662 cm^{-1} . Another band is seen in the spectra of eugenol and geraniol at 1625 cm^{-1} . This data enforces the theory that difference-absorbance spectra predominantly show in change of helical structure due to ligand binding.

However, the bands in the absorbance spectra are only increasing. Additionally there is no sign of major conformational change in the secondary structure (eg. unfolding of helical structure to β -sheet structure) known from X-ray diffraction studies. It is assumed that the structural changes occur in reorientation of the helices and loops into a more compact form. This is in agreement with Spinelli, who reported a decrease of cavity volume from $415\text{ \AA}^3$ to $360\text{ \AA}^3$ after binding of eugenol [62].

Chapter 5

Discussion

5.1 Characterization of the rGO-FET

The hitherto used graphene oxide in the fabrication of sensors in our group was synthesized according to Larisika et al [39]. The method makes use of a “quick and dirty” variation of Hummers method. Although the produced rGO-FETs were functional and were used to determine binding kinetics of OBP14 ligands, the GO Larisika showed a low yield of usable sensors, high average electrical resistance and low reproducibility. Several parameters which influence these properties were examined and the fabrication of the rGO-FET was characterized with various surface sensitive methods. Several alternative fabrications methods were additionally investigated. In a first step, three different GO products were examined to evaluate them in order to achieve high sensor surface coverage in low incubation times. GO Larisika was concluded to not be usable for FET fabrication due to its small flake size, low oxidation and high batch to batch variation in coverage. Additionally, the FTIR spectrum did not show any similarity to literature spectra of GO. Two alternative GO products were tested: a protocol proposed by Marcano et al., that is widely used for graphene oxide synthesis and the GO Feicht protocol that uses a fast and easy purification method [138]. Using those GO products lead to a decrease of resistance and higher reproducibility (personal communication with Reiner-Rozman). According to SEM pictures, the GO Feicht flakes were the largest and showed very consistent size and coverage. Marcano GO consisted of large GO-flakes but did not yield full sensor surface coverage. Consequently, it is proposed that GO Feicht (0.5 mg/ml) is used in the fabrication and that the incubation time should not exceed 5 minutes.

The reduction method influences the functionality of the rGO-FET since it restores the properties of graphene by removing the oxidations which disrupt the lattice. An optimal reduction method leads to low electrical resistance and is highly reproducible. The standard reducing method in our group was the usage of hydrazine at 80°C for 18h, which is widely used by other groups [135, 218]. Many different reducing agents are used in literature but hydroiodic acid was shown to have the best reducing properties [132]. The reducing efficiency was examined via contact angle measurements since it is an easy method and provides information about the surface energy which is related to the oxidation of GO. The reduction was done successfully using three different methods, where HI proved to be the best reducing agent when it is directly dropcasted on the GO. Hydrazine and HI gas showed slightly lower contact angles which were attributed to higher oxidation. However, it did not restore a complete graphene structure which caused lower static water contact angles.

The pyrene anchor is assumed to adsorb via $\pi - \pi$ stacking on the GO-surface although it was not proven. However it is adsorbing on the surface in THF. IR-measurements in water showed no decrease of C=C aromatic vibrations which could be assigned to the desorption of the pyrene anchor. The protein binds within 40 minutes on the PBSE-linker, which is four times faster than just in water. Similar results were determined by Dordi et al. when binding a protein to a NHS-ester [236]. It is concluded that the protein is bound by the linker to the surface.

In his PhD-thesis Reiner-Rozman claims that Ta₂O₅ reduces the unspecific interaction of the sample with the rGO and consequently decreases the unspecific signal [211]. Since the passivation approaches were very unusual they were tested and compared with blank rGO. Reiner-Rozman presents QCM results which imply that Ta₂O₅ reduced the adsorbed mass on the sensor when using a corn extract (filtrate of a blended corn sample in PBS buffer pH 5). However, this could not be reproduced when using a protein solution. A protein solution was used because it seemed to be a better test solution since the corn extract was not totally homogeneous and small flakes affected the QCM measurements. When washed with a lysozyme solution, Ta₂O₅ showed the strongest unspecific adsorption compared to rGO or GO. This result indicates that the improvement of the FET signal is not due to passivation properties of Ta₂O₅ but due to its electronic properties as resistor. Also the standard passivation molecule PEG proved to be inefficient to reduce unspecific adsorption on rGO. However, this could be explained by an inadequate preparation protocol. It was shown that tantal pentoxide can be functionalized via silanes. These functionalization could further be used to bind proteins. The preparation via reactive sputtering is not only time consuming but also causes oxidation of the reduced graphene oxide which leads to a very inconsistent resistance of the rGO-FET.

Since the passivation via Ta₂O₅ is very sophisticated and does not yield in reduction of unspecific binding, more simple methods are proposed as used by other groups. Liu et al. uses ethanolamine to block the unreacted PBSE and to reduce unspecific adsorption on the reduced grapheneoxide [151]. High k -value metals like Al₂O₃ are also used by other groups for passivation [151, 280]. However, those studies always show the reduction of unspecific signal but not that this is the result of the reduction of unspecific adsorption.

5.2 Quantification of OBP14 and PBSE

The quantification on graphene required some modifications of the protocol. GO was used instead of rGO to reduce the interference of the water bending band with the amide bands. However the results are in good agreement with literature [234]. It was shown, that the PBSE linker forms a mono or bilayer on the GO-surface. Additionally calculation of the average angle of C=C vibrations and dichroic spectra showed that the pyrene aromatic rings are aligned parallel to the graphene.

All determined protein concentrations are collected in table 5.1 and Fig. 5.1. The surface concentration of OBP14 on a GFET was calculated to be 9.68 ± 1.23 pM/cm² which is corresponds to a surface coverage of approximately 60%. Similar coverage is resulting after BSA incubation ($\Gamma = 2.21 \pm 0.31$ pM/cm², $\approx 60\%$ coverage). Since there is no literature data for graphene surfaces, protein-concentrations on functionalized germanium were used for comparison [48, 53, 281, 282]. These results are in good agreement with random sequence adsorption packing, which is the highest possible covering for adsorption without rearrangement in a mono-

Figure 5.1: Protein concentrations of (A) bovine serum albumin and (B) OBP14 on prepared surfaces. For comparison literature values are displayed. The calculated maximum BSA and OBP14 concentration is used to calculate the relative protein coverage.

Table 5.1: Quantitation of BSA and OBP on the Si surface at different stages of rGO-FET construction. In literature, also alkaline phosphatase (AP) and human serum albumin (HSA) is used, which have similar molecular weights.

	Γ (pM/cm ²)	Γ (ng/cm ²)	R	mol. area (Å ²)
BSA on Si	1.81	120	2.17	9180
BSA on GO	3.51	233	1.62	4728
BSA on PBSE	2.21 ± 0.44	147 ± 30	1.22 ± 0.28	7820 ± 1550
BSA on gold	4.46 ± 0.13	296 ± 10		
BSA calculated	3.89	258		
AP on Ge [281]	1.74	113		
BSA on Ge [48]	0.717	47.6		
HSA on Ge [53]	5.31 ± 0.49.	352 ± 33		
BSA on Ge [282]	5.70 ± 0.49	378 ± 33		
OBP14 on GO	9.77 ± 0.57	158 ± 13	1.21 ± 0.05	1628 ± 280
OBP14 on PBSE	9.68 ± 3.03	157 ± 50	1.33 ± 0.43	1870 ± 600
OBP14 on DTNTA	5.24 ± 0.76	84.9 ± 12.3	1.15 ± 0.07	3234 ± 440
OBP14 calculated	16.6	268		

layer. Consequently, it can not be demonstrated that the protein binds only to the linker but also adsorbs to the surface. The immobilization of His-tag modified OBP14 on a NTA-functionalized ZnSe ATR-crystal resulted in a concentration of $\Gamma = 5.24 \pm 0.31 \text{ pM cm}^2$ which is equivalent to a surface coverage of 32%. It can be assumed that the protein is predominantly bound via the His-tag to the surface and that nonspecific adsorption plays a minor role.

5.3 Modulated infrared spectroscopy

pH-modulation has been successfully applied to OBP14 and PBP1. Infrared spectra showed significant changes in the helical and disordered structure.

2D-correlation spectroscopy and PSD proved to be very useful to get more information about the peak position during modulation. Both methods detect bands in the spectral regions which are assigned to α -helical and disordered structures.

It is assumed that the protonation of anionic acidic residues of OBP14 causes an increase in bond length and consequently an upshift of the amide bonds. Additionally the salt bridge between the seventh and first helix is broken which causes the opening of the binding pocket and formation of more helical structure. This might correlate with a change in binding affinity at lower pH, which has also been described for other OBPs like PBP1 of *Bombyx mori* [104] or ASP1 of *Apis mellifera* [63].

In comparison, the modulated measurements of PBP1 show a high similarity with the OBP14 modulation although the structural change was expected to be very different. Additionally, the intensity of the disordered structure decreases much more than the increase in helical structures although changes were expected to be of equal intensity. This can be attributed to different extinction coefficients of different secondary structures.

In conclusion, OBP14 and PBP1 also show a reversible conformational change due to pH-change, though the structural effects are different. A mechanism for uptake and release of the ligand has been suggested, which proposes that the pH change causes the protein is in closed state at pH 6.5 and in open state in pH 4.5. The open state allows uptake and release of the ligand while the closed state cages the ligand.

Ligand modulated infrared spectroscopy was used to provide insight into the binding mechanism and kinetics. Although significant spectral changes could be observed, the exact determination of structural change proved to be difficult due to low signal-noise-ratio. It is assumed that the change is caused by a rearrangement of the helices which was also observed in X-ray diffraction studies [62]. The quality of the infrared spectra was not sufficient to provide information about the binding kinetics.

Chapter 6

Summary and Outlook

The aim of this thesis was to characterize the graphene-based field-effect transistor with various surface sensitive methods and to examine the structural changes of OBP14 due to pH and ligand modulation.

Every step of the preparation of the GFET was characterized via infrared and Raman spectroscopy, scanning electron microscopy, quartz microbalance and contact angle measurements. These methods enabled characterization of the efficient modification of the surface and to provide further information about the kinetics of several preparation steps and the overall efficiency. This is extremely valuable as several steps have a critical role in the function of the device. Furthermore, it was possible to provide information about the effective concentration and surface coverage of the pyrene linker and the protein. This information can be used to further optimize the preparation conditions to develop a GFET with uniform and consistent resistances.

Three different GO products were characterized by their composition, thickness and appearance. GO Larisika showed the lowest oxidation grade, lowest defect density and smallest flake size. Additionally it showed a high tendency to form aggregates and is stable for only a few weeks. A big difference in quality from batch to batch makes it impossible to prepare homogeneous graphene layers from GO Larisika. It is concluded that the preparation method is not suitable for the fabrication of a GFET.

In contrast, the GO produced according to Marcano et al. showed the highest oxidation, lower defect density and polydisperse flake size. Some flakes showed a diameter of more than $80\mu\text{m}$ which is sufficient to cover the gap between the source and the drain electrodes of the planned GFET device ($50\mu\text{m}$).

GO Feicht showed similar results as GO Marcano. The graphene coverage was dependent on the graphene preparation and incubation time. It was shown that the GO Feicht yielded the highest surface coverage and graphene properties. Within five minutes the surface was almost completely covered. SEM images exhibit a dense but not complete coverage which consisted of thin graphene layers and only few multilayers and aggregates. Additionally, it was the only product which had a high reproducibility in binding kinetics and coverage. In comparison, GO Marcano binding was not reproducible, took longer and the surface coverage was lower.

Contact angle measurements confirmed the increase in hydrophobicity. Reduction using hydroiodic acid was superior to hydrazine. Dropcasting HI directly on the chip is most efficient, but due to further cleaning processes not very practical. Hydrazine and HI vapor reduce GO but the latter showed higher contact angle which is attributed to better reduction. This correlates

with results of Reiner-Rozman, where using GO Feicht and reducing GO with hydroiodic acid led to the most reproducible resistances and the highest yield of rGO-FET chips. It is therefore proposed to use this combination for further development of the rGO-FET.

The PBSE linker showed to successfully adsorb to the graphene surface and it was stable in aqueous solution. Polarized measurements indicate, that the C=C-aromatic and C=O-carboxyl vibrations are oriented parallel to the surface. This is according to the theory that the pyrene anchor interacts with the graphene via $\pi - \pi$ -stacking. The concentration of 330-550pM/cm² exceeds the calculated theoretical monolayer concentration, but it is comparable to concentrations of similar linker molecules known from literature (520pM/cm²). OBP14 was covalently bound on the PBSE at a surface concentration of 9.68pM/cm² which is equivalent to a coverage of approximately 60%. In order to have a high protein concentration on the surface, the incubation time of protein binding should be at least 30 minutes. The infrared spectra, which are taken during binding showed no visible removal of the PBSE linker.

QCM measurements were applied to examine the passivating effect of tantalum pentoxide to reduce the nonspecific adsorption which were claimed by Reiner-Rozman [211]. The use of a protein solution did not show any reduction of adsorption. Covering the rGO causes a drastic increase of nonspecific adsorption but it is lower than the adsorption on Ta₂O₅. It is advised to use a different anti-fouling method to increase the specificity of the sensor.

Infrared spectroscopy also proved to be a very efficient method to characterize structural changes of OBP14 due to pH and ligand modulation. OBP14 was immobilized on an ATR-crystal using His-NTA-coupling. The composition of the infrared spectrum of the protein was compared to literature spectra and the band assignment of Schweighofer et al. [109] was revised in order to be closer to X-ray diffraction studies. Additionally, the concentration was determined to be 5.24pM/cm² (32% coverage). This significant lower concentration compared to the OBP14 bound to PBSE is caused by a lower protein-binding efficiency of hexa-His-tag than deca-His-tag [251] or a PBSE linker.

Periodic changes of the environment caused the protein to change its structure. These changes were observed via rapid scan infrared spectroscopy. In order to provide more information about the band positions 2D-correlation spectroscopy and phase sensitive detection was applied. Furthermore, phase sensitive detection helped to increase the signal to noise ratio.

It was shown that the protein undergoes structural changes due to pH change. The intensity of the band, which is assigned to helical structures is increasing, implying an increase of helical structure, while a band, which is assigned the disordered structure is decreasing. A theoretical mechanism for the uptake and release of ligands was proposed. The breaking of the salt bridge between Glu33 and Lys114 causes the reorientation of the seventh helix. The binding pocket gets opened and ligand can bind. In addition to this reorientation and stretching of H-bonds helical structures are formed. This information is important because it implies that the FET could be restored by rinsing it with a buffer with different pH.

In order to confirm the proposed mechanism, further studies have to be done. X-ray diffraction studies of OBP14 at pH 4.5 and pH 6.5 would show changes in the protein structure. Additional kinetic measurements are necessary to examine the effect of pH on the binding affinity of different ligands.

The structural change due to the binding of a ligand could be observed. However, the structural changes were very small. This led to a very small spectral change, which was mostly obscured by background noise. The small changes were observed in the helical and disordered structure. Since the expected conformational change was very small it can not be excluded, that the observed spectral change is due to a change in H-bond strength in the protein or increase of extinction coefficient. However, the secondary goal to determine the binding kinetics of OBP14 to different ligands was not accomplished as the signal to noise ratio was not high enough.

In summary, infrared spectroscopy showed its high potential to characterize surface processes and modifications. In addition to qualitative information it was possible to determine the surface concentration of molecules in vitro. Using a simple setup, even small conformational changes of proteins were observed. Utilization of various tools for band deconvolution proved to be efficient in finding single components of a band. However, the measurements also showed limitations. The setup is sensitive to detect very small changes of the protein structure but it was not possible to allocate them to specific secondary structures. One way to overcome this problem is to further increase the measuring time. However, since the measuring time for one experiment was already 22h this is not very practical. Another approach to get more information about the structural changes due to ligand binding could be to use coatings that enhance surface sensitivity prepared on the ATR crystal. SEIRAS was already applied successfully for the examination of cytochrome *c* oxidase [51, 52]. For this, a simple rough gold surface was used. A more sophisticated surface structure are nanostacks which can be tuned to the wavenumber range of interest. These were introduced by Sraier et al. to improve the sensitivity of FTIR [283]. A gold surface is covered by a grating of cylindrical shaped nanostructures, which have the same size and also have the same grating constant. By varying the size and the grating constant, the plasmon can be tuned to specific wavenumbers. By tuning the plasmon to the amide I region, the signal can be increased by up to 125 fold. This opens the way for more sophisticated studies on proteins. Beside the study of protein-ligand interactions, the study of protein-protein interactions like OBP-OR-interactions could be done. This could provide useful information to understand the exact mechanism for how the OBP triggers the receptor and if there is an exchange of ligand or not.

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Glossary

APTES - amino-propyl-tri-ethoxy-silane
ASP - antenna specific protein
ATR - attenuated total reflection
AU - absorbance units
BSA - bovine serum albumin
cAMP - cyclic adenosin mono phosphate
CaCC - Ca-activated Cl-channel
CGG - cyclic nucleotide gated channel
DTNTA -
DTP - Dithio-propionic acid
ELISA - enzyme linked immunosorbant assay
FET - field effect transistor
FTIR - Fourier transform infrared spectrometer
GO - graphene oxide
GOBP - general odorant binding protein
GPCR - G-protein coupled receptor
GFET - graphene field effect transistor
HSA - human serum albumin
IRE - internal reflection element
LOC - lab on a chip MIRE - multiple internal reflection element
MOE - main olfactory epithel
NHS-ester - N-hydroxy-succinimide reactive ester
OBP - odorant binding protein
OR - olfactory receptor
ORN - olfactory receptor neuron
PBP - pheromone binding protein
PBSE - pyrene butyl succinimidyl ester
PCR - polymerase chain reaction PEG - polyethylen glycol
pp - parallel polarisation
PSD - phase sensitive detection
QCM-D - quartz microbalance with dissipation monitoring
rGO - reduced graphene oxide
SEIRAS - surface enhanced infrared absorption spectroscopy
SEM - scanning electron microscope
SIRE - single internal reflection element
SPS - sample point spectrum
THF - tetrahydrofuran

tr - time resolved

VOC - volatile organic compound vp - perpendicular polarisation

2DCS - 2D-correlation spectroscopy

2DMWCS - 2D-moving window correlation spectroscopy

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5.1 Quantitation of BSA and OBP on the Si surface at different stages of rGO-FET construction. In literature, also alkaline phosphatase (AP) and human serum albumin (HSA) is used, which have similar molecular weights. 105

Appendices

Electron Transfer to Light-Activated Photosynthetic Reaction Centers from *Rhodobacter sphaeroides* Reconstituted in a Biomimetic Membrane System

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ABSTRACT: Photosynthetic reaction centers (RCs) from *Rb. sphaeroides* with a genetically engineered 7-his-tag at the C-terminus of the M-subunit are bound to a Ni-NTA-modified gold surface. Subsequently, the bound RCs are subjected to in situ dialysis in the presence of lipid micelles to form a protein-tethered lipid bilayer membrane (ptBLM). Redox properties of the RC thus immobilized are investigated by cyclic voltammetry. Photocurrents are generated in the range of $10 \mu\text{A cm}^{-2}$, however, different from previous studies at potentials of -200 and -300 mV, and without cytochrome *c* as a mediator. The unexpected behavior is explained in terms of an interprotein reaction between RC molecules promoted by the lipid bilayer, which we had previously detected by surface-enhanced infrared absorption spectroscopy.

INTRODUCTION

Efforts toward capture and conversion of solar energy are often inspired by photovoltaic materials provided by nature. The most efficient of these materials are photosynthetic reaction centers (RCs) produced by certain bacteria, e.g., *Rb. sphaeroides*, which have a quantum yield near unity.¹ Therefore, attempts have been made to use these proteins for hybrid bioelectronic solar cells.² A prerequisite for utilizing the RCs in such a device is their immobilization in a uniform orientation, thereby retaining their natural quantum efficiency. Equally important, however, is the efficient electron transfer to the electrode. For a review of existing RC-functionalized nanocomposite films, see ref 3. Effective electronic wiring of proteins to conducting surfaces is of interest not only from the point of view of application but also for purposes of basic research. In combination with spectroscopic methods, this methodology can provide interesting mechanistic insights.⁴

Investigations of light-activated RCs in the context of biologically inspired light energy conversion were mostly directed toward electron transfer (ET) to the primary electron donor (special pair, P870 or P), converted by illumination into the oxidized species P870⁺ (P⁺). For this purpose, the group of Lebedev was the first to immobilize RCs on gold electrodes via his-tag technology on top of NTA-terminated linker molecules of different lengths.^{5–7} Photocurrents in the range of nA were recorded after light excitation, mediated by cytochrome (cyt) *c* and ubiquinone-2, (Q-2). Cyt *c* and Q-2 were considered as intermediate electron acceptors between the working electrode

and P⁺, and between the reference electrode and ubihydroquinone, QH₂, respectively. Later, this group increased the sensitivity of their method by employing arrayed carbon nanotube electrodes.⁸ Cyt *c* as a mediator was also used by the group of Frese, who employed RCs and RCs with light harvesting LH1 complexes directly adsorbed on bare gold electrodes.^{9–11} They were able to raise photocurrent densities to the level of $45 \mu\text{A cm}^{-2}$ by optimizing illumination intensities among other factors. A very interesting photoelectrochemical system has been presented by a large group around Strano, who employed RCs incorporated in lipid bilayer nanodiscs,¹² which autonomously regenerate around carbon nanotubes. Photocurrents in the nA cm⁻² range were mediated by ferro/ferricyanide and ubiquinone/quinol, respectively.^{13,14}

Recently, we have investigated the photoexcitation of RCs from *Rhodobacter sphaeroides* by surface-enhanced infrared absorption spectroscopy (SEIRAS).¹⁵ RCs were immobilized on structured gold surfaces similar to the method used by Trammel et al.,^{6,7} namely, on NTA-terminated linker molecules via a poly his-tag engineered at the C-terminal end of the M-subunit. However, different from their work, we have reconstituted the RCs into a lipid bilayer by *in situ* dialysis. The result is a protein-tethered lipid bilayer membrane (ptBLM), which has been used with other membrane proteins

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before.^{16,17} Under continuous illumination, we detected P870⁺ and QH₂ generated in equal amounts.¹⁵ This was surprising because, under these conditions, RCs usually yield QH₂ only if P⁺ is reduced back to P by an external electron donor.^{18,19} This prompted us to investigate the electrochemical reduction of P⁺ of the special pair after photoexcitation of RC embedded in the ptBLM.

EXPERIMENTAL METHODS

Reagents. Deionized water was used from a Sartorius-Stedim system (Goettingen, Germany) with a resistivity of 18 MΩ cm. Argon 4.8 was obtained from Linde Gas GmbH (Stadl-Paura, Austria). 3-Mercaptopropyltrimethoxysilane (MPTES, 95%) was purchased from ABCR GmbH (Karlsruhe, Germany). Gold granules (99.99%) for evaporation were obtained from Mateck GmbH (Juelich, Germany). Biobeads (20–50 mesh) were purchased from Bio-Rad Laboratories GmbH (Vienna, Austria). 1,2-Diphytanoyl-*sn*-glycero-3-phosphocholine (DiPhyPC, >99%) was provided by Avanti Polar Lipids (Alabaster, AL). Dithiobis (nitriloacetic acid butylamidyl propionate) (DTNTA, ≥95.0%) was obtained from Dojindo Laboratories (Kumamoto, Japan). Hydroxylamine hydrochloride (NH₂OH·HCl, 99%), gold(III) chloride hydrate (HAuCl₄·xH₂O, 99.999%), dimethyl sulfoxide (DMSO, puriss., dried over molecular sieve), 3,3'-dithiodipropionic acid (DTP, 99%), dodecyl-β-D-maltoside (DDM, ≥98%), nickel(II) chloride (NiCl₂, 98%), 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinone (Q-2), and cytochrome *c* from horse heart were purchased from Sigma-Aldrich (Steinheim, Germany). All chemicals were used as purchased.

Immobilization of the Protein and Reconstitution into a Lipid Bilayer. Wild-type *Rhodobacter sphaeroides* RCs with a genetically engineered 7-his-tag at the C-terminus of the M-subunit were expressed from a strain kindly provided by S.G. Boxer.²⁰ RCs were purified according to a modification of the original method.²¹ The immobilization of RC was performed on the template stripped gold (TSG) slides prepared according to ref 22. The TSG slides were immersed in a solution of 2.5 mM DTNTA and 7.5 mM DTP in dry DMSO for 20 h. After rinsing with ethanol and purified water, the surface was immersed in 40 mM NiCl₂ in acetate buffer (50 mM, pH 5.5) for 30 min, followed by thorough rinsing with purified water to remove excess NiCl₂. The surface was dried under a stream of argon prior to assembly in the measuring cell and rehydrated with DDM-DPK buffer (0.05 M K₂HPO₄, 0.1 M KCl, pH 8, 0.1% DDM) before adsorption of the RCs dissolved in DDM-DPK at a final concentration of 100 nM. After 4 h time of adsorption carried out at 28 °C, the cell was rinsed with DDM-DPK to remove unspecifically adsorbed and bulk protein. In the case of additional Q-2 and cyt *c*, they were added to the aqueous phase to final concentrations of 40 and 10 μM, respectively. For the reconstitution of the RCs into a lipid bilayer, DDM-DPK was replaced by a DiPhyPC/DDM-DPK solution (40 μM DiPhyPC in DDM-DPK) or alternatively by Q-10 solubilized together with DiPhyPC (6 μM Q-10 in DiPhyPC/DDM-DPK). In both cases, DDM was removed by adding Biobeads to the lipid–detergent solution.

Illumination was performed with white light from a Fiber-Lite DC950 illuminator (150 W, quartz halogen lamp) provided with an optical fiber obtained from Dolan-Jenner (Boxborough, MA) with a light intensity of 200 mW/cm² (at a wavelength of 800 nm).

Electrochemical Measurements. Electrochemical impedance spectroscopy, cyclic voltammetry, and chronoamperometry were performed using a potentiostat from Autolab (PGSTAT12 with GPES 4.9 from Metrohm, Herisau, Switzerland) in a three-electrode configuration with TSG as the working electrode, a Ag|AgCl|KCl_{sat} reference, and a platinum wire as the counter electrode. The geometrical area of the working electrode was 0.875 cm². All electrode potentials are quoted versus the standard hydrogen electrode (SHE). Cyclic voltammetry measurements were done in DPK buffer (0.05 M K₂HPO₄, 0.1 M KCl, pH 8) flushed with N₂ and with vertex potentials V₁ = +700 mV and V₂ = −300 mV at a sweep rate of 20 mV s^{−1}. In the absence of the lipid layer, measurements were performed with DDM (0.1%) added to the DPK buffer,

RESULTS AND DISCUSSION

Impedance spectra were recorded in a frequency range from 100 kHz to 10 mHz with an excitation amplitude of 10 mV, first after immobilization of RCs and then after reconstitution in the ptBLM (Figure 1). Data were subsequently analyzed by the fitting algorithm using an equivalent circuit (inset in Figure 1A) based on the Boukamp model and supplied in the data processing software package Nova (Version 1.7). Parameters obtained from the fitted data are presented in the inset of Figure 1B.

Figure 1. Impedance spectra of RCs immobilized on the NTA-functionalized gold surface (black) and after reconstitution in the ptBLM (red). (A) Bode plot. (B) Frequency-normalized admittance plot. Full lines represent the fitted curves. The equivalent circuit and the fit parameters are inserted in (A) and (B), respectively.

A substantial increase in the resistance of the layer on the gold surface can be observed in the Bode plot (Figure 1A) after formation of the ptBLM, indicating that the voids between the proteins are filled with a lipid bilayer, thus impeding the flow of ions. Furthermore, there is a significant decrease in the capacitance of the layer after reconstitution (Figure 1B), which can be understood in terms of the smaller dielectric constant of lipid than that of water and protein molecules, thus confirming the formation of the ptBLM.

Cyclic voltammograms (CVs) of the immobilized RCs were recorded in the dark before and after reconstitution in the ptBLM. The reduction/oxidation of P870/P870⁺ was indicated by the peaks at +350/+600 with a midpoint potential of +475 mV (Figure 2). The midpoint potentials (E_m) of cofactors in RC have

Figure 2. CV of RCs immobilized on the gold surface before (solid line) and after (dashed line) formation of the ptBLM, and after addition of oxidized cyt *c* and Q-2 (dotted line) to final concentrations of 40 and 10 μ M, respectively. The inset shows the CV with an expanded y axis.

been quoted (mostly at pH $\approx$ 7) as follows: P/P⁺ E_m = +460 mV (by CV),^{23,24} Q_A/Q_A⁻ E_m = 0 to -150 mV,^{25,26} Q_B/Q_B⁻ E_m = 50 to -50 mV,²⁶ BPhe/BPhe⁺ E_m = -300 to -500 mV.²⁷ The midpoint potential that we have found (+475 mV) can thus be attributed to the primary donor, and corresponds closely to the values of (+475–510 mV) found by Trammel et al.^{6,7} In the presence of the lipid bilayer, a clear anodic peak appeared at 650 mV, whereas a very large cathodic peak, in the range of 50 μ A cm⁻², appeared at -250 mV. A similar cathodic peak was also recorded at -150 mV without reconstitution into a ptBLM, however, only in the range of 5 μ A cm⁻² (Figure 3). Addition of Q-2 to the RCs encapsulated in a ptBLM resulted in slight shifts of the peaks at +600 and -150 mV (Figure 2). Addition of oxidized cyt *c* had no appreciable effect (not shown). On the basis of the known midpoint potentials, the large cathodic peaks between -150 and -250 mV could possibly be attributed to a cofactor different from P such as Q_A or BPhe. However, the anodic counterpeak is still around +600 mV, which would not be in accordance with a reversible redox couple. Summarizing, we observe not only peaks around the midpoint potential of the special pair but also, in addition, a large cathodic peak in the negative range of potentials between -150 and -250 mV.

In order to elucidate this unexpected behavior, the system was investigated by chronoamperometry. Photocurrents were measured after application of the selected potential when the charging current had reached a stationary state. Small photocurrents in the range of nA were found at around 0 mV in the presence of oxidized cyt *c* and Q-2, however, different from

Figure 3. CV of RCs immobilized on the gold surface without reconstitution into a ptBLM before (solid line) and after addition of oxidized cyt *c* (dashed line) and Q-2 (dotted line) to final concentrations of 40 and 10 μ M, respectively.

Trammel et al.^{6,7} in the anodic direction (Figure 4). Similar anodic photocurrents were reported by Kong et al., a group that

Figure 4. Photocurrents of RC immobilized on NTA-modified TSG and reconstituted in a ptBLM, at 0 mV (red), +100 mV (black), and +200 mV (green) in the presence of oxidized cyt *c* and Q-2. The arrows indicate when the light is switched on (↑) and off (↓).

has specialized in RCs electrostatically bound to self-assembled monolayers of amino-terminated thiols.^{3,28} They interpreted the anodic photocurrents in terms of the RCs oriented with QH₂ species facing the electrode. In our case, this explanation is hardly conceivable, since the RCs are bound with the special pair directed to the electrode, whereas quinone/quinol can diffuse freely within the lipid bilayer. Therefore, we consider QH₂, formed during light excitation, being reoxidized at the gold working electrode giving rise to the anodic currents. Permanent currents are considered as photocurrents, rather than the upward and downward spikes preceding them; see for a detailed discussion of such spikes further below.

As far as cathodic photocurrents are concerned, they were obtained in the order of magnitude of μ A cm⁻², in accordance with voltammetry in the negative potential range, with an optimum at -300 mV and lasting for more than 15 min. Photocurrents were still more enhanced in the presence of the membrane (Figure 5), particularly after an overnight incubation with Q-2 (Figure 6), but were not appreciably affected by oxidized cyt *c* (not shown). Photocurrent measurements after

Figure 5. Photocurrents of RC immobilized on NTA-modified TSG measured before (black) and after (red) formation of the ptBLM, at -200 mV (open squares) and -300 mV (full squares); green lines are fits of the data to a monoexponential function. The arrows indicate when the light is switched on ($\uparrow$) and off ($\downarrow$). The inset shows an enlarged version of the photocurrent in the region of the double layer charging, when the light is switched on.

Figure 6. Photocurrents of RC immobilized in a ptBLM, in the absence of oxidized cyt *c*, measured at -200 mV before (black) and after (red) an overnight incubation with Q-2 ($10 \mu\text{M}$). The arrows indicate when the light is switched on ($\uparrow$) and off ($\downarrow$).

light excitation could be repeated several times (Figure 7). Permanent photocurrents in the presence of the lipid membrane were preceded by transient current spikes; see particularly Figure 5, the red as compared to the black curves. The spikes are considered in terms of charging of an electrochemical double layer as a consequence of the initial charge separation within the RC. In the presence of the lipid bilayer membrane encapsulating the RC molecules (see the TOC graphic), a small aqueous submembrane layer is formed defined by the length of the linker molecules, which is separated from bulk DPK buffer. If the membrane/water interphase is positively charged due to the generation of P^+ , the redistribution of ions in this submembrane layer will give rise to a charging current at the gold electrode. Hence, spikes are observed in the presence of the lipid bilayer rather than in its absence. We have fitted the spikes separately from the photocurrent generation using the same monoexponential function, also used by Trammel et al.⁸ Likewise, when the light is switched off, the relaxation of the current occurs in two phases; the first one indicates the relaxation of the double layer charging in the submembrane layer, the second the relaxation of the photocurrent. However, the clear separation of photocurrent generation and double layer charging observed in Figure 5 is lost,

Figure 7. Photocurrents of RC immobilized on NTA-modified TSG measured after formation of the ptBLM, at -200 mV in the presence of oxidized cyt *c* and Q-2 (final concentrations of 40 and $10 \mu\text{M}$, respectively). The arrows indicate when the light is switched on ($\uparrow$) and off ($\downarrow$).

when the system is excited repeatedly (Figure 6 and 7). This can be explained by an insufficient relaxation of the double layer charging between excitation cycles, indicated by an elevated $k_{\text{off},1}$ versus $k_{\text{on},1}$; see below for an explanation. Therefore, we have concentrated on the first cycles depicted in Figure 5, from which we obtain four kinetic constants, two k_{on} and two k_{off} . The most relevant parameters $k_{\text{on},2}$ and $k_{\text{off},2}$ represent the kinetics of photocurrent generation and relaxation, whereas $k_{\text{on},1}$ and $k_{\text{off},1}$ represent the double layer charging and discharging. Values of these parameters are collected in Table 1 together with the final current densities. $k_{\text{on},2}$ values are in the range of $50\text{--}100 \text{ s}^{-1}$, the same order of magnitude as in Trammel et al., decreasing in the presence of the membrane (Figure 5, Table 1).⁸ Current densities are lower, however, in the same order of magnitude as reported by Hollander et al. using RC–LH1 complexes adhered to gold electrodes.^{9,10}

In order to explain these observations, we have to consider that the primary charge separation after light excitation of RCs results in the formation of P^+ and Q_A^- , with subsequent electron transfer to Q_B forming the Q_B^- semiquinone. A second electron cannot be transferred to Q_A^- unless P^+ has been rereduced to P .^{18,19} This reduction must be faster than the recombination of P^+ and Q_B^- back to the PQ_B ground state, which occurs with a half-time of $1\text{--}2 \text{ s}$.^{29,30} Hence, appreciable photocurrents can be generated only when the electrochemical reduction of P^+ is faster than the charge recombination. However, in our SEIRAS studies of RCs within the same membrane architecture, we had found an alternative to the fast recombination, particularly in the presence of a lipid bilayer.¹⁵ This alternative pathway was deduced from the slow accumulation of the species P^+ and QH_2 during continuous illumination. The concerted formation of P^+ and QH_2 was discussed in terms of the interprotein ET reaction between two molecules of RC in the excited state

in which protonation is coupled to the double reduction of Q_B to QH_2 , followed by release of QH_2 into the membrane and rebinding of quinone from the quinone pool. Consequently, the process was strongly affected by the concentration of Q-10 in the lipid phase. Mechanistically, the disproportionation reaction (eq 1) is likely to include the transient state $\text{P}^+\text{Q}_\text{A}^-\text{Q}_\text{B}^-$.³¹ The disproportionation was shown to occur upon prolonged illumination of relatively high intensity (200 mW cm^{-2}) and to

Table 1. Kinetic Parameters of Photocurrents of RC Immobilized on NTA-Modified Gold Surfaces

applied potential/mV	bilayer	$k_{\text{on},1}/\text{s}^{-1}$	$k_{\text{on},2}/\text{s}^{-1}$	$k_{\text{off},1}/\text{s}^{-1}$	$k_{\text{off},2}/\text{s}^{-1}$	current density/ $\mu\text{A cm}^{-2}$
−200	without		142.9			4
	with	2.7	78.7	124.1	86.3	7
−300	without		101			7
	with	3.1	54.6	80.6	84.1	13

be reversible when the light is switched off. We conclude that we do not record electron transfer to P^+ after the initial charge separation, when the resulting excited states are $\text{P}^+\text{Q}_\text{A}^-$ and $\text{P}^+\text{Q}_\text{B}^-$. This conclusion is consistent with the finding that cathodic photocurrents are not recorded near 0 V (note that Figure 4 shows anodic currents). Instead, the desired cathodic photocurrents are measured at around −200 mV, with an anodic counter peak at around +600 mV, particularly in the presence of the lipid bilayer. The cathodic currents are considered to represent electron transfer to the species $\text{P}^+\text{Q}_\text{B}$ generated in the course of the interprotein reaction described by eq 1. Since $\text{P}^+\text{Q}_\text{B}$ is much longer lived, in the time scale of minutes, as compared to $\text{P}^+\text{Q}_\text{A}^-$ or $\text{P}^+\text{Q}_\text{B}^-$, vanishing in the time scale of seconds, higher current densities can be attained, albeit on the same surface architecture as in Trammel et al.'s work.^{5–8} However, the electroreduction of $\text{P}^+\text{Q}_\text{B}$ occurs at a potential shifted in the negative direction, thus restoring the ground state PQ_B and/or PQ_A . The special pair in the ground state, P, may then give rise to electrooxidation, indicated by an anodic peak at around +600 mV, just like the RC before any illumination. We conclude that, in our setup, photocurrents are dominated by the disproportionation reaction, promoted by the lipid bilayer. The fact that cyt *c* is not needed indicates direct rather than mediated ET to $\text{P}^+\text{Q}_\text{B}$, which is possibly due to the difference in linker concentration between Trammels and our work. We had found earlier that the efficiency of electronic wiring of multiredox center proteins bound to NTA-terminated linkers depends strongly on the packing density of the proteins. A mixing ratio of 2:8 (NTA linker versus DTP dilution molecule) had been found to be the optimum in the case of other proteins.¹⁷ The lower linker concentration appears to minimize the 2 nm long tunneling distance determined by the length of the linker between electron acceptor and electrode. We can only speculate that, under these conditions, linkers and proteins are present on the surface in a more flexible configuration, thus facilitating direct electron transfer to P^+ . Nevertheless, we have indications that an aqueous layer mentioned above may still be present between electrode and lipid bilayer. In addition, the presence of the lipid bilayer leads to a stabilization of the protein molecules in an upright position, further facilitating direct electron transfer to P^+ . We consider these conditions as the main reason for an effective ET without the need of a mediator.

As to the role of Q-2, this is considered to become part of the quinone pool after prolonged incubation, thus replacing Q-10 co-reconstituted in our SEIRAS study.¹⁵ Even without any lipid phase, some quinone is always left within the RC protein bulk phase. The kinetics of the interprotein reaction eq 1 had been shown to depend strongly on the concentration of Q-10 in the lipid phase. In the present investigation, we deliberately omitted additional Q-10 and instead investigated the role of Q-2, since his had been promoted as a mediator between the RC and the counter electrode. We found no immediate effect of Q-2 in the aqueous phase, but instead an increase in photocurrent after overnight incubation (Figure 6). We conclude from this that the water as well as lipid-soluble Q-2 distributes between aqueous

and lipid phase and thus facilitates rebinding of quinone from the quinone pool in eq 1, similar to Q-10 co-reconstituted with the lipid seen in our previous work. Hence, we do not share the notion of Q-2 as a mediator between counter electrode and RC, at least in our setup. Instead, QH_2 formed according to eq 1 is considered to become reoxidized at the gold working electrode. However, the resulting anodic currents are considerably smaller as compared to the cathodic ones. This could suggest that they are due to the products of the primary charge separation rather than the follow-up interprotein reaction (eq 1). Nevertheless, the charging spikes go in the same direction (positive under light-on conditions) as in the case of the cathodic photocurrents, in agreement with the discussion given above.

CONCLUSION

The results presented in this study support the notion of an interprotein reaction put forward in our previous work.¹⁵ The reason why such a reaction has never been observed before originates from different experimental conditions employed in other investigations. Either the lipid bilayer was missing, e.g., in FTIR investigations of RCs, where P^+ is reduced back to P by an external electron donor,¹⁸ or RCs were investigated in combination with bc_1 complexes, e.g., in chromatophores.³² In the latter case, P^+ is reduced back to P in a circular fashion by reduced cyt *c* generated by the bc_1 . As to the possible biological significance of the interprotein reaction, one could think of it as a kick-off reaction for the circular process in chromatophores. In the artificial setup, photocurrents of around $10 \mu\text{A cm}^{-2}$ have been attained with a surface architecture similar to the work of Trammel et al, however, with an optimized packing density combined with the reconstitution into a lipid bilayer. RCs thus immobilized on solid surfaces still do not reach the level of the conversion of light into electric currents, as in the case of RC/LH1 complexes directly absorbed on gold electrodes reported by Frese et al.^{9,10} However, the new format has the advantage of an unmediated ET. Moreover, devices employing RCs stabilized by the lipid bilayer may have a longer shelf life compared to a Langmuir–Blodgett (LB) layer transferred to the surface of an electrode. Packing density in their case is reported to be 60–70%; hence, proteins present in the LB film have a certain flexibility but they are not protected by a lipid bilayer.¹⁰ This bilayer is significant in the context of the interprotein reaction, which is held responsible for the boost in photocurrent versus RCs simply bound to the electrode via undiluted NTA linkers, where recombination is the limiting factor.

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Notes

The authors declare no competing financial interest.

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Review

Biomimetic Membranes for Multi-Redox Center Proteins

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Abstract: His-tag technology was applied for biosensing purposes involving multi-redox center proteins (MRPs). An overview is presented on various surfaces ranging from flat to spherical and modified with linker molecules with nitrile-tri-acetic acid (NTA) terminal groups to bind his-tagged proteins in a strict orientation. The bound proteins are submitted to *in situ* dialysis in the presence of lipid micelles to form a so-called protein-tethered bilayer lipid membrane (ptBLM). MRPs, such as the cytochrome c oxidase (CcO) from *R. sphaeroides* and *P. denitrificans*, as well as photosynthetic reaction centers (RCs) from *R. sphaeroides*, were thus investigated. Electrochemical and surface-sensitive optical techniques, such as surface plasmon resonance, surface plasmon-enhanced fluorescence, surface-enhanced infrared absorption spectroscopy (SEIRAS) and surface-enhanced resonance Raman spectroscopy (SERRS), were employed in the case of the ptBLM structure on flat surfaces. Spherical particles ranging from μm size agarose gel beads to nm size nanoparticles modified in a similar fashion were called proteo-lipobeads (PLBs). The particles were investigated by laser-scanning confocal fluorescence microscopy (LSM) and UV/Vis spectroscopy. Electron and proton transfer through the proteins were demonstrated to take place, which was strongly affected by the membrane potential. MRPs can thus be used for biosensing purposes under quasi-physiological conditions.

Keywords: multi-redox center proteins; protein-tethered bilayer lipid membrane; cytochrome c oxidase; photosynthetic reaction centers; surface-enhanced infrared absorption spectroscopy; surface-enhanced resonance Raman spectroscopy; laser-scanning confocal microscopy; UV/Vis spectroscopy; his-tag technology; proteo-lipobeads

1. Introduction

Biosensing based on multi-redox center proteins (MRP) has often been performed on solubilized proteins. The most prominent example of this approach is protein film voltammetry [1,2]. Important information about the electrochemical properties of proteins has thus been obtained. The methods of preparation were mostly the immobilization of micelles. Similar methods have often been applied for use with FTIR spectroscopy. Proteins were immobilized on the surface of an ATR crystal via detergent depletion, drying and rehydration [3]. A large body of valuable information has been assembled, but uncertainties remain as to whether the proteins maintain their full functionality under these conditions. Therefore, designing biomimetic membrane systems on surfaces has been attempted, which would be suitable to entrap MRPs into a functionally active bilayer lipid membrane (BLM) [4,5].

The oriented immobilization of MRPs on surfaces modified with nitrile-tri-acetic acid (NTA) via his-tag technology has proved to be very auspicious in this context, particularly if the immobilization was followed by *in situ* dialysis in the presence of lipid micelles. The proteins are thereby incorporated into a BLM to form what was called a protein-tethered bilayer lipid membrane (ptBLM) [6,7]. The system was later employed by other groups to investigate MRPs such as membrane photoreceptors

and complex I from *E. coli* [8,9], whereas we concentrated on the cytochrome *c* oxidase (CcO) from *R. sphaeroides*. The protein was genetically engineered with his-tags attached to subunit II. In this configuration, the first electron acceptor Cu_A points toward the electrode so that the enzyme can be activated by direct electron transfer (ET) [10]. The CcO was investigated via fast scan voltammetry and electrochemical titration followed by surface-enhanced infrared absorption spectroscopy (SEIRAS) and surface-enhanced resonance Raman spectroscopy (SERRS) [11–13]. The enzyme could be shown to undergo conformational changes through the uptake of four electrons and to translocate protons across the membrane [14]. These results prompted us to conduct time-resolved tr-SEIRAS on this and other proteins, such as the reaction centers (RCs) and the *bc*₁ complex of photosynthetic bacteria.

Moreover, we made attempts to expand the range of methodologies applicable to MRPs incorporated in lipid membranes from vibrational to fluorescence and UV/Vis spectroscopies. In this context, we exchanged flat surfaces *versus* particles from μm sized gel beads down to nanoparticles. The concept of the ptBLM was thus shown to be applicable also to particle surfaces.

Hence we have demonstrated that the concept of the ptBLM offers the advantage of the immobilization of MRPs in a strict orientation on various surfaces, an important feature if it comes to the investigation of transport parameters. Hence a particular MRP can be induced to transport ions and/or electrons outside-in or inside-out of the BLM, provided the MRP is available with his-tags attached to either side of the protein. This was available to us in the case of the CcO and the photosynthetic reaction centers from *R. sphaeroides* and *P. denitrificans*, respectively. Pros and cons of the different approaches are discussed.

2. Results and Discussion

2.1. Multi-Redox Center Proteins Immobilized on Planar Surfaces

The most advanced techniques applicable to proteins on planar surfaces are surface-enhanced IR and Raman spectroscopies. The surfaces are enhanced by nano-structures such as nanoparticles (NPs) attached to the plane surface that modulate the electromagnetic field around them. The enhancement comes about by the excitation of localized surface plasmon resonances (LSPR), which are electromagnetic waves that enhance the electromagnetic (EM) field near the metallic nanostructures [15,16]. This EM field rapidly decreases into the liquid phase so that only molecules a few nm away from the metallic surface are affected. A review on surface-enhanced Raman spectroscopy for biomedical diagnostics and imaging can be found in ref. [17]. The theory of SEIRAS and SERRS comprises the high sensitivity of bands whose components are located at a short distance from the surface but also the surface selection rule, which predicts that adsorbed molecules are reflected the stronger the more the transition dipole moment is oriented perpendicular to the surface [16,18]. This means that a component may be presented only in one orientation whereas other orientations are not represented. We had previously developed two-layer gold and silver surfaces with NPs generated *in situ* that were suitable to apply not only electrochemical techniques but also SEIRAS and SERRS to our ptBLM structures. CcO incorporated into a ptBLM had thus been investigated by fast scan voltammetry and electrochemical titration followed SEIRAS as well as SERRS [11–13]. The same surface was then employed to perform time-resolved tr-SEIRAS [19].

2.1.1. Time Resolved tr-SEIRAS of Cytochrome *c* Oxidase (CcO) from *R. sphaeroides*

CcO with a his-tag engineered to the C terminus of subunit II was immobilized on the surface of the silicon ATR crystal of an IR spectrometer, covered with a structured gold film. On top of the ATR crystal, a flow cell was mounted and filled with a buffer solution appropriate for the immobilization and subsequent reconstitution into a ptBLM using *in situ* dialysis. Tr-SEIRAS was performed in the step-scan mode under anaerobic conditions by applying periodic potential pulses stepping between –800 and +400 mV at a modulation frequency of 100 Hz [19].

As a result, a broad band was obtained in the region of the amide I bands (Figure 1), which, however, contained many overlapping bands, as revealed by a treatment called phase sensitive detection (PSD) (Figure 2A) [20]. PSD allowed us to subtract a large water band at 1643 cm^{-1} which otherwise would have obscured the bands of the secondary structures. The absorbance of this band was determined independently by measuring a SEIRA spectrum of a droplet of water on top of a Ni-NTA modified nano-structured gold surface. Details can be found in the supplementary information of reference [19]. Figure 2A shows PSD-deconvoluted tr-SEIRA spectra. The band areas obtained from Figure 2A were plotted as a function of time; see examples in Figure 2B–D used for the fitting routine, all of which are negative bands. The negative sign means they represent reduced species, which gradually disappear in the first half period of the potential pulse, going from -800 to $+400$ mV, and re-appear in the second half period, from $+400$ to -800 mV. The bands located at 1603 , 1610 and 1625 cm^{-1} , assigned to Cu_A , heme a , and heme a_3 , respectively, were selected to be fitted to the kinetic model of the CcO, which is described below and in detail in reference [21,22]. See also Figure 3.

Figure 1. Time-resolved surface-enhanced infrared absorption spectroscopy (tr-SEIRAS) spectra of the activated cytochrome c oxidase (CcO) under anaerobic conditions. Spectra of the first excitation half period (oxidation) are shown in red, and those of the second half period (reduction) are shown in black. Spectra were taken over time intervals of $200\text{ }\mu\text{s}$. Reproduced with permission from Schwaighofer *et al.* (2013) [19].

Figure 2. Cont.

Figure 2. (A) Simulated spectra of deconvoluted bands revealed by phase sensitive detection (PSD) at an excitation frequency of 100 Hz. Subtraction of the OH bending band reveals positive and negative bands that are attributed to oxidized and reduced species, respectively. Spectra obtained in the first and second excitation half periods are shown in red and black, respectively; (B–D) Plots of band areas *vs.* time (black squares) of the bands at 1603.2 (Cu_A), 1610.7 (heme *a*) and 1625.7 cm^{−1} (heme *a*₃). The fitted curves of band areas *vs.* time to the sequential four-electron transfer model of cytochrome *c* oxidase (CcO) are given as solid red lines. Reproduced with permission from Schwaighofer *et al.* (2013) [19].

Figure 3. Kinetic scheme for sequential electron transfer in CcO under anaerobic conditions. The four centers are arranged in the order Cu_A, heme *a*, heme *a*₃, and Cu_B, which can be present either in the reduced (r) or oxidized (o) state. All of the r and o states can be protonated or deprotonated. Hence, the enzyme can exist in 16 different redox states and 256 protonation states. The vertical arrows with red branches represent electron uptake from the electrode, whereas the black horizontal arrows indicate second-order reactions between redox centers. The blue arrows indicate proton uptake and release steps likely to occur as a consequence of the second-order ET reactions between redox centers. Reproduced with permission from Schwaighofer *et al.* (2013) [19].

The same bands were found as in our titration study, although the distribution between the two types of secondary structures was quite different. In the titration, the helical structures are more

prominent, while in the tr-SEIRA spectra the β -sheets are more strongly represented (see the bands at 1603, 1615, 1619, 1625 cm^{-1}) while the band at 1653 cm^{-1} , which is characteristic for α -helices, is weak in comparison. These findings agree well with the higher flexibility of β -sheets found in molecular dynamics (MD) studies of high-resolution crystallographic structures [23,24]. The greater rigidity of the α -helices, on the other hand, prevents these structures from following the excitation at high modulation frequencies, resulting in a lower absorbance in the respective IR-signals. Substantial conformational changes were also recently observed in separate high-resolution crystallography images of the reduced and oxidized form of CcO [25]. Bands at 1603.2, 1610.7 and 1625.7 cm^{-1} were assigned to changes in the redox state of Cu_A , heme a , and heme a_3 , respectively.

The time-resolved absorbances of these bands were fitted to a kinetic model of proton transport through CcO (Figure 2). In this model, we use chemical reaction kinetics to explore proton transport coupled to electron transport (ET) in terms of a sequence of protonation-dependent second-order redox reactions, first presented in reference [21]. We thereby assume fixed rather than shifting dissociation constants of the redox sites. A schematic of the model is shown in Figure 3.

Bayesian inference was used to obtain the set of parameters that achieved the best agreement of experimental and simulated data. As fitting parameters, we chose the inherently pH-independent standard redox potentials and the pK values of the four redox centers in the order of sequential ET: Cu_A , heme a , heme a_3 , and Cu_B . As for the kinetic parameters, we must distinguish between electrochemical ET to Cu_A , which is characterized by the electrochemical rate constant, and the chemical reaction kinetics between consecutive redox centers. Finally, τ , the time constant of charging the interface also has to be included. The kinetic constants of reactions between consecutive redox centers were found to be in the ms time scale and limited by electrochemical excitation. For further details, we refer to the original publication [19].

In this investigation, direct ET to a selected redox center of an MRP has thus been demonstrated to mimic the natural pathway of electrons through the enzyme. Electrochemical excitation has been shown as an alternative to the photolytic CO dissociation otherwise used to initiate enzyme turnover. The kinetics of enzyme reduction and oxidation, however, is limited by the ET rate to the electrode.

Later, we extended these studies to other multi-redox center proteins such as light-activated photosynthetic reaction centers from *Rhodobacter sphaeroides*.

2.1.2. Photosynthetic Reaction Centers from *R. sphaeroides*

Reaction centers (RCs) with 7-his tags attached to the C-terminus of the M-subunit were reconstituted into a ptBLM as described. In this configuration, the special pair P/P⁺ is directed toward the gold film, whereas the Q_A and Q_B sites are directed toward the aqueous phase, and the ubiquinone pool is freely mobile within the lipid bilayer. As a further benefit, additional liposoluble Q_{10} can be co-reconstituted in controlled quantities. The formation of the entire structure was followed by electrochemical impedance (EIS) and SEIRA spectroscopy because the thin gold film can also be used as an electrode [26].

Light Activation of Reaction Centers (RCs) Followed by SEIRAS

In this configuration, light-minus-dark SEIRA spectra were measured while the RCs were excited by continuous illumination under a halogen lamp first without any additional Q_{10} . The term “light-minus-dark spectra” means that these are difference spectra that use spectra in the dark as references, which are subtracted from the spectra under illumination. A small number of prominent bands appeared, increasing over time in a time scale of minutes (Figure 4).

Figure 4. Light-minus-dark absorption spectra of reaction centers (RCs) encapsulated in the protein-tethered bilayer lipid membrane (ptBLM) taken at 5 min time intervals without added Q₁₀ under continuous illumination. The term “light-minus-dark spectra” means that these are difference spectra using the spectra in the dark as references, which are subtracted from the spectra under illumination. The spectra were recorded every 5 min. Reproduced with permission of Nedelkovski *et al.* (2013) [26].

A steady state was not attained within a time scale of seconds as in previous investigations [27,28]. Nevertheless, the bands shown in our spectra could be correlated with characteristic groups found in previous studies. The tentative assignment of bands is shown in Table 1. The most prominent of these bands (1282 and 1434 cm^{−1}) could be assigned to the oxidized form of the primary electron donor or special pair of the RC, P⁺, and ubihydroquinone, QH₂, respectively, the products of light activation of the RCs. This was a surprising result, because in previous studies, appreciable amounts of QH₂ could only be attained in the presence of an artificial electron donor, such as ascorbate P⁺, as it was reduced back to the reduced form of the special pair, P [29].

Table 1. Tentative band assignments of SEIRAS spectra of RCs encapsulated in the ptBLM recorded under continuous illumination [26]. Reproduced with permission of Nedelkovski *et al.* [26].

Band (cm ^{−1})		Tentative Assignment	
Experimental	Literature	Species	Component
1282	1282	P ⁺	(complex)
1360	1355, 1365	Q _A	−CH ₃
1434	1433	QH ₂	
1643	1640, 1641, 1642 [31]	quinone Q, Q _B	C=O
1685	1682, 1683	9-keto group of P	C=O
3400	3485	Q _B [−] /Q _B or P ⁺	H ₂ O
3629	3632	Q _B [−] /Q _B or P ⁺	H ₂ O

The growth of the band areas was fully reversible, when the light was switched off, as shown in Figure 5B. Most band areas are reduced in the course of the relaxation with the exception of the bands at 1234 and 1685 cm^{−1}, which are restored. This unexpected behavior combines several features characteristic for the SEIRA spectra of the ptBLM. Above all, the general pattern of the spectra is obviously different from the pattern of FTIR difference spectra usually displayed in the literature, which is mostly composed of narrow peaks and troughs [30].

Figure 5. Band areas of the absorbances of characteristic bands of the RCs in the ptBLM under continuous illumination taken at different time intervals with added Q_{10} when the light is switched on (A) and off (B), *i.e.*, during relaxation. Reproduced with permission of Nedelkovski *et al.* (2013) [26].

The effect was even more pronounced when additional Q_{10} was co-reconstituted. The evolution of band amplitudes over time in the presence of additional Q_{10} is shown in Figure 5.

The reduced number of bands in the SEIRA spectra can be explained in terms of the surface selection rule, which comprises the high sensitivity of only those bands whose components are located at a short distance from the surface and the dependency of the sensitivity from the orientation of the transition dipole moments. The sensitivity is higher the more the component is oriented perpendicular to the surface [16,18]. According to this rule it depends on the orientation of a component whether it is strongly represented or not at all. This explains why the same functional group is represented by a certain band whereas other bands are missing. *e.g.*, QH_2 is usually represented by the bands 1434, 1491, 1470 cm^{-1} , but only the band at 1434 cm^{-1} is definitively seen. This effect is amplified by the pre-orientation of the MRP molecules on the surface. Moreover, most of the bands appear to point in one direction, as seen in Figure 2A (CcO) and Figure 3 (RC). In the case of the CcO, this directionality can be explained by the superposition by the large water band. After subtraction, the bands point in both directions. In the case of the RC, all the bands are positive except for the 1685 cm^{-1} band, which is consistent with the fact that they represent products of the illumination. The only exception is the ground state of P, which is an educt. Another specific feature of the SEIRA spectra can be seen in the relatively large full width at half maximum (FWHM) of the bands, as compared to the sharp bands found in FTIR spectra of molecules present on the surface in a random orientation [30]. The FWHM is associated with the freedom of movement of the particular structure represented by the component [32]. In previous studies, proteins were investigated mostly with redox mediators present. In our SEIRAS titration study of cytochrome *c* oxidase, we recorded a marked minimizing of the FWHMs in the presence of mediators [11,13]. This effect might be explained by the difference in internal dipole moments of the activated protein after the redox sites had been equilibrated with the mediators.

Irrespective of these general features of the SEIRA spectra, the slow evolution of species such as P^+ and QH_2 , found in the case of the RC, is unexpected. In previous FTIR work, the release of QH_2 from its binding pocket and its replacement by a quinone molecule from the quinone pool has been observed under the continuous illumination of chromatophores from *R. sphaeroides*, but only in the presence of ascorbate or other compounds to reduce P^+ back to P [29]. The slow generation of species such as QH_2 (and/or Q_BH_2) and P^+ , in the absence of an electron donor, can be explained by the reaction between two excited molecules of the RC.

followed by protonating Q_B^{2-} , releasing QH_2 into the membrane and then rebinding Q_B from the quinone pool. The model is supported by the fact that all of the light-induced SEIRA bands exhibit

similar kinetics. Further support is derived from by the reversal of the original disproportion during relaxation (Figure 5B):

Thus, this study has demonstrated that inter-protein reactions are possible when the MRP is incorporated into the ptBLM. SEIRA spectra were again shown to be dominated by the surface selection rule, whose effects are strongly enhanced by the strict orientation of the protein perpendicular to the surface.

Direct Electron Transfer to Photosynthetic Reaction Centers from *R. sphaeroides*

The RCs were immobilized into a ptBLM as described above, with a genetically engineered 7-his tag at the C-terminus of the M-subunit such that the primary electron donor (the special pair P) was directed toward the gold electrode, in this case a smooth gold electrode. After illumination, P is converted into the oxidized species P^+ . Direct electron transfer (ET) to P^+ was investigated, particularly in the context of generating photocurrents [33].

A very similar system had previously been introduced by the group of Lebedev and Trammel [34–36], who used linker molecules of different lengths to immobilize RCs on gold electrodes via his-tag technology, but without reconstitution into a ptBLM. Photocurrents were recorded in the range of nA after illumination of the RCs, but only when the photocurrents were mediated by cytochrome *c* (cc) and ubiquinone-2 (Q2). In our attempt to repeat these measurements in the presence of the BLM, we found dramatically different behavior. Substantial photocurrents lasting for more than 15 min in the range of μA were obtained in the cathodic direction in the range of negative potentials with an optimum at -200 mV. Photocurrents were even larger in the presence of the ptBLM (Figure 6) but not appreciably affected by the mediators Q2 and cc.

Figure 6. Photocurrents of RCs immobilized on a nitrilo-tri-acetic acid (NTA) modified template stripped gold (TSG) measured before (black) and after (red) formation of the ptBLM, at -200 mV (open squares) and -300 mV (full squares). Green lines are the monoexponential fits of the data. Reproduced with permission of Gebert *et al.* (2015) [33].

These comparatively high photocurrents were explained in terms of the disproportionation reaction (Equation (1)) discussed above. The direct electron transfer was attributed to the species P^+Q_B rather than P^+ . This is consistent with the fact that we did not detect the photocurrents found by Trammel *et al.* at potentials of approximately 0 mV. We conclude that the photocurrents measured at -200 and -300 mV are due to direct ET to the species P^+Q_B . The corresponding oxidation peak is

again between +600 and +650 mV, which can be explained in terms of the electrochemical reduction of P^+Q_B , which restores the ground state of the RC primary donor.

In summary, photocurrents of remarkable magnitude and stability have been generated, amplified by an inter-protein reaction in addition to the usual high energy conversion efficiency in RCs.

2.2. From Flat Surfaces to Particles

Flat surfaces are well designed for the application of electrochemical and surface-enhanced techniques such as surface plasmon resonance, surface plasmon-enhanced fluorescence and vibrational spectroscopies. Localized fluorescence effects, however, are not easily accessible on flat surfaces. Methods such as laser scanning confocal microscopy (LSM) are more suitable for 3D particles. Therefore, we transferred the ptBLM technique to commercially available particles of different sizes from μm down to nm, modified with NTA-terminated linkers. We referred to the obtained structures as proteo-lipobeads (PLBs) (Figure 7).

Figure 7. A schematic view of a proteo-lipobead (PLB). The agarose or silica bead is shown in dark grey, the NTA-terminated linker in yellow and the CcO in red, which is bound to the NTA-linker via a his-tag attached to the cytoplasmically oriented C-terminus of subunit I. The lipid bilayer is shown in light grey. The layered structure is not drawn to scale. Reproduced with permission of Frank *et al.* (2015) [37].

2.2.1. Cytochrome *c* Oxidase from *P. denitrificans* Incorporated in Proteo-Lipobeads (PLBs) Based on Agarose Beads

CcO from *Paracoccus denitrificans* with a his-tag engineered to the C-terminus of the subunit I was bound to agarose beads (HisPur Ni-NTA Resin, 50–150 μm , Thermo Scientific, Schwerte, Germany), followed by *in situ* dialysis in the presence of lipid micelles to form PLBs [37].

The formation of the lipid layer was demonstrated by LSM, employing fluorescence-labeled lipids such as 1,2-dihexadecanoyl-sn-glycero-3-phospho-(*N*-4-nitrobenz-2-oxa-1,3-diazolyl)ethanolamine (NBD-PE) and the potential-sensitive dyes di-8-ANEPPS (di-8-butyl-amino-naphthyl-ethylene-pyridinium-propyl-sulfonate) and the red-shifted dye di-4-ANBDQBS (4-(1-[2-(di-n-butylamino)-6-naphthyl]-4-butadienyl)-1-(4-butylsulfonate) quinolinium betaine) (Figure 8). Hemicyanine-based membrane probes, such as di-8-ANEPPS and di-4-ANBDQBS, fluoresce strongly when bound to BLMs whereas they exhibit negligible fluorescence in aqueous solution [38]. Figure 8 illustrates a BLM layer with regular fluorescence intensity around the bead.

In the configuration above, the cc binding site of the CcO is pointing toward the outside of the PLB. Hence proton transport can be initiated by photoactive electron donors such as the Ru complex Ru_2C ($[(\text{bpy})_2\text{Ru}(\text{diphen})\text{Ru}(\text{bpy})_2](\text{PF}_6)_4$), which after light-activation acts as an electron donor, bound to the cc binding site. Ru_2C was mixed with aniline as a sacrificial electron donor and 3CP (3-carboxy-PROXYL) to prevent proton release from the aniline [39]. The pH changes

at the outer surface of the PLB were detected by fluorescein DHPE (1-(8-((3',6'-dihydroxy-3-oxo-spiro(isobenzofuran-1(3H),9'-(9H)xanthen)-5-yl)amino)-3-hydroxy-8-thioxo-2,4-dioxo-7-aza-3-phosphaoct-1-yl)-1,2-ethanediyl ester, P-oxide), a sensor molecule that incorporates into the distal leaflet of the lipid bilayer. The decrease in fluorescence intensity indicates a decrease in pH in the outer solution. The fluorescence intensities were measured after continuous illumination with a halogen lamp with and without a mixture of Ru₂C, 3CP and aniline. Fluorescence intensities decreased to an extent of 10% and 42% of the initial value in the buffered and unbuffered KCl solution, respectively. No decrease was observed in the presence of valinomycin and FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone), *i.e.*, under uncoupling conditions. The intensity decreased to 55% of the initial value with only valinomycin present, which is explained by the collapse of the membrane potential (Figure 9). This behavior is in accordance with an active transporter controlled by the membrane potential.

Figure 8. Confocal laser scanning microscopy (LSM) images of PLBs based on agarose beads. PLBs are labeled with the potential-sensitive dye di-4-ANBDQBS (4-(1-[2-(di-n-butylamino)-6-naphthyl]-4-butadienyl)-1-(4-butylsulfonate) quinolinium betaine). Reproduced with permission of Frank *et al.* (2015) [37].

Figure 9. Change in the relative fluorescence intensity of membrane-bound pH-sensitive fluorescein DHPE ((1-(8-((3',6'-dihydroxy-3-oxo-spiro(isobenzofuran-1(3H),9'-(9H)xanthen)-5-yl)amino)-3-hydroxy-8-thioxo-2,4-dioxo-7-aza-3-phosphaoct-1-yl)-1,2-ethanediyl ester, P-oxide) over time, before and after continuous illumination with a halogen lamp. (A) Comparison of unbuffered (40 mM KCl) (blue) and buffered (35 mM KCl, 5 mM Tris-HCl) (red) PLB solutions, each of them containing Ru₂C ([([bpy]₂Ru(diphen)Ru(bpy)₂](PF₆)₄), 3CP (3-carboxy-PROXYL) and aniline to enable light excitation, and buffered PLB solution without the Ru₂C, 3CP and aniline (black); (B) Change in the relative fluorescence intensity of pH-sensitive membrane bound fluorescein DHPE over time, before and after continuous illumination with a halogen lamp with Ru₂C, 3CP and aniline in a 40 mM KCl solution (blue), in the presence of valinomycin alone (red) and with both valinomycin and FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone) (black). The pH was adjusted to 8 before all measurements. Reproduced with permission of Frank *et al.* (2015) [37].

2.2.2. Cytochrome *c* Oxidase from *P. denitrificans* Incorporated in PLBs Based on Silica Nanoparticles

The size of the agarose beads (50–150 μm) described above, is very well suited for the application of LSM. However, they are not designed for the application of optical spectroscopy because they would suffer from serious interferences by light scattering of the particles. Therefore, we used commercially available 25 nm sized Ni-NTA-modified silica nanoparticles (NPs) to prepare PLBs designed for UV/Vis measurements [40].

Particle size was measured by dynamic light scattering in the course of PLB preparation, starting with the NPs before and after CcO binding and after incorporation of the lipid bilayer. The size distribution increases regularly from 25 to 40 and 50 nm, for the NPs, the proteobeads and the PLBs, respectively (Figure 10). This increase is in accordance with crystallographic data for CcO, which predicts a height of 12 nm in the upright position [41].

Figure 10. Particle size of PLBs, measured by dynamic light scattering of Ni-NTA-functionalized nanoparticles (NPs) (black), after CcO binding (red) and after dialysis (blue). Reproduced with permission of Schadauer *et al.* (2015) [40].

UV/Vis spectra of CcO solubilized and CcO encapsulated in PLBs, before and after reduction with dithionite are shown in Figure 11. Light scattering effects are indicated by a sloping baseline, particularly at lower wavelengths. However, there is no interference of light scattering with the spectroscopic bands used for the determination of the concentrations of CcO, obtained using an extinction coefficient $\epsilon_{606-630\text{ nm}}^{\text{reduced-oxidized}} = 23.4\text{ mM}^{-1}\cdot\text{cm}^{-1}$ [42].

Figure 11. UV/Vis spectra of solubilized CcO (A) and PLB-bound CcO (B) oxidized with potassium ferricyanide (solid line) and reduced with sodium hydrosulfite (dashed line), respectively, at a CcO concentration of 1.1 μM . Reproduced with permission of Schadauer *et al.* (2015) [40].

The activity of the incorporated CcO was assessed using a model based on the Michaelis-Menten equation. To this end, the reaction of the enzyme with reduced cc was followed, using solubilized CcO as well as the protein incorporated in proteobeads and PLBs. The measurements of the PLBs were also performed with 0.5 μM valinomycin and 1.2 μM FCCP present. The initial rates plotted against the cc concentration were fitted to the Michaelis-Menten equation. Fit parameters were the turnover number, k_{cat} , and the substrate affinity, K_{M} . The kinetic constants are collected in Table 2.

Table 2. Kinetic parameters derived from Michaelis-Menten kinetics for CcO incorporated in PLBs. Reproduced with permission of Schadauer *et al.* (2015) [40].

Parameter	CcO, Solubilized	CcO, in Proteobeads	CcO, in PLBs	
			−Val./FCCP	+Val./FCCP
K_{M} (μM)	5.8 ± 0.4	1.6 ± 0.4	2.6 ± 0.4	2.7 ± 0.6
k_{cat} (s^{-1})	264.1 ± 7.4	32.4 ± 0.5	11.9 ± 0.8	33.4 ± 8.8
Number of preparations		3	3	3
Number of measurements	3	3	3	3

$k_{\text{cat}} = 264 \text{ s}^{-1}$ of solubilized CcO was found to reflect literature values [43]. It was found to decrease to a $k_{\text{cat}} = 32$ and 11 s^{-1} for proteobeads and PLBs, respectively. k_{cat} of CcO incorporated in PLBs recovered in the presence of valinomycin and FCCP to $k_{\text{cat}} = 33 \text{ s}^{-1}$. The reduction of $k_{\text{cat}} = 32 \text{ s}^{-1}$ was considered as being due to the immobilization of the proteins. The further reduction to $k_{\text{cat}} = 11 \text{ s}^{-1}$ within the PLBs was considered in terms of the lipid membrane. This conclusion seems justified in view of $k_{\text{cat}} = 33 \text{ s}^{-1}$ found under uncoupling conditions. From these values, the respiratory control ratio (RCR), was calculated to be 2.8. This is well in accordance with the RCR of 2.7 observed in proteo-liposomes [44]. The binding constant $K_{\text{M}} = 5.8 \mu\text{M}$ of solubilized CcO was found to agree with literature data [43]. It decreased to 1.6 and 2.6 μM for proteobeads and PLBs, respectively. Summarizing, CcO within the PLBs suffers from a drastic decrease in activity, however, exhibiting improved binding properties.

3. Conclusions

These results demonstrate that the ptBLM structure is useful as a tool for biosensing. The main advantage of the ptBLM over traditional systems such as solubilized proteins or proteoliposomes is the strict orientation of the proteins embedded in the lipid bilayer. Other methods may be feasible in this context, such as electrostatic or antibody binding. Antibodies, however, usually have much larger dimensions compared with the combined his-tag and NTA-linkers. Hence, the proteins under investigation would be located outside the region of the evanescent wave of the EM field and thus would not be amenable to surface enhancement techniques such as SEIRAS and SERRS. Larger distances between the electrode and protein would also seriously hinder direct ET, which is relevant, for example, in the context of photocurrents. Electrostatic binding, on the other hand, is not as specific as his-tag binding. Moreover, purified proteins are now generally available genetically engineered with his-tags.

A method particularly well designed for use with ptBLMs is SEIRA spectroscopy. Electrochemical and light activation can be easily applied in a periodic fashion so that high quality S/N ratios can be easily obtained in tr-FTIR measurements. The surface selection rule, which works in favor of certain vibrations while others are silenced, presents a drawback. This feature, however, may actually be an advantage if the orientation of certain secondary structures is an aspect of interest. In any case, conformational changes in secondary structures with time can be observed, which is an important issue, particularly because time-dependent X-ray spectroscopy is not yet available.

The ptBLMs may also be considered for biosensing MRP inhibitors. They are frequently applied as herbicides because of their function as inhibitors, for example against complex III of the respiratory chain. There is high interest in biosensing these compounds with high sensitivity and selectivity.

The ptBLMs are not only significant in the context of biosensing but also in the context of basic research. We have strong evidence that under the conditions described here, the natural pathway of electrons all the way through the protein is maintained, particularly in the case of the CcO.

As for the methods to be applied, ptBLMs on flat surfaces allow the application of external electric fields and surface sensitive techniques such as surface plasmon resonance, surface plasmon-enhanced fluorescence and vibrational spectroscopies. PLBs based on spherical particles, on the other hand, allow for fluorescence microscopy and classical UV/Vis measurements. Fluorescence methods applied to PLBs open the way to bioassays for screening purposes including high-throughput screening. Hence, a whole range of methods is available to study MRPs under nearly physiological conditions.

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Electronic Olfactory Sensor Based on *A. mellifera* Odorant-Binding Protein 14 on a Reduced Graphene Oxide Field-Effect Transistor

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Abstract: An olfactory biosensor based on a reduced graphene oxide (rGO) field-effect transistor (FET), functionalized by the odorant-binding protein 14 (OBP14) from the honey bee (*Apis mellifera*) has been designed for the *in situ* and real-time monitoring of a broad spectrum of odorants in aqueous solutions known to be attractants for bees. The electrical measurements of the binding of all tested odorants are shown to follow the Langmuir model for ligand–receptor interactions. The results demonstrate that OBP14 is able to bind odorants even after immobilization on rGO and can discriminate between ligands binding within a range of dissociation constants from $K_d = 4 \mu\text{M}$ to $K_d = 3.3 \text{ mM}$. The strongest ligands, such as homovanillic acid, eugenol, and methyl vanillate all contain a hydroxy group which is apparently important for the strong interaction with the protein.

Odorant-binding proteins (OBPs) are small acidic proteins (ca. 13–16 kDa) highly concentrated in the lymph of the chemosensillae of insects or in the nasal mucus of vertebrates.^[1] They act as carriers for volatile organic compounds, VOCs, (air-borne odorants) shuttling them from the air–water interface to the membrane-integral odorant receptor. Although their full function has not been completely clarified

yet, OBPs certainly play a major role in detecting and recognizing olfactory stimuli.^[2]

A large number of OBPs has been expressed in bacterial systems and their ligand-binding properties have been investigated in solution by a fluorescent ligand displacement assay.^[3] Generally, dissociation constants, K_d , are in the upper nanomolar or lower micromolar range for strong odorants.^[1c,d] An important characteristic of OBPs for technical applications is their stability to extreme temperatures, solvents, and proteolysis, making such proteins ideal elements for biosensors to be used in medical application, for example, in breath analysis for cancer diagnostics, for food quality control, for crop-disease detection, or in general environmental monitoring.^[4a–f]

Various biosensor devices mimicking the olfactory system (artificial noses) have been developed but only few studies have used OBPs as functional elements for the design of a “bio-electronic nose”.^[5] Herein we present the fabrication and functional characterization of a label-free biosensor for odorant detection based on a reduced graphene oxide field-effect transistor functionalized with the odorant-binding protein 14 (OBP14) from the honey bee *Apis mellifera*.

Reduced graphene oxide field-effect transistor (rGO-FET) devices were fabricated according to established methods, schematically given in Figure 1A.^[6] A scanning electron microscopic image of rGO flakes assembled onto the gate substrate before any further surface functionalization is shown in Figure 1B. IR spectra of the linker monolayer, 1-pyrenebutanoic acid succinimidyl ester (PBSE), that is typically used for protein immobilization on graphene substrates,^[7] with partial covalent immobilization of OBP taken at different times during the assembly from solution to the gate are given in Figure 1C, while Figure 1D summarizes the quantitative analysis of the functionalization process by monitoring the time-dependent increase of the amide I and II bands of the protein and the corresponding decrease of the band at 1738 cm^{-1} , assigned to the cleavage of the active ester group of the linker molecules. The entire fabrication process of the olfactory biosensor device, including the successful reduction of GO to rGO, the linker binding, and more details of the protein attachment are described in the Supporting Information. The cloning, expression, and purification of OBPs, the preparation of odorant solutions and the performance of the electrical measurements are also described in the Supporting Information.

The mode of operation of the device as a field-effect transistor is demonstrated in Figure S5. Recording the I_{SD} versus V_G scans under different bulk solution conditions, in

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Figure 1. A) Schematic illustration of the individual fabrication steps of the graphene biosensor device. B) Scanning electron microscopic image of the rGO-FET before the functionalization with 1-pyrenebutanoic acid succinimidyl ester (PBSE) linker. C) Infrared spectra of the PBSE linker attached to gate area of the rGO surface, and OBP14 immobilized for 5 and 35 min, respectively, as indicated (spectra measured in ATR configuration and water corrected). D) Time-dependent increase of the amide I and II bands, upon binding of OBP14 to the linker molecules at the gate surface, and the corresponding decrease of the band at 1738 cm⁻¹ upon cleavage of the active ester of the linker molecules during the protein immobilization (cf. also Figure S6). The dashed red curves are guides to the eye.

particular, in aqueous solutions with different ligand concentrations results in a slightly modified slope of the cathodic branch. We attribute this change to a slight modification of the dipolar layer upon binding of the ligands to the free binding sites in the OBP which act as receptors. The OBP protein monolayer is immobilized on the graphene gate and ligand binding causes a slight reorientation of its alpha-helical parts. At the selected gate voltage of $V_G = -0.6$ V the concentration dependency of the slopes in the I_{SD} versus V_G curves could be used to measure the binding of odorants to OBP14 in real-time, resulting in the quantitative determination of the kinetic rate constants for the association process, k_{on} , and for the dissociation process, k_{off} as well as for

the affinity constant K_A and the dissociation constant K_d . As an example, Figure 2 A shows a global analysis measurement, that is, the time dependence of the change of the source-drain current, ΔI_{SD} , of the FET as a function of time upon binding of methyl vanillate, a strong binder, from solution to the OBP14-functionalized gate surface at increasing and decreasing odorant bulk concentrations. The error of the kinetic rate

Figure 2. A) Real-time biosensor measurement of the binding of methyl vanillate to OBP14: the current increases with the bulk concentration of methyl vanillate increasing (from 100 nM to 200 μM) and then saturates. Blue arrows indicate runs with pure buffer, red arrows indicate experiments with methyl vanillate solutions. Red curves are the fitting of the raw data by kinetic simulations of the association and dissociation processes based on the Langmuir model. (For the estimation of the error limits, see the Supporting Information). B) Analysis of the reaction rate constants, k , obtained from the fitted data in (A) as a function of methyl vanillate concentration; different symbols from three different devices; error bars are ±20%. C) Langmuir adsorption isotherm, obtained for three different samples; the red fit curve gives $K_d = 20 \mu M$ (plotted are also error limits for K_d of 20%).

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constants, k , obtained was estimated to be around 20 % (cf. also Figure S8 in the Supporting Information).

Upon plotting the resulting reaction rate constants k , as they were derived from the fits to the kinetic traces as a function of the bulk ligand concentration, $c_0 = c_{\text{Methyl vanillate}}$ (Figure 2B), gives a straight line, the slope of which, according to $k = k_{\text{on}}c_0 + k_{\text{off}}$ with k_{on} being the association and k_{off} the dissociation rate constant, respectively, yields $k_{\text{on}} = 235 \text{ M}^{-1} \text{ s}^{-1}$, and the intersection with the y-axis gives $k_{\text{off}} = 0.01 \text{ s}^{-1}$. According to the Langmuir model the ratio $k_{\text{on}}/k_{\text{off}}$ gives the affinity constant $K_A = 2.3 \times 10^4 \text{ M}^{-1}$, which can be compared to the value derived from the equilibrium titration experiment given in Figure 2C: according to $\theta = K_A c_0 / (1 + K_A c_0)$ the fit to the data (full red curve plus the error limits of $\pm 20\%$) gives the affinity constant $K_A = 5 \times 10^4 \text{ M}^{-1}$ which compares quite well with the value obtained from the kinetic experiments thus confirming the Langmuir model for this binding process.

To exclude false signals arising from, in particular, non-specific binding, several control experiments were performed. Firstly, a sensor was prepared which was covered only with linker molecules, without the coupling of the odorant-binding protein. Even high concentrations of the strong binder homovanillic acid (cf. Figure S7A) or other ligands (Figure S7B) did not result in a significant signal. Coating the gate with OBP14 but exposing it to a totally uncorrelated small molecule, biotin, gave no signal (Figure S7C). And finally, immobilizing a protein (OBP9A from the red flour beetle, *Tribolium castaneum*) that is structurally similar but is not a receptor for these ligand odorants also gave a very weak signal (Figure S7D).

Further evidence for the specificity of the ligand receptor binding originates from a direct comparison of the binding of eugenol and methyl eugenol to the same chip, functionalized with OBP14. Figure 3A shows a real-time current trace recorded during the addition of methyl eugenol at $50 \mu\text{M}$, then rinsing with pure buffer, and then a $5 \mu\text{M}$ solution of eugenol being rinsed through the flow cell. Despite the minor chemical variation between the two ligands a large difference in the affinity of their binding reaction to OBP14 confirms the specificity of the sensor. Figure 3B summarizes the Langmuir isotherms for both ligands, demonstrating the significantly

Figure 3. A) Real-time sensor response to the injection of a $50 \mu\text{M}$ solution of methyl eugenol, subsequent buffer wash, and the injection of $5 \mu\text{M}$ eugenol solution. B) Langmuir isotherms of eugenol and methyl eugenol.

different dissociation constants: $K_d = 40 \mu\text{M}$ for eugenol and $K_d = 1400 \mu\text{M}$ for methyl eugenol.

Table 1 summarizes the quantitative data measured for a series of ligands and gives the kinetic rate constants, k_{on} and k_{off} , obtained as well as the dissociation constant K_d . It could be confirmed also for this label-free sensing format that the odorant binding affinity to OBP14 gradually decreases from homovanillic acid to citral and to geraniol.^[8] Molecules which are structurally related to eugenol also showed strong affinities to OBP14.

Table 1: Dissociation constants, K_d , association rate constants, k_{on} , and dissociation rate constants, k_{off} , for a variety of odorants binding to OBP14 as obtained by the global analysis.

Odorant	Homovanillic acid	Methyl vanillate	Eugenol	Citral	Methyl eugenol	Geraniol
structural formula						
$K_d [\times 10^{-6} \text{ M}]$	4	20	40	800	1400	3300
$k_{\text{on}} [\text{M}^{-1} \text{ s}^{-1}]$	1130	235	170	9	6	3
$k_{\text{off}} [\text{s}^{-1}]$	0.008	0.01	0.006	0.003	0.006	0.008

Interestingly, also other odorant-binding proteins bind their odorants with affinities in the same order of magnitude.^[9] It is remarkable to note that for all the ligands investigated the dissociation rate constants k_{off} , differ by less than a factor of 2.5. The strongly differing affinity constants, varying by nearly three orders of magnitude, can be almost exclusively attributed to the differences in the association rate constants k_{on} , (cf. Table 1).

The Spinelli group used X-ray diffraction analysis to determine the structure of the eugenol-OBP14 complex. They found that the hydroxy group of eugenol interacts with the cavity wall of the OBP14 binding pocket by forming two hydrogen bonds.^[10]

It has been speculated that the hydroxy group, together with the substituted aromatic backbone, plays a key role for the strong binding which is in agreement with the high affinities also of homovanillic acid and methyl vanillate (cf. Table 1).^[10] This hypothesis is further supported by the lower affinity measured for methyl eugenol, in which the hydroxy group is replaced by a methoxy group.

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Supporting Information

Electronic Olfactory Sensor Based on *A. mellifera* Odorant-Binding Protein 14 on a Reduced Graphene Oxide Field-Effect Transistor

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1 Materials and Methods

All reagents were purchased from Sigma-Aldrich unless otherwise indicated and used without further purifications.

1.1 Cloning and purification of OBPs

Odorant binding proteins (OBP14) were expressed in bacterial systems using established protocols.^[1] Purification of the proteins was accomplished using a combination of conventional chromatographic techniques followed by a final gel filtration step on Superose-12 (GE-Healthcare) as previously described in standard protocols.^[2] The purity of the proteins was checked by SDS-PAGE and a size of ~14.0 kDa was determined for OBP14 (cf. Figure S1).^[1b]

Figure S1. Bacterial expression and purification of OBP14 wt of *A. mellifera*. Purification was accomplished by anion-exchange chromatography on DE-52, followed by a second step on QFF, or gel filtration on Sephacryl-12, as indicated. Molecular weight markers (M) are, from the top, 66, 45, 29, 20 and 14 kDa. Pre: bacterial pellet before induction; I: bacterial pellet after induction with IPTG.

1.2 Preparation of odorant solutions

Odorants were prepared as 5 mM stock solutions in phosphate buffer (1 mM PBS, pH = 8.0) with an addition of 0.01 % ethanol (purity ≥ 99.9 %) and further diluted (from 10^{-3} M to 10^{-7} M) by successive dilutions in PBS buffer. All solutions were freshly prepared on the day of the experiment.

A broad spectrum of ligands was tested known as typical pollen odorants like the benzenoids Eugenol (4-Allyl-2-methoxyphenol) and Methyl eugenol (1,2-Dimethoxy-4-prop-2-enylbenzene).^[3]

Moreover, a few floral volatiles, known to be attractive for bees, were tested. These include Citral ((2E)-3,7-dimethylocta-2,6-dienal) and Geraniol ((2E)-3,7-Dimethylocta-2,6-dien-1-ol), which were also identified as Nasonov pheromone compounds. In addition, two honey flavours, namely Homovanillic acid (2-(4-Hydroxy-3-methoxy-phenyl)acetic acid) and Methyl vanillate (Methyl 4-hydroxy-3-methoxybenzoate) were also tested.

1.3 Setup

Figure S2. Schematic illustration of the graphene based FET biosensor device with gold source and drain electrodes and an Ag/AgCl gate electrode. The area between the gold electrodes is covered by OBP14 proteins immobilized with a bifunctional linker. Eugenol can be captured into the binding pocket of the protein.

Figure S3. Electrical connections and flow inlet and outlet are realized by a customized polymer block.

1.4 Characterization of the electrical performance of the devices

Electrodes were applied consisting of gold (60-100 nm) with an adhesive layer of chrome (2-3 nm) using a standard evaporation process with a shadow mask. A chip-holder was designed for this process, ensuring the central positioning of the electrodes as well as reducing electrode geometry glazing because of unwanted shadow offset during the evaporation process. Success of the used reduction technique via hydrazine was probed before (Larisika et al., 2012), so resistance of the fabricated devices was measured for check-up of graphene-oxide reduction quality using a Fluke Multimeter "87 V True RMS Multimeter". To assure conductivity of the used electrolyte not being higher than the conductivity of used graphene layers, all devices displaying higher conductivity than ~800 ohm were dismissed, equivalent to the resistance of 170 mM PBS buffer at room temperature. A silver-silver chloride reference electrode (Flex ref, World Precision Instruments) was used to operate the FET device in liquid gate configuration with a constant gate bias (V_g) of -0.6 V and a constant source-drain bias (I_{SD}) of 0.05 V. The general procedure of the whole titration experiment started with continuously flushing the detection area with pure buffer (1 mM PBS, pH = 8.0), until a stable baseline of drain current was established.

2 Fabrication process of OBP-based biosensor device

2.1 FET fabrication

Silicon substrates with a 300 nm oxide layer were chosen as basal layer for the FETs. The SiO_2 substrates were cleaned with a standard RCA cleaning procedure. The substrates were then submerged in a 2% aminopropyltriethoxysilane (APTES) solution in ethanol for 1 h, APTES forming a self-assembled monolayer used to increase the adsorption of graphene oxide sheets. After rinsing with ethanol, the substrates were heated to 120°C for two hours and afterwards cooled to room temperature. Graphene oxide sheets were prepared using a variation of the Hummers method derived for the application on FETs.^[4] The obtained graphene oxide flakes were applied onto the Si-Wafer via drop casting of the top portion of the graphene-oxide solution. The devices were then treated in hermetically sealed glass petri dishes with hydrazine at 70°C overnight to accomplish the graphene-oxide reduction, forming the graphene structure consisting of sp^2 -hybridized bonds. Flake distribution was first checked with an optical microscope and selected devices then characterized using SEM.

2.2 Reduced Graphene Oxide Gate

The entire fabrication process of the olfactory biosensor device, including successful reduction of GO to rGO, linker binding and protein attachment was carefully examined using Raman and Fourier transform infrared (FTIR) spectroscopy. Raman measurements were done using a Horiba LabRAM HR Raman confocal microscope (a laser with a wavelength of 532 nm (52 mW, 10% filter) and a laser spot size of $0.41 \mu\text{m}^2$ was used).

FTIR Microscope measurements were performed with 256 scans using a Bruker Hyperion 3000 Microscope. For Raman and FTIR Microscope measurements, silicon wafers were used as substrates. Samples were prepared as described previously.^[4] In order to follow the protein immobilization, FTIR measurements were done using a Bruker Vertex 70 V FTIR Spectrometer and a multiple reflection silicon ATR crystal (angle of incidence $\Theta = 45^\circ$, 12.5 active reflections). Spectra were recorded every 5 minutes with a spectral resolution of 4 cm^{-1} in double-sided acquisition mode; the mirror velocity was set to 80 kHz.

The Raman spectrum of GO shows two prominent bands at 1342 cm^{-1} (D-Band) and 1581 cm^{-1} (G-Band) and three smaller bands at 2680 cm^{-1} (2D Band), 2930 cm^{-1} (D+G Band) and 3230 cm^{-1} (2D' Band), respectively (cf. Figure S4).

Figure S4. Raman spectra of graphene oxide (GO) and reduced graphene oxide (rGO).

Table ST1. Band positions of Raman spectra.

Band	Graphene oxide		Reduced grapheme oxide	
	Experimental [cm ⁻¹]	Literature [cm ⁻¹]	Experimental [cm ⁻¹]	Literature [cm ⁻¹]
D	1342	1341 ^[4]	1338	1341 ^[4]
G	1581	1595 ^[4]	1573	1595 ^[4]
2D	2680	2700 ^[5]	2671	2726 ^[6]
D+G	2930	2950 ^[5]	2927	2960 ^[5]
2D'	3230		3215	3220 ^[5]

After reduction, the G and 2D bands are shifted to lower wave numbers. The D/G ratio of GO increased from 0.9 to 2.1 after the reduction process. This can be explained by an increase of sp² domains in the carbon.^[7] Table ST1 compares band positions of Raman spectra of GO and rGO with literature data.

2.3 Operation of the FET device

The mode of operation of the device as a field-effect transistor is given in Figure S5. In the I_{SD}-vs-V_G scan one can clearly distinguish between the two ambipolar branches typical for graphene FETs: the cathodic scan which is dominated by the hole mobility and the anodic scan determined by the electron mobility. The Dirac voltage is seen at ca. V_G = 0.4 Volts. Recording these I_{SD}-vs-V_G scans at different bulk solution conditions, in particular, in aqueous solutions of different ligand concentrations results in a slightly modified slope of the cathodic branch which we attribute to a slight modification of the dipolar layer with the OBP protein monolayer immobilized onto the graphene gate surface upon partial binding of the ligand to the free binding sites in the OBP acting as receptors. Figure S5 shows this effect for different Eugenol concentrations ranging from 100 nM to 250 μM. Therewith we selected a negative gate voltage of V_G = -0.6 V for all ligand binding measurements.

Figure S5. Current-voltage characteristics of the OBP14 based biosensor device without Eugenol (PBS buffer) and with 100 nM - 250 μM Eugenol.

2.4 Hydrolysis of the active ester (PBSE linker)

Figure S6 shows FTIR measurements of the immobilized PBSE linker, incubated in PBS buffer (A). The bands of the active ester (1738 , 1781 , and 1815 cm^{-1} , respectively) decrease as a function of time because of spontaneous hydrolysis. Figure S6 B shows the exponential decrease of all three bands. For the attachment of the protein to the sensing area, the graphene surface was chemically modified by a bi-functional linker, 1-pyrenebutanoic acid succinimidyl ester (PBSE). On one end the linker firmly

Figure S6. (A) Hydrolysis of the active ester in PBS leads to a decrease of the bands at 1738 , 1781 and 1815 cm^{-1} , respectively. (B) The area of the 1738 , 1783 , and 1812 cm^{-1} band, respectively, decrease as a function of time. The dashed red curve guides the eye.

attaches to the graphene surface through π - π interactions with a pyrene group and on the other hand covalently reacts with the amino group of the protein to form an amide bond. Therefore, 20 μL of a 5 μM PBSE solution in Tetrahydrofuran was placed onto the rGO-FET channel. Fast evaporation of Tetrahydrofuran allowed immediate deposition of 30 μL of a 10 μM OBP14 solution in PBS buffer (1 mM ; $\text{pH} = 8.0$) onto the detection area and incubated for 2 h at 4°C . Loosely or unbound linker/OBP14 was ensured by rinsing the detection area with same PBS buffer. The entire fabrication process of the olfactory biosensor device, including successful reduction of GO to rGO, linker binding and protein attachment was carefully examined using spectroscopic methods, as described in chapter Fabrication process of OBP-based biosensor device.

3 Control measurements

Figure S7 shows different types of control measurements that were performed in order to demonstrate that the response signals obtained by the olfactory biosensor are specific and thus clearly assignable as protein-ligand interactions rather than non-specific interaction of the tested odorants with the rGO surface. Firstly, rGO-FET devices were fabricated and functionalized as described above but without any odorant binding protein (OBP) immobilized. Figure S7 A demonstrates that after addition of different concentrations of a phenolic structured odorant like Homovanillic acid, no sensor signal could be observed. Similar observations were made for other ligands (Figure S7 B).

Figure S7. (A) Real-time response of PBSE-functionalized rGO-FETs to different concentrations of Homovanillic acid. (B) ΔI_{SD} vs. time measurement of PBSE-functionalized rGO-FETs to high concentrations (mM range) of different odorants. (C) Real-time response of OBP14-functionalized biosensor to different concentrations of a nontarget ligand, i.e., biotin. (D) Real-time response of an OBP9-functionalized biosensor to different Eugenol concentrations.

Figure S7 C demonstrates that introducing a small but unnatural ligand like biotin to the OBP14 functionalized sensor surface, does not show a distinct response signal. And finally, after immobilizing an odorant binding protein from a different species, namely OBP9 from *Tribolium castaneum* to the rGO surface, different concentrations of Eugenol do not cause a detectable change in the source-drain-current, whereas this odorant proved to be an extremely strong ligand to OBP14 from the honeybee (cf. Fig. 7 D).

In conclusion, all negative control experiments have clearly demonstrated that although it seems likely that π -orbitals of phenolic compounds like Eugenol or Homovanillic acid are easily stacked on the rGO surface similar to pyrene derivatives (e.g., PBSE), the sensor response signals clearly reflect the

affinity between OBPs and their ligands and is not caused by unspecific interactions of odorants with the rGO surface.

4 Measurements of all odorants

In the following Figures S9 – S13, individual real-time biosensor measurements of the binding of different ligands (one example each) to OBP14 are summarized: In each case, (A) shows the current increase upon increasing the bulk concentration of the ligands in solution (red arrows). Blue arrows indicate runs with pure buffer.

Figure S8. On the determination of the rate constant, k , and its error limits.

An example of how we determine the error limits of the rate constants is given in Figure S8. Shown are the experimental data of the current change, ΔI_{SD} , after injection of a 30 μM solution of Methyl vanillate into the flow cell, together with 3 fit curves: red, $k=0.01872 \text{ s}^{-1}$, dark blue with $k'=k+20\%$, light blue, $k''=k-20\%$. This error limit is added to all rate constants in the following figures.

(B) Plotting the reaction rate constants as derived from the fits to the kinetic traces (full red curves shown in (A)) as a function of the respective bulk ligand concentration, $c_0=c_{\text{ligand}}$, (for all ligands given in (B)) one obtains a straight line, the slope of which yields k_{on} , and the intersection with the y-axis gives k_{off} , respectively. According to the Langmuir model the ratio $k_{\text{on}}/k_{\text{off}}$ gives the affinity constant K_A . The obtained values for all ligands are indicated. (C) According to $\theta=K_A c_0/(1+K_A c_0)$ one obtains from the fit to the equilibrium surface coverage, θ , ($=\Delta I_{SD}/\Delta I_{SD,\text{max}}$) (full red curve) the affinity constant K_A , which typically compares quite well with the value obtained from the kinetic experiments thus confirming the Langmuir model for this binding process. Obtained affinity constants are given for each.

Figure S9. Homovanillic acid

Obtained affinity constants:

Figure S9 A $k_{off} = 0.008\ s^{-1}$; $k_{on} = 1126\ M^{-1}\ s^{-1}$; $K_d = 7.1 \times 10^{-6}\ M$
 Figure S9 B $K_d = 4 \times 10^{-6}\ M$

Figure S10. Eugenol

Obtained affinity constants:

Figure S10 A $k_{off} = 0.006 s^{-1}$; $k_{on} = 170 M^{-1} s^{-1}$; $K_d = 3.5 \times 10^{-5} M$
Figure S10 B $K_d = 4 \times 10^{-5} M$

Figure S11. Citral

Obtained affinity constants:

Figure S11 A $k_{off} = 0.0033 \text{ s}^{-1}$; $k_{on} = 8.9 \text{ M}^{-1} \text{ s}^{-1}$; $K_d = 3.1 \times 10^{-4} \text{ M}$
Figure S11 B $K_d = 8 \times 10^{-4} \text{ M}$

Figure S12. Methyl eugenol

Obtained affinity constants:
Figure S12 A $k_{off} = 0.0058\ s^{-1}$; $k_{on} = 5.9\ M^{-1}\ s^{-1}$; $K_d = 1.1 \times 10^{-3}\ M$
Figure S12 B $K_d = 1.4 \times 10^{-3}\ M$

Figure S13. Geraniol

Obtained affinity constants:

Figure S13 A $k_{off} = 0.0078 \text{ s}^{-1}$; $k_{on} = 2.5 \text{ M}^{-1} \text{ s}^{-1}$; $K_d = 3.25 \times 10^{-3} \text{ M}$

Figure S13 B $K_d = 3.3 \times 10^{-3} \text{ M}$

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