Optimization of Interfaces for Genosensors Based on Thiol Layers on Gold Films

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CONTENTS

1. INTRODUCTION

The design and assembly of sensors for the detection of proteins or DNA is the basic prerequisite for many applications, including identification of pathogens, gene expression monitoring, diagnosis of genetic disorders, forensic or pharmaceutic applications as well as product and food control [Wolfbeis, 1991], [Patolsky et al., 1999], [Berney et al., 2000]. By definition, a biosensor is an analytical device that combines the specificity of a biological sensing element (the *receptor*) with a *transducer* to produce a signal proportional to target analyte concentration [Junhui et al., 1997].

The transducers may be based on mass changes [Okahata et al., 1998], acoustic wave formation [Zhang et al., 1998], electrochemical [Palecek et al., 1998], [Marrazza et al., 1999] or optical [Piunno et al., 1994] events. The biosensors may be prepared in the form of electrodes, chips, or crystals.

Sensor chips are a combination of an inorganic support (like glass or silicon) and biological material. In order to combine these two components, an anchoring layer is necessary which, on the one hand, is attached to the inorganic support and, on the other hand, allows the immobilization, i.e. the tethering of the receptor to this solid support. There are various possibilities to couple biological material to inorganic supports.

One frequently used method is the application of alkanethiol molecules as the joining material between inorganic substrates and organic substances. Thiols (R-SH), as well as other sulfur-containing compounds, are able to form a self-assembled monolayer (SAM) on gold [Bain et al., 1989a], [Nuzzo et al., 1990], [Finklea, 1996] and various other metals [Bryant and Pemberton, 1991], [Mebrahatu et al., 1988].

This property can be used to prepare an anchoring layer showing the characteristics mentioned above, consisting of two very thin layers: a film of metal, for example gold, sputtered onto the silicon or glass support, serving as an inorganic basis for the monolayer of alkanethiols.

In order to get areas with different properties (e.g. hydrophilic and hydrophobic regions or domains where different biological substances can be selectively immobilized at the same sensor chip), one possibility is to use thiols with differing functional tail groups, forming monolayers of mixed composition. Several methods have been described in the literature to obtain mixed monolayers or monolayers with patterns, e.g. by coadsorption [Hayes et al., 1997], [Yang et al., 1997] or photolithographic techniques using UV-irradiation [Gillmor et al., 2000], or by micro-contact printing [Kumar and Whitesides, 1993], [He et al., 2000].

Once the SAM of alkanethiols is formed (as a uniform or a mixed monolayer), it is possible to use the tail groups of the thiols, pointing away from the gold film, to immobilize organic substances (the "receptor") via chemical reactions. These techniques are described for a wide range of different organic substances [Hermanson, 1995]. However, immobilization of DNA will be the only focus of this work.

There is a special interest in the use of DNA as the biological recognition element of affinity biosensors because of the ability of a single stranded DNA molecule (ssDNA) to "seek out", or hybridize to, its complementary strand with high selectivity.

All these techniques mentioned above are meant to lead to a tethering of organic substances to the surface, and once the biological receptor like DNA (or proteins) are immobilized, the sensor chip is ready for detection.

However, in order to build up a sensitive and selective biosensor, several obstacles (e.g. defects in the thiol layer, suboptimal immobilization conditions, or nonspecific adsorption of receptor or target) have to be overcome. In this interdisciplinary thesis (involving chemistry, physics, and biology), most of these problems will be solved by optimization. This is illustrated especially for genosensors (DNA biosensors) and a capacitance-based detection method. In the following short overview, the separate steps of optimization will be depicted:

At first, in chapter 2, the composition of capacitance-based genosensors will be presented and it will be shown where the crucial points of maintaining high quality can be found. Then (in chapter 3) the methods utilized in this thesis will be introduced.

Capacitance-based detection methods, as will be pointed out in chapter 2, require densely packed alkanethiol layers at the surfaces of electrodes. The improvement of the thiol layer on metal surfaces like gold or palladium utilizing a heating procedure as an electrode pretreatment will be shown in chapter 4 (for gold: cf. chapter 4.1, for palladium see chapter 4.2).

The quality of a (DNA-)biosensor depends on the efficiency of the immobilization technique. However, it is not very easy to accomplish perfect immobilization of the receptor: properties of the solution in which the immobilization is performed influence not only the coupling reagent itself but also the receptor. In order to achieve a good coupling of the biological substances to the inorganic support covered by thiols, there are two possible starting-points for optimization which are studied here.

The first is, that an unnecessary loss of coupling reagent has to be prevented. A loss of coupling reagent in unwanted side reactions decreases the yield of the desired reaction. To study this loss, a technique will be shown (cf. chapter 6) for monitoring the concentration

of EDC (used here as a coupling reagent). With the help of this technique, the effects of electrolyte components on EDC concentration are determined.

The second is, that the immobilization conditions have to be optimized for each special application (e.g. for DNA biosensors on gold electrodes) in order to increase the yield of chemically attached molecules. A study of immobilization conditions and washing procedures to remove nonspecifically adsorbed oligomers will be presented in chapter 7. This study applies an alternative detection method based on scintillation counting, the results of which are compared to those obtained after immobilization, according to the protocols of two companies producing biosensors.

In addition to the two problems concerning immobilization mentioned above, there is the problem of nonspecific, physical adsorption of DNA at the surface of biosensors which can occur not only during immobilization but also during the actual analysis. Nonspecifically adsorbed molecules lead to a perturbation of the measurements. Especially label-free methods like capacitance-based methods (as well as many others, like the use of a quartz crystal microbalance, QCM), are sensitive to them. These labe-free methods detect dielectric thickness (or mass) of specifically immobilized molecules and of nonspecifically adsorbed ones, implicating the risk of misinterpretation of signal changes. Hence, the prime goal must be to minimize the amount of nonspecifically adsorbed molecules, as described in chapter 5.

The method illustrated therein focuses on building up a mixed self-assembled-monolayer (mixed SAM) of alkanethiols with different functional tail groups, one kind of them providing the possibility of a coupling between thiol and DNA-oligomer, the other kind preventing nonspecific adsorption of DNA molecules.

As a summary, the results of chapters 4 to 7 will be integrated in chapter 8 and the optimized conditions for the surface preparation of genosensors in combination with a capacitance-based detection method will be offered.

2. PRINCIPLES

The optimization of immobilization conditions for DNA biosensors (genosensors) can be useful in various detection methods. The focus of this thesis will be on capacitance-based measurements. The following chapter will therefore demonstrate in detail the detection principle of these kinds of measurements. The coupling reaction by which the receptor molecules (DNA-oligomers) are tethered to the surface will be shown, as will be possible problems during assembly of a capacitance-based genosensor.

2.1. CAPACITANCE-BASED MEASUREMENTS

In the case of biosensors based on capacitive measurements, the capacitor is formed by a combination of the working electrode, a second, virtual plate of the capacitor consisting of the electrolyte itself and between them, a dielectric layer formed by alkanethiols (cf. chapter 2.2).

The capacitance (C) of the layers of alkanethiols and other organic molecules depend on several parameters (eq. 2.1): the surface (S) of the working electrode covered by the layer, the thickness (d) of the dielectric layer, and the relative dielectric constants (ε_r) of the molecules forming the layer (alkanethiols and other molecules); ε_0=8.9 $\cdot$ 10^{-12} C/Vm is the permittivity of free space.

(2.1) $$C=\frac{S}{d}\varepsilon_r\varepsilon_0$$

On applying an AC voltage $u(t)$ between two electrodes (here: the working electrode and the reference electrode of a capacitance-based

biosensor), the result is a current *i*(*t*), shifted in phase compared to the applied voltage (Fig. 2.1). This behavior can be represented in general by a circuit consisting of a resistor and a capacitor in parallel. The capacitor and the resistor here correspond to capacitive and conductive properties of the layers of alkanethiol or other organic molecules.

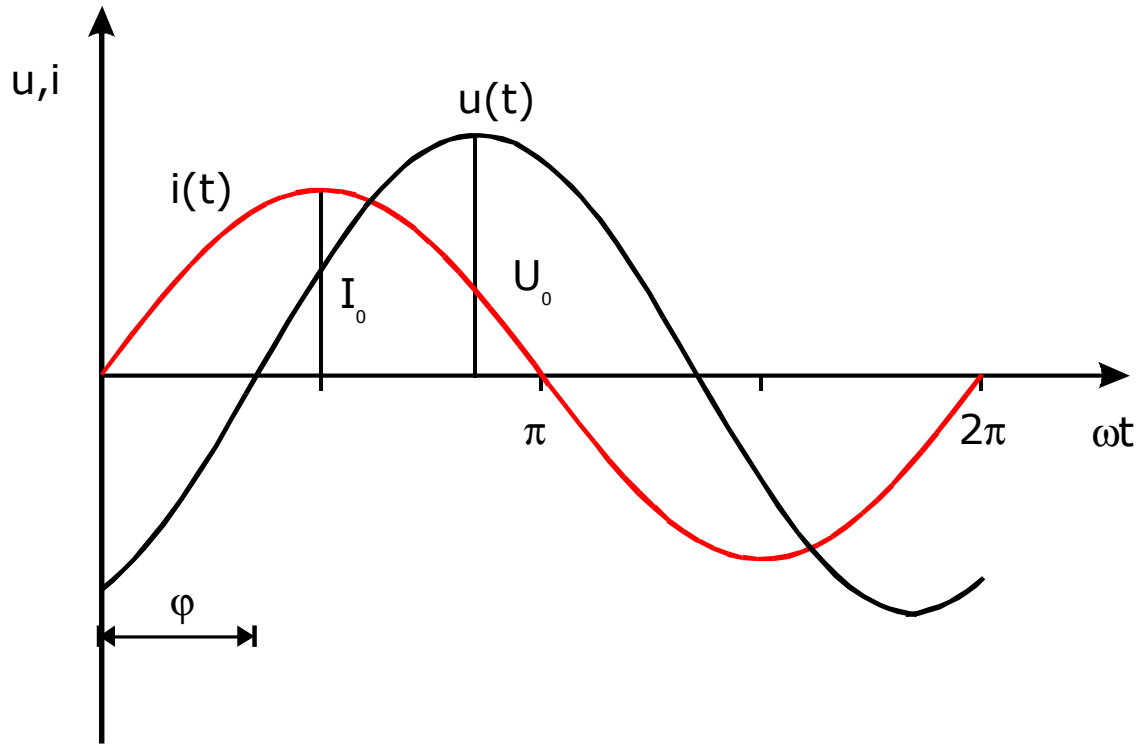


Fig. 2.1. Phase shift φ between applied voltage u(t) and resulting current i(t) for a parallel arrangement of a resistor and a capacitor.

The impedance (*Z*) is the virtual resistance of the whole system and can be described in complex coordinates (Fig. 2.2). The reciprocal impedance, 1/*Z*, is the admittance and Re(1/*Z*) equals to the conductivity (the reciprocal resistance, 1/*R*) of the system and Im(1/*Z*) is proportional to the capacitance [Lindner et al., 1986].

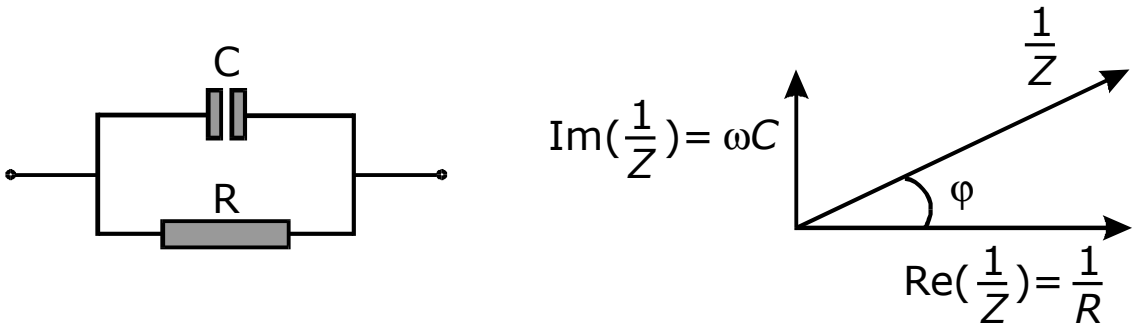


Fig. 2.2. Admittance (reciprocal value of the impedance) (1/*Z*) of a parallel arrangement of a resistor and a capacitor in complex coordinates. The real part, Re(1/*Z*), corresponds to the reciprocal value of the resistance (1/*R*), the imaginary part, Im(1/*Z*), is proportional to the capacitance. φ corresponds to the phase shift between applied voltage u(t) and resulting current, i(t), cf. Fig. 2.1.

Purely capacitive behavior of a biosensor hardly ever occurs. This implies that the influence of the resistance is still present and not negligible. The goal of optimization here would be to diminish the influence of the ohmic resistance. Presuming that the phase angle $\varphi = 90°$, thus implying that $R = \infty$, an ideal capacitor without loss at an ohmic resistance would be given.

2.2. IDEAL ASSEMBLY OF CAPACITANCE-BASED BIOSENSORS

The detection principle of capacitance-based biosensors utilizes a capacitor formed by a dielectric layer (composed of organic substances) between two charged surfaces. Modifications of this dielectric layer, for example by adsorption of molecules, lead to capacitance changes which are detected.

Fig. 2.3 shows the formation of such a capacitor with a dielectric layer consisting of a self-assembled monolayer (SAM) of alkanethiols. At

first, two electrodes, one working electrode (gold) and one reference electrode (e.g. Ag/AgCl), are immersed into an electrolyte. An electric potential is applied between these two electrodes, the gold electrode being positively charged.

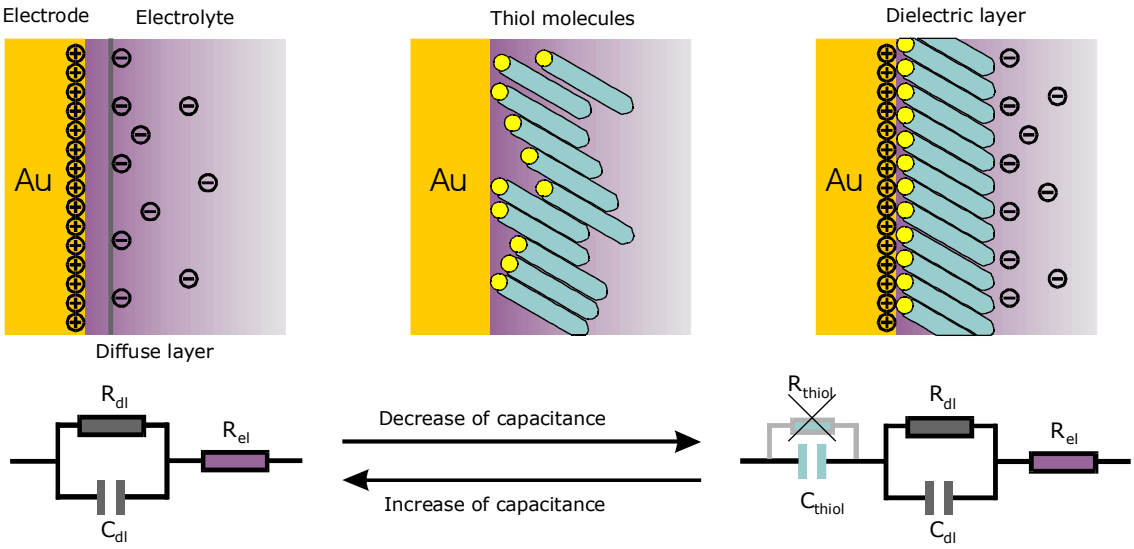


Fig. 2.3. Formation of a diffuse electric double layer out of the bulk solution of the electrolyte by applying a positive potential at the gold electrode. After self assembly of an alkanethiol layer the diffuse layer of the negative charge is shifted further away from the gold electrode. The additional, dielectric layer of alkanethiols reduces the total capacitance of the whole arrangement. The first circuit chart in this simplified model is a combination of capacitance and resistance of the ionic double layer (C_{dl}) and (R_{dl}) in parallel combined with the resistance of the electrolyte (R_{el}) represented by a resistor in series. After formation of the thiol layer, the second chart describes the situation. In addition to the combination of capacitor and resistors of the ionic double layer and the electrolyte, a capacitor for the thiol layer is inserted (C_{thiol}), which causes the total capacitance to decrease. Considering the thiol layer as defect-free, faradayic currents and therefore its resistance (R_{thiol}) can be neglected. When thiols desorb from the gold surface, the total capacitance increases.

Anions gather in the bulk solution near the positively charged gold electrode until they form a diffuse layer of negative charge. A sort of steady state is reached between the attraction of the positively charged gold film and the rejection of other anions. This diffuse layer represents

the second, virtual plate of the capacitor. The electric properties of the diffuse layer can be described in a simplified model by a circuit chart: a capacitor and a resistor in parallel. The capacitance of this electric double layer (C_{dl}) depends on several parameters, for example electrode potential, temperature, ionic concentration, types of ions, oxide layers and electrode roughness. R_{el} signifies the resistance of the electrolyte.

By adding alkanethiols into the electrolyte they form a SAM with insulating properties at the gold surface (cf. Fig. 2.4). The attachment is a result of the strong affinity of the sulfur containing thiol head group for the gold surface, while the organization results from the favorable interactions between close-packed alkane chains (van der Waals forces) [Finklea, 1996]. The alkane chains are tilted with an angle of inclination in the range of 20 to 30 degrees. This is the case as the packing of the sulfur atoms on the gold substrate is less dense than the packing of the alkane chains that would optimize interchain van der Waals attractive forces (5 vs. 4.4 Å nearest neighbor distances). By tilting, the three-dimensional density of the monolayer is increased [Bensebaa et al., 1998].

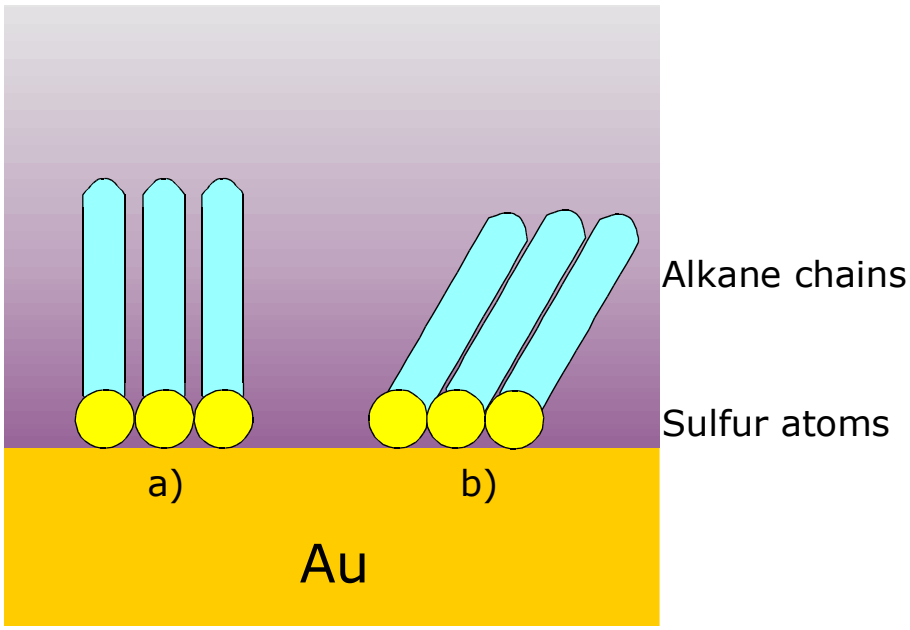


Fig. 2.4. Packing of alkanethiols on gold. The packing of the sulfur atoms is less dense than the packing of the alkane chains (a). The alkane chains optimize their van der Waals interchain attractive forces by tilting (b) with an angle of inclination of about 20 to 30 degrees. The three-dimensional density is increased.

By insertion of the dense layer of alkanethiols, the negatively charged electric layer is shifted away from the gold electrode, leading to a decrease of the capacitance of the system (cf. eq. 2.1). The circuit chart can be completed by an additional capacitor (C_{thiol}) in series, which summarizes the capacitive properties of the thiol layer. Assuming that the dielectric insulating layer of the alkanethiols is ideally formed (without defects), no current through the thiol layer (faradaic current) is observed and so the resistance of the thiol layer (R_{thiol}) can be neglected.

Once the self-assembly of the alkanethiols is completed, further layers of molecules can be immobilized at the surface, for example, receptors like proteins or DNA strands, which are meant to recognize

the target molecules in an assay. This immobilization process, or coupling, is performed by a chemical reaction (cf. chapter 2.4).

Fig. 2.5 shows such a coupling of biological molecules to the thiol layer. The ideal result is a defect-free monolayer of immobilized molecules.

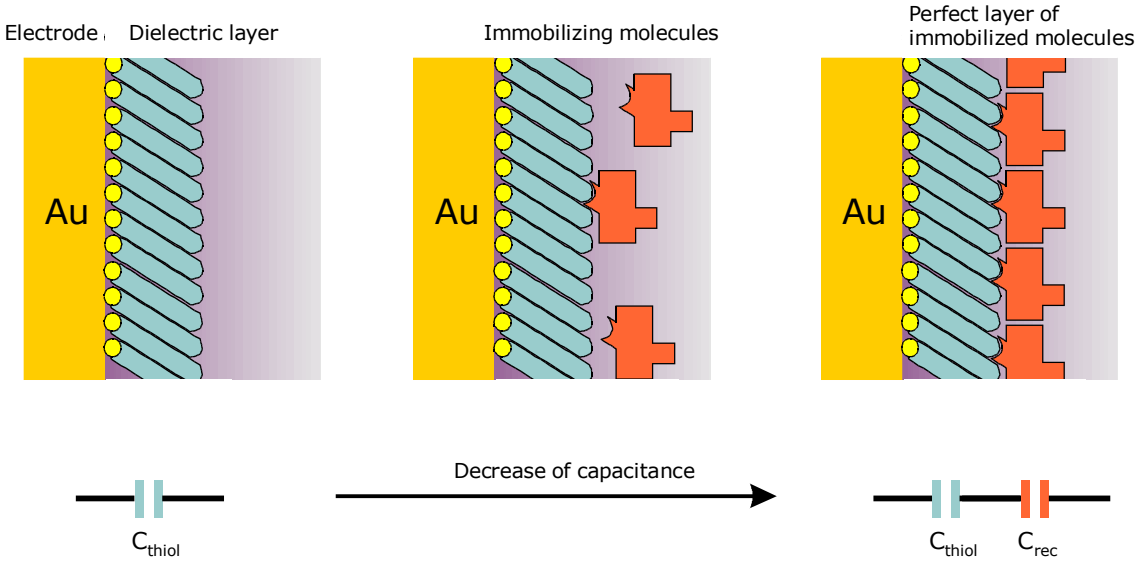


Fig. 2.5. Immobilization of receptor molecules such as proteins or DNA at an ideal, defect-free alkanethiol monolayer. The resulting layer of biological material is also considered to be defect-free. Then the ohmic resistances of the resulting layers are very large and the circuit chart of the layers can be simplified to a combination of two capacitors (C_{thiol} and C_{rec}) in series.

In this simplified model only the capacitive properties of the alkanethiol layer (an ideal case without the influence of an ohmic resistance – cf. chapter 2.1) are considered (first picture in Fig. 2.5). All other influences are neglected and the circuit charts can be further simplified now only showing the capacitance of the dielectric layer.

Similar considerations are correct for an ideal, defect-free monolayer of immobilized biological molecules (third picture in Fig. 2.5). The situation is described by two capacitors in series, one summarizing the purely capacitive properties of the alkanethiol layer (C_{thiol}) and the

other those of the layer of immobilized receptors (C_{rec}). The total capacitance (C_{total}) of the whole arrangement decreases while the biological substances are immobilized, according to the law for serially arranged capacitors:

$$\frac{1}{C_{total}} = \frac{1}{C_{thiol}} + \frac{1}{C_{rec}} . \qquad (2.2)$$

2.3. REAL SITUATION

In the last section only ideal conditions were considered. In reality, the situation is quite different, there are a lot of possible disturbances as for instance defects in the thiol layer, suboptimal conditions for immobilization, nonspecific adsorption of DNA (or proteins). The influences of these disturbances on capacitive measurements will be discussed in the following.

At first, the thiol layers are seldom perfect in reality [Diao et al., 2000] (Fig. 2.6). Therefore, not only purely capacitive current but also conductive current occurs (i.e. $R < \infty$). As a consequence of these defects of the thiol layer, the influences of ion adsorption, electrolyte activity or of redox active substances are no longer negligible [Riepl, 2000]. The results of the measurements are altered by each of these effects. So, in order to get undisturbed capacitive measurements, one goal is to reduce the defects in the thiol layer and thus get an approximation to the ideal situation. This will be the subject of chapter 4.

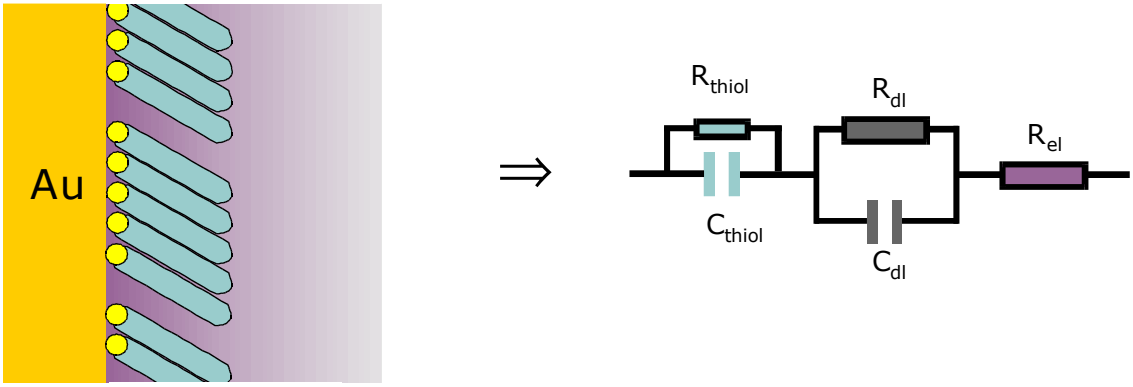


Fig. 2.6. Actual defects in the thiol layer cause conductive currents which disturb the results of capacitive measurements. Then R_{thiol} is no longer negligible.

Further problems can occur during immobilization, even if the monolayer of alkanethiols is defect-free.

The conditions of immobilization may not be optimal for the coupling reagent and/or for the molecules to bind to the surface (cf. Fig. 2.7 a). Both facts could lead to a lower yield of immobilized molecules. So the immobilization conditions have to be tested and optimized. This will be presented in chapter 6 and 7.

Another problem is that of nonspecific adsorption. Biological molecules like proteins or especially DNA, tend to attach to various surfaces, e.g. to gold but also to other surfaces. This nonspecific adsorption gives false positive results and therefore has to be diminished by appropriate methods. These methods are described in chapter 5 and 6.

In the following, the effects of nonspecifically adsorbed molecules and imperfect immobilization on the value of the measured capacitance will be identified. In Fig. 2.7 b immobilized and nonspecifically adsorbed receptors are schematically gathered.

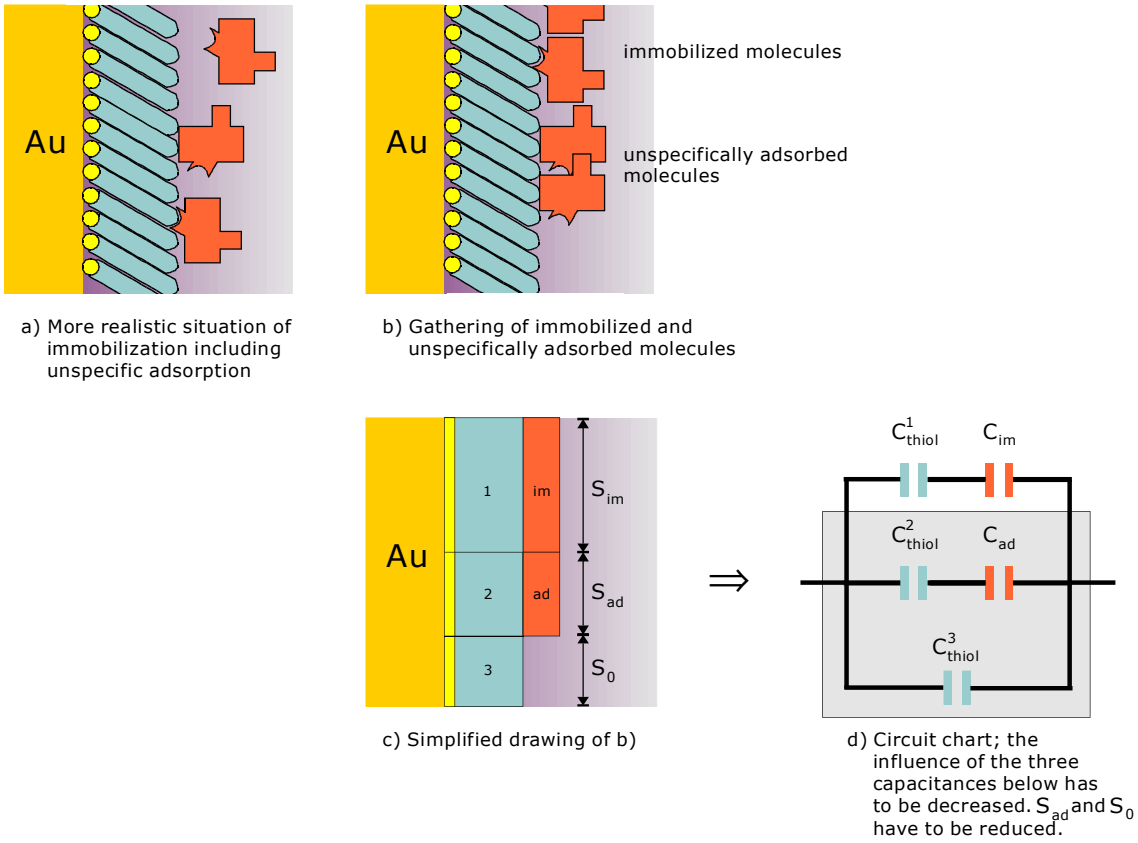


Fig. 2.7. (a) Problems which can occur during immobilization are nonspecific adsorption (in the middle of chart a) and not optimized conditions for binding of molecules to the surface (above in chart a). For a quantitative consideration, the immobilized and nonspecifically adsorbed molecules are schematically gathered (b and c) and a circuit diagram for this situation is given (d).

The capacitance of the whole system can be described as a combination of five capacitors (Fig. 2.7 d): one capacitor for the uncovered thiol layer (C^3_{thiol}) in parallel to two sets of serially arranged capacitors. One set summarizes the properties of the thiol layer covered by *immobilized receptors* (C^1_{thiol} and C_{im}) and the other (C^2_{thiol} and C_{ad}) represents the properties of the thiol layer covered by *nonspecifically*

adsorbed receptors. The capacitances of Fig. 2.7 can be calculated with equation 2.1. as

(2.3 – 2.7) $$C_{im}=\frac{S_{im}}{d_{im}}\varepsilon_{r(rec)}\varepsilon_0\,,\; C_{ad}=\frac{S_{ad}}{d_{ad}}\varepsilon_{r(rec)}\varepsilon_0\,,$$

$$C^1_{thiol}=\frac{S_{im}}{d_{thiol}}\varepsilon_{r(thiol)}\varepsilon_0\,,\; C^2_{thiol}=\frac{S_{ad}}{d_{thiol}}\varepsilon_{r(thiol)}\varepsilon_0\,,\text{ and }\; C^3_{thiol}=\frac{S_0}{d_{thiol}}\varepsilon_{r(thiol)}\varepsilon_0\,,$$

with d_{thiol} being the thickness of the thiol layer as well as d_{im} and d_{ad} the thicknesses of the layers of immobilized and nonspecifically adsorbed receptors, $\varepsilon_{r(thiol)}$ and $\varepsilon_{r(rec)}$ are the relative dielectric constants of alkanethiols and receptors ($\varepsilon_{r(thiol)}$ = 2 - 3 [Kuchling, 1989], $\varepsilon_{r(rec)}$ = 4 - 20 for proteins [Beck, 1999] and $\varepsilon_{r(rec)}$ = 20 - 400 for DNA [Brabec et al., 1997]). S_{im} and S_{ad} are the surfaces covered by immobilized and nonspecifically adsorbed receptors and S_0 is the surface free of receptors.

Equation 2.2 together with the equation for capacitors arranged in parallel

(2.8) $$C_{total}=C_1+C_2+...+C_n$$

the total capacitance of the whole system in Fig. 2.7 d amounts to

(2.9) $$C_{total}=\frac{C^1_{thiol}C_{im}}{C^1_{thiol}+C_{im}}+\frac{C^2_{thiol}C_{ad}}{C^2_{thiol}+C_{ad}}+C^3_{thiol}$$

which can be simplified to the following equation:

(2.10) $$C_{total}=C^1_{im}+C^2_{ad}+C^3_{thiol}$$

with $C^1_{im}=\frac{C^1_{thiol}C_{im}}{C^1_{thiol}+C_{im}}$ and $C^2_{ad}=\frac{C^2_{thiol}C_{ad}}{C^2_{thiol}+C_{ad}}$.

As the value ε_r for immobilized and nonspecifically adsorbed receptors is identical (the molecules are the same), only the different

surfaces covered and the thickness of the layers are responsible for the magnitude of C_{im}^{1} and C_{ad}^{2}.

For bioanalytical applications of the biosensor, only the density of coverage by immobilized molecules is important, i.e. the ratio of $S_{im} : S_{total}$. It is therefore a prime goal to lessen the amount of nonspecifically adsorbed molecules, that is to reduce S_{ad}, in order to diminish false positive results. Furthermore, S_0 has to be decreased.

Now it is clear that prevention of nonspecific adsorption and the optimization of the immobilization are crucial points for capacitive measurements. All of the problems mentioned above (defects in the thiol layer, suboptimal immobilization conditions, nonspecific adsorption of receptor or target molecules) affect the results of label-free methods, including capacitive-based detection methods.

2.4. IMMOBILIZATION REACTIONS

A common way for immobilization of receptors to the surface of a biosensor is the linkage of a carboxy group and an amino group to form a peptide bond. Those two moieties can be introduced at the end of the thiol and at the (accordingly modified) DNA, respectively. The coupling reaction is mediated by a carbodiimide, e.g. 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide (EDC) [Zhang et al., 1991], often used together with a succinimide, e.g. the likewise water-soluble N-hydroxy-sulfosuccinimide (S-NHS) [Hermanson, 1995] Various protocols to perform such kind of immobilization have been developed.

Also other possibilities for immobilization are described in the literature. In the following, a short overview is given.

One (less direct) possibility is to immobilize (strept-)avidin at the SAM, making use of the immobilization technique mentioned above, and

utilize biotinylated DNA oligomers which are captured by the protein at the surface [Junhui et al., 1997]. This method has the advantage of a high binding capacity between (strept-)avidin and biotin [Yang et al., 1997]. The disadvantage is the necessity of one additional experimental step. Another, very direct technique is to modify the DNA oligomer by a thiol linker which can be used for direct binding to the metal surface in order to form self-assembled layers. Despite the attractive nature of this approach, thiol modified DNA oligomers probably cannot form densely packed layers, due to the large hydrophilic nucleic acid group and therefore the stability of the layer is questionable, especially at higher temperatures [Yang et al., 1997].

In this work, two methods for immobilization were used. For most of the experiments, the water soluble zero-length crosslinker EDC alone was used. Only in some experiments of chapter 7.4, a two-step reaction of EDC in combination with the likewise water-soluble compound sulfo-NHS (*N*-Hydroxysulfosuccinimide) was performed.

The reactions are simple and can proceed in aqueous solution at room temperature, which is important especially for manipulations with biological material. The two reaction principles used here are demonstrated in Fig. 2.8 and Fig. 2.9. In the first approach, a carbodiimide reacts with the carboxyl group of the first molecule to form a highly reactive *O*-acylurea which then reacts with nucleophiles such as primary amines to form an amide (peptide) bond. An urea component is formed as the by-product of the crosslinking reaction (Fig. 2.8).

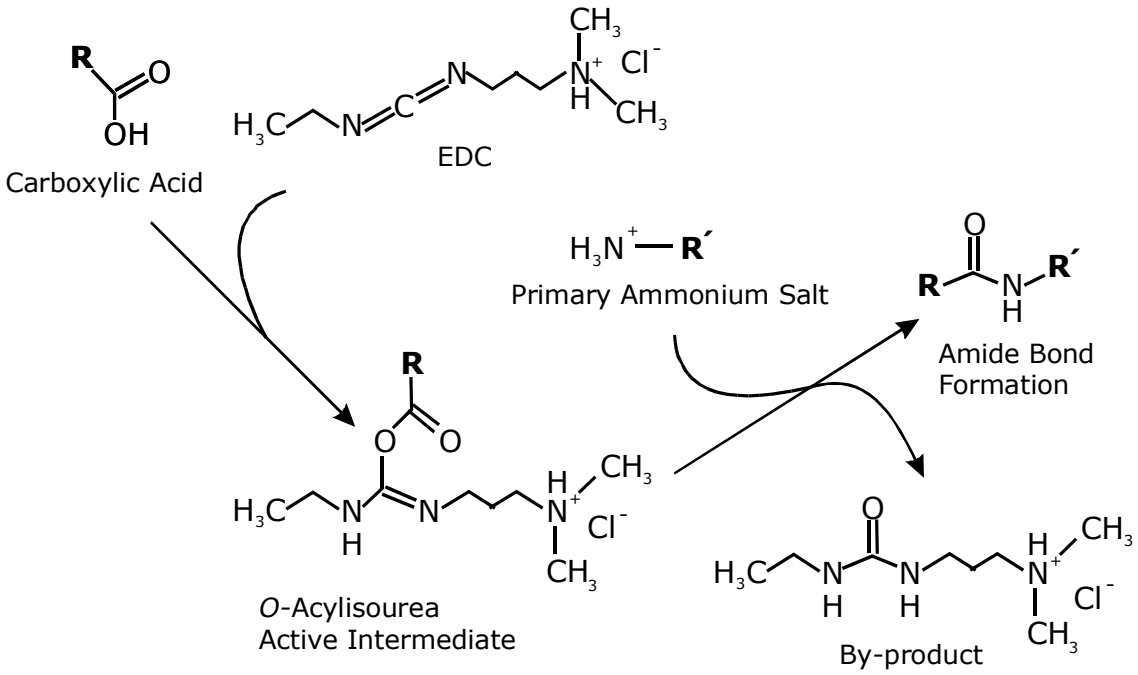


Fig. 2.8. Reaction of EDC with a carboxylic acid to create an active-ester intermediate. In the presence of an amine nucleophile, an amide bond is formed and an urea by-product is released. After [Hermanson, 1995].

In the second approach (Fig. 2.9), EDC is used to form active ester functional groups with carboxylate groups using sulfo-NHS. Sulfo-NHS esters are hydrophilic active groups that react rapidly with amines on target molecules [Staros, 1982], [Jennings and Nicknish, 1985].

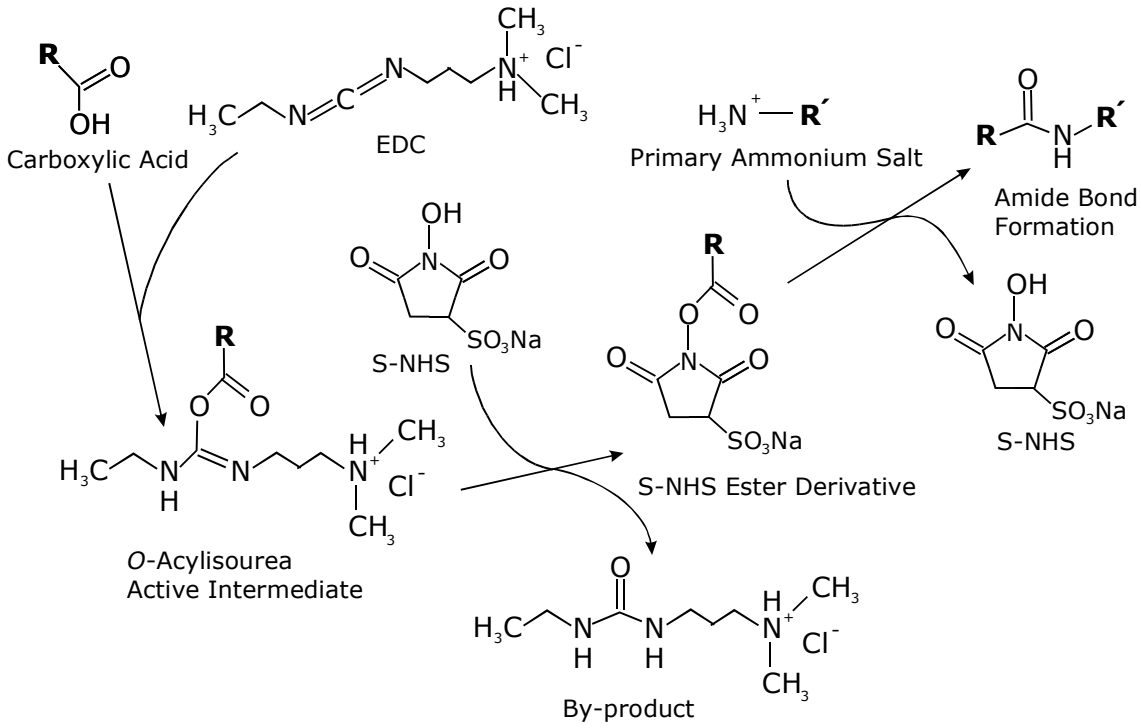


Fig. 2.9. Reaction of EDC with a carboxylic acid followed by the formation of a sulfo-NHS ester intermediate. The sulfo-NHS ester survives in aqueous solution longer than the active ester formed from the reaction of EDC alone with a carboxylate. In the presence of an amine nucleophile, an amide bond is formed. After [Hermanson, 1995].

The advantage using sulfo-NHS esters which are long-lived and hydrolyze relatively slowly in water is that the stability of the active intermediate is increased. However, in the presence of amine nucleophiles that can attack at the carbonyl group of the ester, the *N*-hydroxysulfosuccinimide group rapidly leaves, creating a stable amide linkage with the amine [Hermanson, 1995].

3. MATERIALS AND METHODS

In chapter 2, the principles of capacitance-based genosensors and possible problems during assembly and measurement were explained. In chapter 3, the materials and devices used for capacitance measurements with gold or palladium, for the scintillation counting experiments, and for UV spectroscopy will be presented.

3.1. CAPACITIVE MEASUREMENTS

All electrodes used in this work for capacitive experiments (discussed in chapters 4 and 5) were prepared at the Department of Microelectronics, Faculty of Electrical Engineering, Slovak University of Technology. The design of the electrodes for capacitance measurements is shown in Fig. 3.1.

A thin film (150 nm in thickness) of gold is sputtered onto a 3 mm x 10 mm x 0.3 mm silicon support covered on the surface by an insulating layer consisting of silicon dioxide. Titanium and palladium (50 nm in thickness each) serve as adhesion agent between those two layers of silicon dioxide and of gold. The working part of the gold electrode is a square of 2.4 mm^2 connected via a wire of 10 μm to a soldering pad where the electrodes can be contacted. Palladium electrodes appeared identical but there was no adhesion layer between the layers of silicon dioxide and palladium.

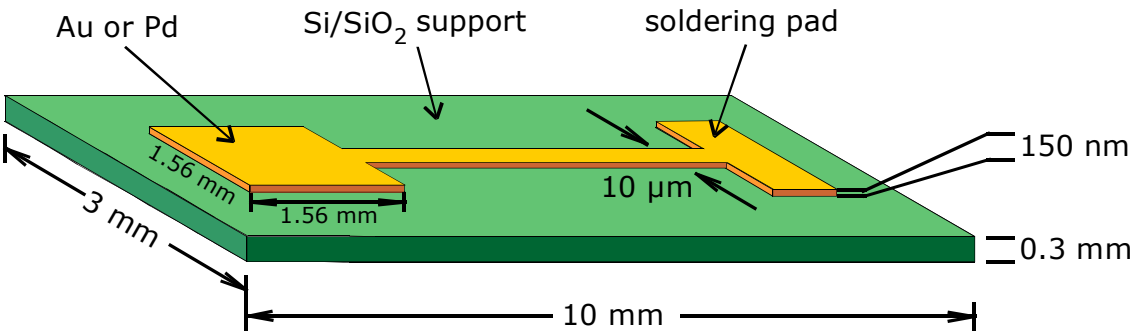


Fig. 3.1. Design of gold or palladium electrodes used for capacitive measurements.

Water was purified in an ion-exchanger purification train (> 18 MΩ with low organic content; Millipore plus 185).

For the preparation of the self-assembled monolayers (SAMs) of thiols, the following substances were used: 11-mercaptoundecanoic acid, 11-mercaptoundecanol and 16-mercaptohexadecanoic acid, purchased from Aldrich and used as received. 16-Mercaptohexadecanol was synthesized by Thomas Hirsch at our institute (unpublished results).

DNA-oligomers with or without modification by amino groups (combined with a C_6-spacer) were received from MWG Biotech or Interactiva as lyophilisates. Before use, they were dissolved in water or buffer. The sequence for the amino-modified DNA-oligomer was 5´-GCA AAG GGT CGT ACA CAT CAT CAT (C_6-spacer)-NH_2-3´, for the unmodified strand 5´-GCA AAG GGT CGT ACA CAT CAT CAT-3´, for the complementary strand 5´-ATG ATG ATG TGT ACG ACC CTT TGC-3´ and for a DNA-oligomer with 3 mismatches 5´-ATG ATG TTG CGT ATG ACC CTT TGC-3´.

All other chemicals were purchased from Merck and used as received. The coupling reagent EDC (1-ethyl-3-(3-dimethylamino-propyl)carbodiimide) was bought from Sigma and stored at -18 °C.

A steel microliter syringe (Hamilton) was used to perform additions to the measuring chambers (1.5 mL Eppendorf cups).

For the measurements a setup was used as shown in Fig 3.2. The setup consists of a combination of a DC and an AC generator, a current amplifier, a lock-in amplifier (Stanford DSP 850 or PAR 121), a x-t recorder and/or a PC, and a thermostat (Haake).

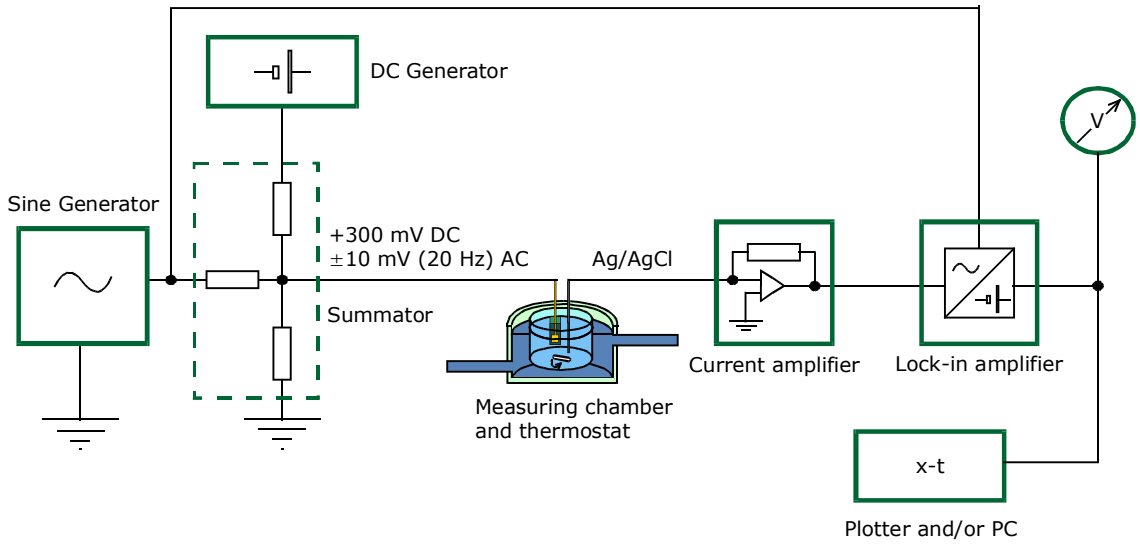


Fig 3.2. Setup for capacitive measurements. The working electrode is provided with a DC voltage of +300 mV (vs. Ag/AgCl) and a sine voltage of 10 mV, 20 Hz added to this DC voltage. The signal, that is the 90° component of the electrical current, is, after amplification, recorded by a x-t writer or a PC. A thermostat is used to adjust the temperature of the stirred electrolyte.

In the following the procedure of preparation, cleaning of electrodes and their using in capacitance measurements is described.

As a first step, a silver plated copper wire (length: 5 cm) was soldered to the contact pad of the electrodes. Before being covered with thiol monolayers, the electrodes had to be cleaned using an ultrasonic bath. The electrodes, immersed in ethanol (p.a. quality), were treated therein twice for 5 min. After each cleaning step, they were dried in a stream of nitrogen. Then, they were dipped for 2 min into hot piranha

solution (30% H_2O_2 : 98% H_2SO_4 = 1 : 3, v/v). After that the electrodes were rinsed with copious amounts of water and blown dry in a stream of nitrogen. The electrodes then were stored *in-vacuo* for at least 1 h (desiccator).

Palladium electrodes were cleaned without dipping them into piranha solution which would oxidize the palladium and would wash it off the surface of the electrode.

For the build-up of the SAMs, electrodes were immersed in a 100 µmol/L solution of 11-mercaptoundecanoic or 16-mercapto-hexadecanoic acid in chloroform (p. a. quality) for at least 12 h at room temperature (22±2 °C). After that, the electrodes were rinsed with chloroform and dried in a stream of nitrogen.

For the mixed SAMs as used in chapter 5, two stock solutions were prepared. These were mixed in different ratios of alkanethiols with carboxy and hydroxy functionalities. The first stock solution consisted of 4 µmol/L of 11-mercaptoundecanoic acid or 16-mercaptohexadecanoic acid and 100 µmol/L of 11-mercaptoundecanol or 16-mercapto-hexadecanol in chloroform (p. a. quality). The other one was a solution of 100 µmol/L of 11-mercaptoundecanoic or 16-mercaptohexadecanoic acid in chloroform (p. a. quality). Thiol monolayers were built up as described above.

After being covered by the desired alkanethiol monolayer, the electrodes were immersed in the respective electrolyte and capacitance was determined.

A DC voltage of +300 mV (vs. Ag/AgCl) and an AC voltage of 10 mV, 20 Hz were applied to the working electrode (gold or palladium). For measuring the capacitance of the electrodes the 90° component of the electrical current was determined. The signal was registered after amplification by a x-t recorder or a PC and calibrated with a capacitor

(49.1 nF). A thermostat (Haake) was used to adjust the temperature of the stirred electrolyte.

In experiments where DNA-oligomers (and EDC) were added into the measuring chamber, the microliter syringe was used. The changes of the capacitance upon these additions were recorded.

3.2. SCINTILLATION COUNTING

The scintillation counting experiments (discussed in chapter 6) were performed in cooperation with Prof. P. Hegemann, Institute of Biochemistry, Genetics, and Microbiology, Regensburg University.

The samples were prepared at the Faculty of Electrical Engineering, Department of Microelectronics, Slovak University of Technology. The design of the samples is shown in Fig. 3.3.

16-Mercaptohexadecanoic acid was purchased from Aldrich and used without further purification. Water was purified in an ion-exchanger purification train (> 18 MΩ with low organic content; Millipore plus 185). EDC (1-ethyl-3-(3-dimethylaminopropyl)carbo-diimide) was from Sigma, S-NHS (*N*-Hydroxysulfosuccinimide) was from Aldrich, HEPES (N-(2-hydroxyethyl)piperazine-N´-(2-ethanesulfonic acid)) was from Serva, other chemicals from Merck. All chemicals were used as received. EDC was stored at -18 °C. DNA-oligomers with modification by an amino group (combined with a C_6-spacer) were received from MWG Biotech or Interactiva as lyophilisates. Before use, they were dissolved in water or buffer.

Measurements were performed on a Beckman LS 6500 multi purpose scintillation counter.

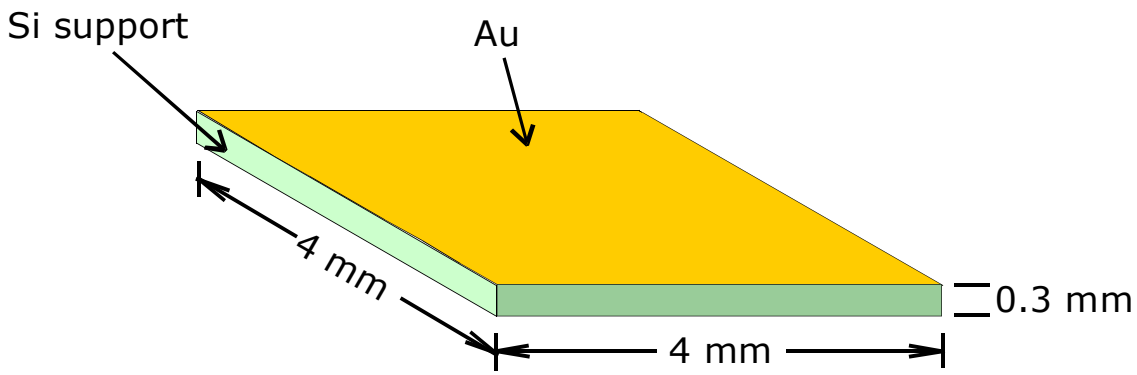


Fig. 3.3. Design of the substrate for the self assembly of alkanethiol monolayers. After immobilization of radiolabeled DNA-oligomers, scintillation counting was performed.

The samples were cleaned as described for gold electrodes in chapter 3.1. Also the SAM of alkanethiols was formed as mentioned above.

Amino-modified DNA-oligomers (5´-GCA AAG GGT CGT ACA CAT CAT CAT-NH_2-3´) were enzymatically labeled with dideoxyadenosine 5´-[α-^{32}P] triphosphate (Amersham Pharmacia Biotech) using a terminal deoxyribonucleotidyl transferase (TdT) and purified so that 1 mmol of DNA-oligomers equaled to 0.315 Ci. This work was performed by Dr. W. Deininger of the Institute of Biochemistry, Genetics, and Microbiology, Regensburg University.

The procedure of the scintillation counting experiments is shown in Fig. 3.4. At first, the amino-modified DNA-oligomers were enzymatically labeled with dideoxyadenosine 5´-[α-^{32}P] triphosphate (① in Fig. 3.4) and the gold plates were covered by a SAM of 16-mercaptohexadecanoic acid (②). The plates were distributed in individual vessels. Always three of the vessels were filled with the same solution of different pH and ionic strength (③) (for the conditions see Table 3.1). Then, ^{32}P-radiolabeled DNA-oligomers were added to each vessel, except for the samples for testing external activation

(immobilization protocol of company 2) to give a concentration of 4 pmol/µL. After that, EDC (concentrations: Table 3.1.) was added to two vessels for each kind of solution conditions, the remaining one for each kind served as a control. At that point, the samples were incubated in a shaker for 90 min (④). (The samples according to company 2, which were incubated for 30 min after addition of EDC, were translocated to new vessels filled with water. Then, radiolabeled DNA-oligomers were added to these samples and they also were incubated in a shaker for 90 min. After this time, the gold plates were taken out, rinsed with copious amounts of water (⑤) and the scintillation counts in 20 mL water were determined (⑥). In order to determine the back-ground counts, the gold samples were removed and the scintillation counts of the vessels were measured (⑦). The plates were then placed into vessels filled with washing solution and were incubated (0.5 h for 0.2 mol/L NaOH, 10 h for 1 mol/L NaCl) in a shaker (⑧). After each of these washing procedures, the samples were rinsed with water and the determination of the scintillation counts was repeated (⑤ - ⑦).

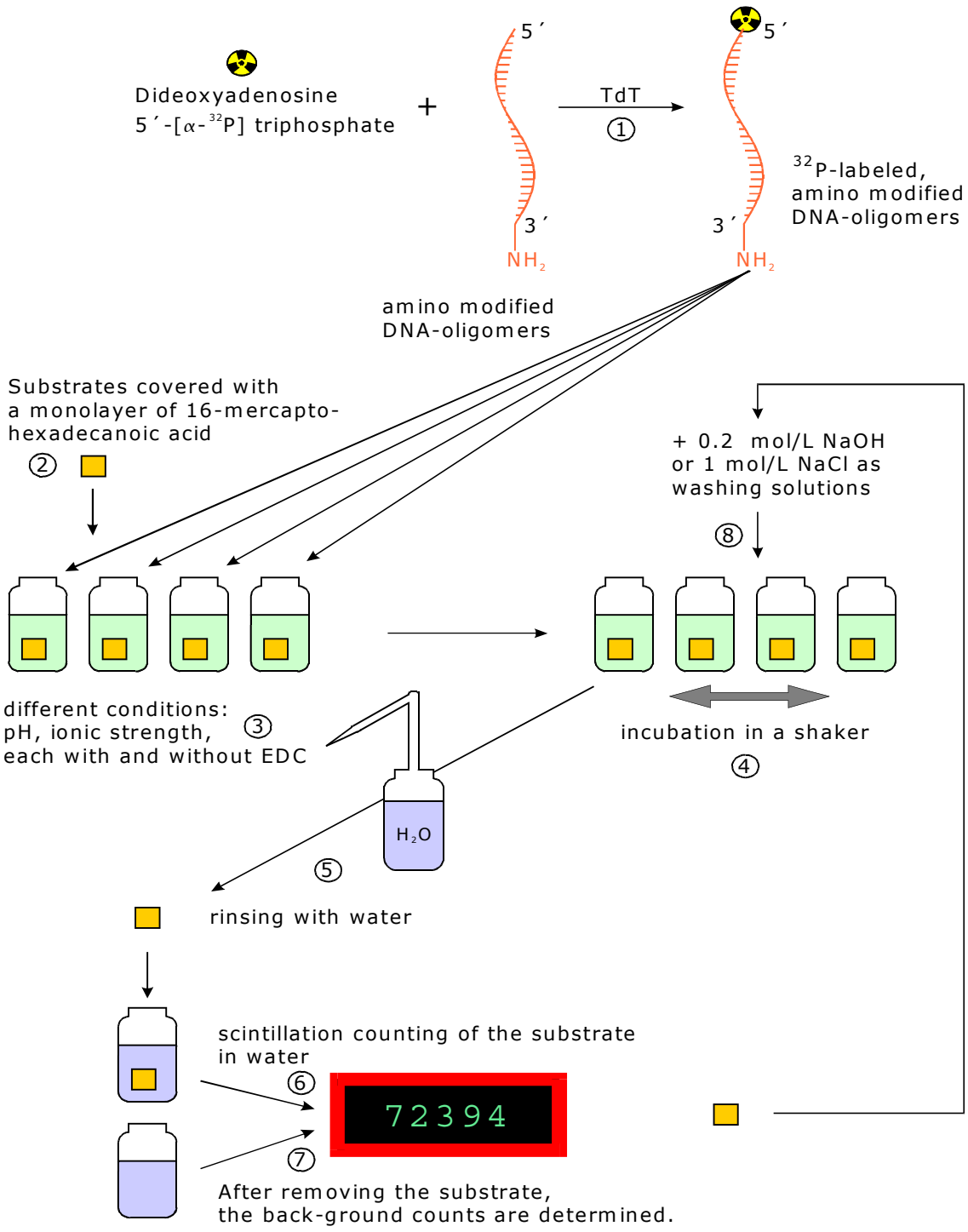


Fig. 3.4. Flow chart of the scintillation counting experiments. After preparation of the radiolabeled DNA-oligomers (using the enzyme TdT) and the coating of the samples with alkanethiols, different immobilization conditions are tested.

Table 3.1. Conditions for immobilization applied in the scintillation counting experiments.

Solution	***pH***	***KCl/NaCl***	***EDC***	***S-NHS***	***Samples***
0.5 mmol/L sodium hydrogen phosphate	2.2	_	400 mmol/L	_	2
0.5 mmol/L sodium hydrogen phosphate	2.2	_	_	_	1
0.5 mmol/L sodium hydrogen phosphate	4.0	_	400 mmol/L	_	2
0.5 mmol/L sodium hydrogen phosphate	4.0	_	_	_	1
0.5 mmol/L sodium hydrogen phosphate	6.9	_	400 mmol/L	_	2
0.5 mmol/L sodium hydrogen phosphate	6.9	_	_	_	1
0.5 mmol/L sodium hydrogen phosphate	9.4	_	400 mmol/L	_	2
0.5 mmol/L sodium hydrogen phosphate	9.4	_	_	_	1
Company 1: 10 mmol/L HEPES, 3.4 mmol/L EDTA	7.4	150 mmol/L NaCl	400 mmol/L	100 mmol/L	2
Company 1: 10 mmol/L HEPES, 3.4 mmol/L EDTA	7.4	150 mmol/L NaCl	_	100 mmol/L	1
Company 2: water	5.8	_	800 mmol/L	200 mmol/L	2
Company 2: water	5.8	_	_	200 mmol/L	1
water	5.8	1 mol/L KCl	400 mmol/L	_	2
water	5.8	1 mol/L KCl	_	_	1
water	5.8	10 mmol/L KCl	400 mmol/L	_	2
water	5.8	10 mmol/L KCl	_	_	1

3.3. UV SPECTROSCOPY

The experiments using UV spectroscopy are discussed in chapter 6.1.

EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) and MOPS (3-(N-morpholino)propanesulfonic acid) were from Sigma, HEPES (N-(2-hydroxyethyl)piperazine-N´-(2-ethanesulfonic acid)) was from Serva, sodium hydrogen phosphate, sodium acetate, citrate, SDS (sodium dodecyl sulfate), and TRIS (Tris(hydroxymethyl)ammino-propane) were purchased from Merck. EDC was stored at -18 °C. All reagents were used as received. Water used in all experiments was purified in an ion-exchanger purification train (Millipore plus 185). The pH was adjusted with 1 mol/L NaOH or HCl.

The measurements were performed on a Hitachi U-3000 spectrophotometer in 1 mm or 10 mm quartz cuvettes at room temperature (22±2 °C).

Before each experiment, EDC was added to give the appropriate concentration in the measuring cell. For the standard curve, an aliquot of a 100 mmol/L EDC solution was added to deionized distilled water (pH 5.8), stirred carefully, and immediately after the absorbance at 213/214 nm was determined. For the kinetic runs, a 9 µL aliquot was added to the solution of buffer or surfactant to give a 3 mmol/L concentration in the measuring cell, and, after stirring, the absorbance was monitored at 213/214 nm over 20 minutes.

4. THIOL LAYER PRETREATMENT AND TEMPERATURE EFFECTS

Capacitance-based detection methods, as pointed out in chapter 2, require densely packed alkanethiol layers at the surfaces of electrodes. In the following, the improvement of the thiol layer on metal surfaces like gold or palladium utilizing an electrode pretreatment is shown (for gold: cf. chapter 4.1, for palladium cf. chapter 4.2). Furthermore, the influence of temperature changes on the capacitance signal is studied.

In the analytical step using genosensors, higher temperatures are often needed in order to improve the selectivity of the sensor (see below). For this reason, a predictable function describing the capacitance changes due to temperature variations is an essential for the alkanethiol layers on gold (or palladium). Offering such a predictable reaction, they can serve as useful basic layers for the interface preparation of capacitance-based genosensors.

As shown in Fig. 4.1, DNA is coiled to form a double helix ("double-stranded DNA", dsDNA) composed of two single strands (ssDNA) and held together by hydrogen bonds. These hydrogen bonds are formed between certain pairs of nucleobases (or simply "bases": adenine, cytosine, guanine, and thymine) opposing each other, and which are arranged like the rungs of a ladder along the sugar-phosphate backbones of the DNA strands [Junhui et al., 1997]. Hydrogen bonds between opposite nucleobases are holding the two strands together. Adenine is pairing off with thymine via two hydrogen bonds, and cytosine is pairing off with guanine via three hydrogen bonds.

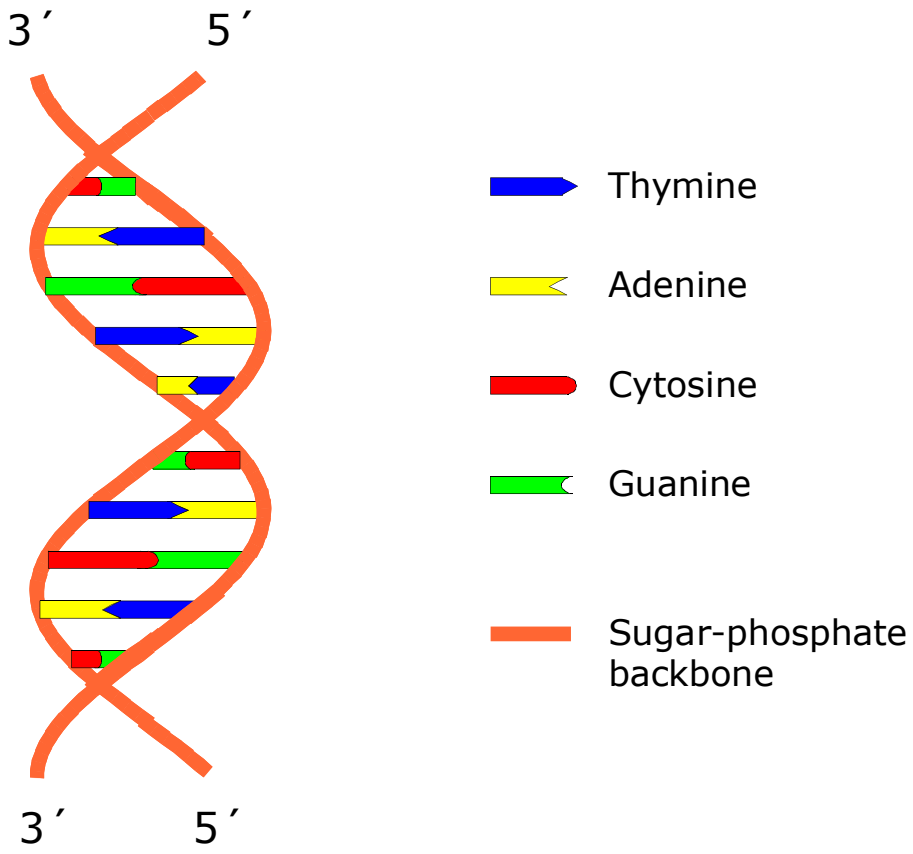


Fig. 4.1. Diagram of a double-stranded DNA (dsDNA) without mismatches. The nucleobases (adenine, cytosine, guanine, and thymine) are attached to the sugar-phosphate backbone, matching bases of the opposite strands pair off via hydrogen bonds.

If the temperature of a solution containing a helical nucleic acid is raised sufficiently, strand separation or "melting" occurs. The melting temperature (T_m) of a double-stranded DNA (dsDNA) is defined as the midpoint of the melting process. It is the temperature at which half of the nucleic acid exist in the helical state and the other half exists in the single stranded state and the two species are in equilibrium [Wilson et al., 1997]. Strand separation involves disruption of the hydrogen bonds between the paired bases and the hydrophobic interactions between the stacked bases. The value of T_m can vary in the range of 0 to 100 °C depending on the size [Lehninger, et al., 1993] and the base

composition of the DNA [Bonner et al., 1973], on the ionic strength of the solution [Schildkraut and Lifson, 1965], and possible denaturing agents [McConaughy et al., 1969]. After breaking the bonds, the result are two single DNA strands (ssDNA) which can re-anneal with decreasing temperature into the double stranded conformation.

For analytical purposes, it is necessary to obtain a high selectivity of the sensor. Concerning genosensors, the analytical step is the hybridization, as mentioned in chapter 1. In order to increase the selectivity of the hybridization, that is to distinguish correctly between the target strand (perfect match) and other DNA strands (mismatch or no match at all), another property of DNA is exploited. Imperfect base pairing, or mismatches, cause a destabilization and therefore the re-annealing occurs at lower temperatures than it would be the case for perfect base pairing (every percentage of mismatch causes a decrease of 1 – 1.5 °C). To utilize this, the analysis is performed mostly at a temperature of about 20 - 25 °C below the melting temperature [Sambrook et al., 1989]. This is a temperature where perfect matching strands will hybridize in a sufficient amount but strands with mismatches will not pair or build only very week pairs with the receptor strands.

In order to use a capacitance-based sensor as a genosensor, the reaction of the capacitance to temperature variations has to be tested. In the following, the dependence of the electric capacitance of thiol layers on temperature is studied.

4.1. TEMPERATURE DEPENDENCE OF THE CAPACITANCE SIGNAL OF THIOL LAYERS ON GOLD

On applying higher temperatures during capacitance-based measurements, it is necessary to know the influence of temperature on self-assembled monolayers (SAMs) of thiols in order to decide whether a measured capacitance change is caused by changes of temperature or by any other event on the electrode surface. So, the dependence of the capacitance signal of SAMs of 16-mercaptohexadecanoic acid on temperature is investigated in the following. Also a procedure to pretreat electrodes in order to increase the density of the thiol layer on gold is shown.

As explained in chapter 2.2, the capacitance of an alkanethiol monolayer decreases with increasing density. This density is achieved by a tilting of the alkane chains of the thiol molecules (Fig. 2.4, p. 11). In literature it was described that the alkanethiol molecules of a SAM are untilted upon heating [Bensebaa et al., 1998]. This untilting is concomitant with a loss of three-dimensional density and for capacitance measurements that would imply an increase in capacitance.

In fact, the capacitance increases with increasing temperature as shown in Fig. 4.2. A gold electrode covered by a SAM of 16-mercaptohexadecanoic acid was immersed in a solution of 10 mmol/L Na_2HPO_4, 50 mmol/L KCl, pH 7.2. The temperature of the electrolyte was shifted stepwise from room temperature to nearly 70 °C, and the capacitance of the thiol layer was monitored. A nonlinear dependence of capacitance on temperature was detected: The capacitance changes were small for changes of temperature up to 40 °C, for temperature steps in the higher temperature (40 – 70 °C) range the capacitance changes were much larger.

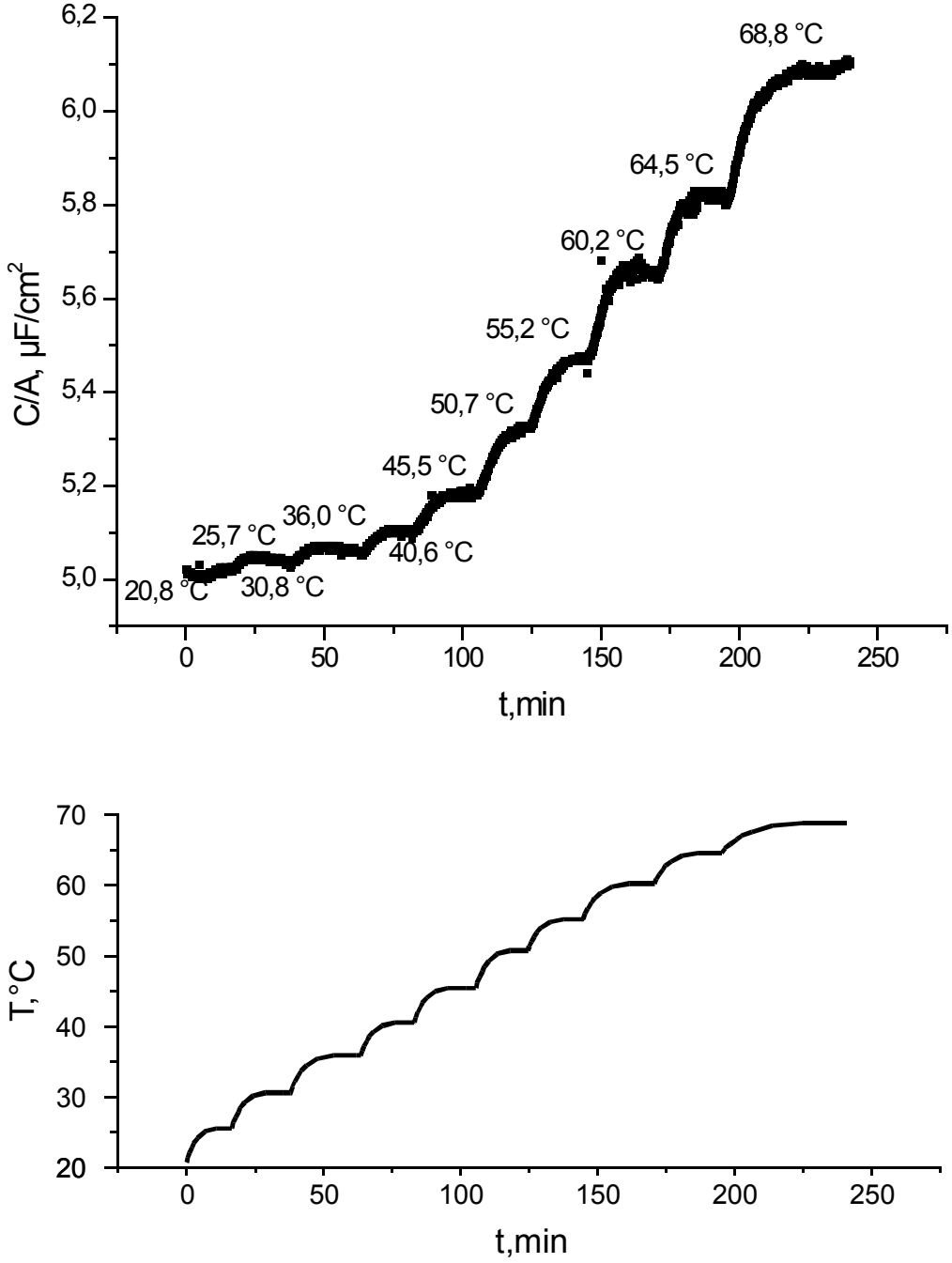


Fig. 4.2. Above: Dependence of the capacitance of a SAM of 16-mercaptohexadecanoic acid on temperature (Au electrode). Capacitance is increasing with increasing temperature.
Buffer: 10 mmol/L Na_2HPO_4, 50 mmol/L KCl, pH 7.2
Below: Change of temperature with time (schematically).

In order to see this dependence more clearly, the capacitance is plotted vs. temperature in Fig. 4.3. Up to 40 °C, there are nearly no changes of capacitance of the thiol layer on increasing temperatures, but then there is a very strong increase of capacitance on increasing temperature. This nonlinear effect can be seen for fast temperature elevation and when the temperatures reached are held for relatively short time periods of about 10 minutes.

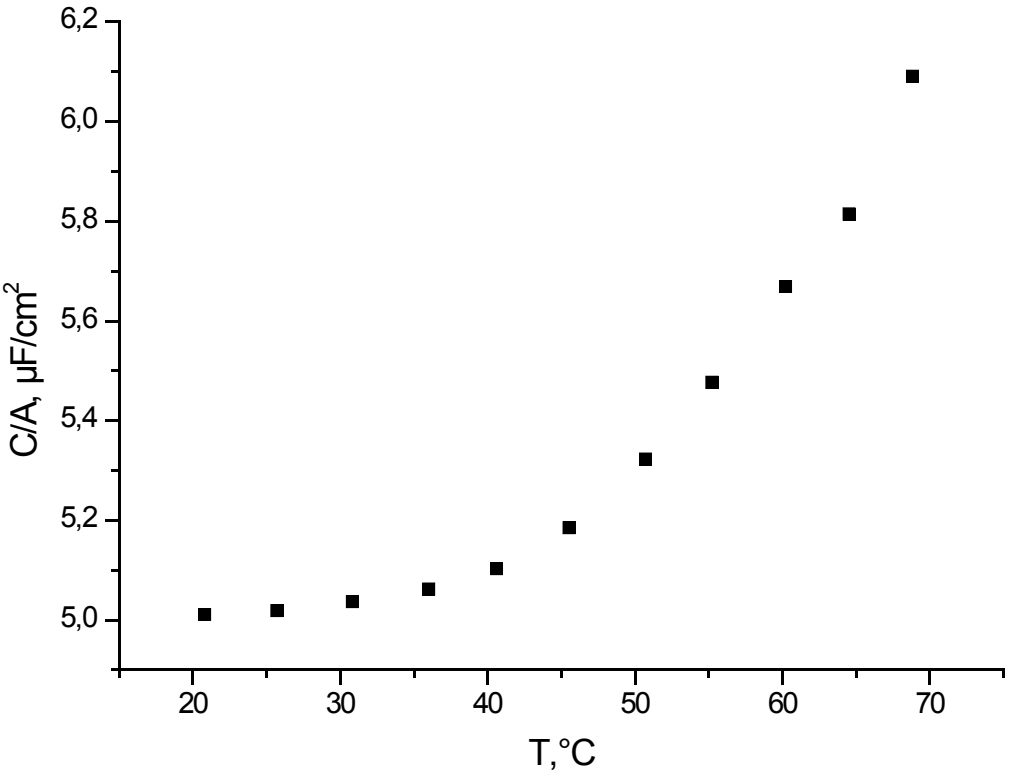


Fig. 4.3. Dependence of the capacitance of a monolayer of 16-mercaptohexadecanoic acid on temperature up to 70 °C (Au electrode). Capacitance is nonlinearly increasing with increasing temperature.
Buffer: 10 mmol/L Na_2HPO_4, 50 mmol/L KCl, pH 7.2

If a higher temperature is applied for a longer time period than the 10 minutes mentioned above, the capacitance of the thiol monolayer at this fixed temperature changes dramatically. This is shown in Fig. 4.4 for another electrode. The temperature of the electrolyte was increased to 56 °C and then stabilized at this value. The capacitance of the monolayer of thiols increased when the temperature was risen. But when the temperature was held at 56 °C for a longer time, the capacitance decreased to reach a value which was far lower (1.3 $\mu F/cm^2$) than the starting value (about 3 $\mu F/cm^2$). When heating of the electrolyte was discontinued, the capacitance further decreased and reached a value of about 1.2 $\mu F/cm^2$.

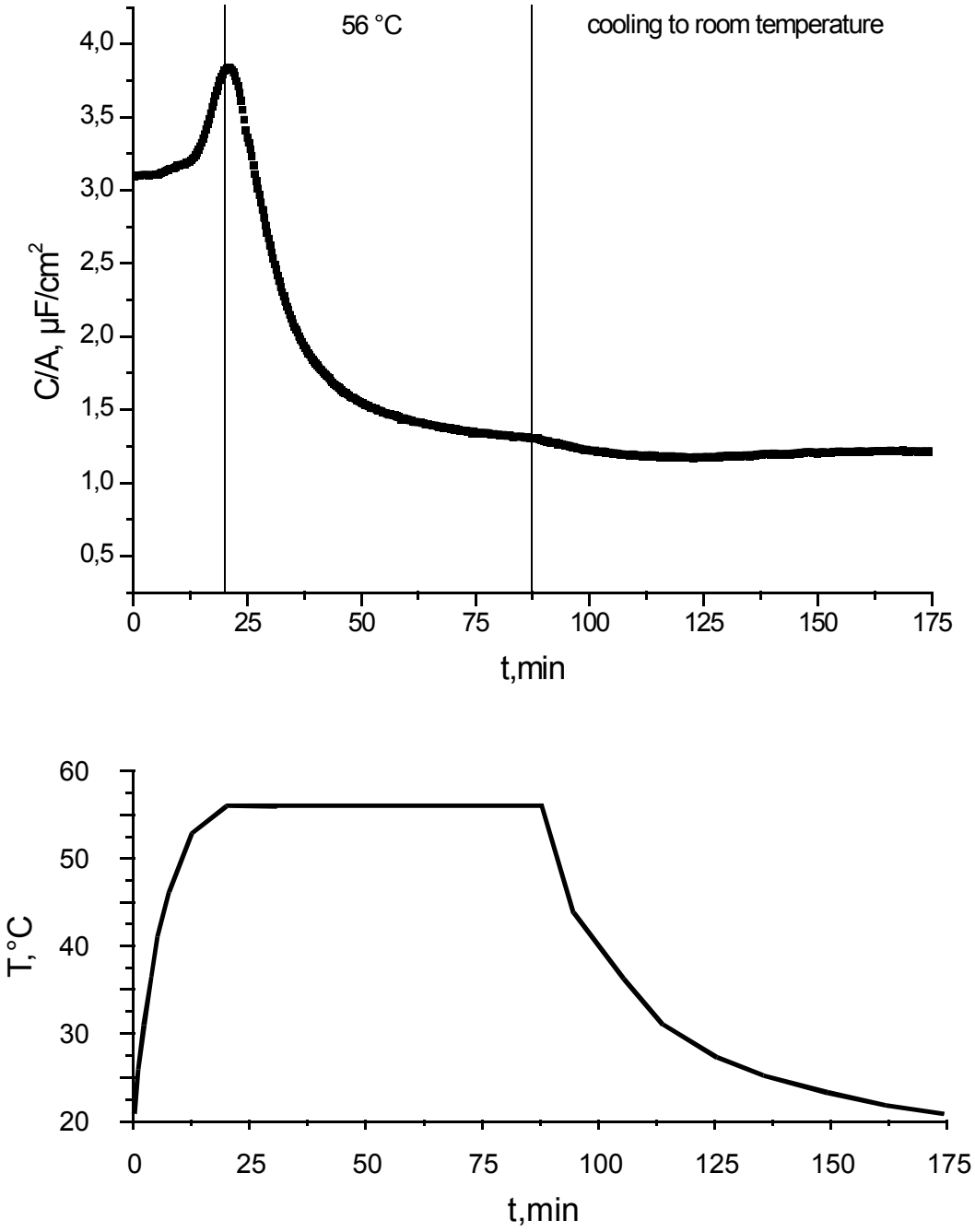


Fig. 4.4. Above: Capacitance change of a SAM of 16-mercaptohexadecanoic acid heated to 56 °C (Au electrode). This temperature was held constant for 70 minutes, and then the electrolyte was cooled down to room temperature.
Electrolyte: 0.1 mol/L HCl, 1 mol/L KCl, pH 1.35
Below: Change of temperature with time (schematically).

Plotting capacitance vs. temperature for this last experiment, the curve displays a nonlinear behavior (Fig. 4.5). At first the capacitance increases with increasing temperature. Holding the temperature at a higher level for longer time leads to a drop of capacitance to reach a value which is notedly below the starting value. This capacitance further decreases when the temperature is shifted back to room temperature.

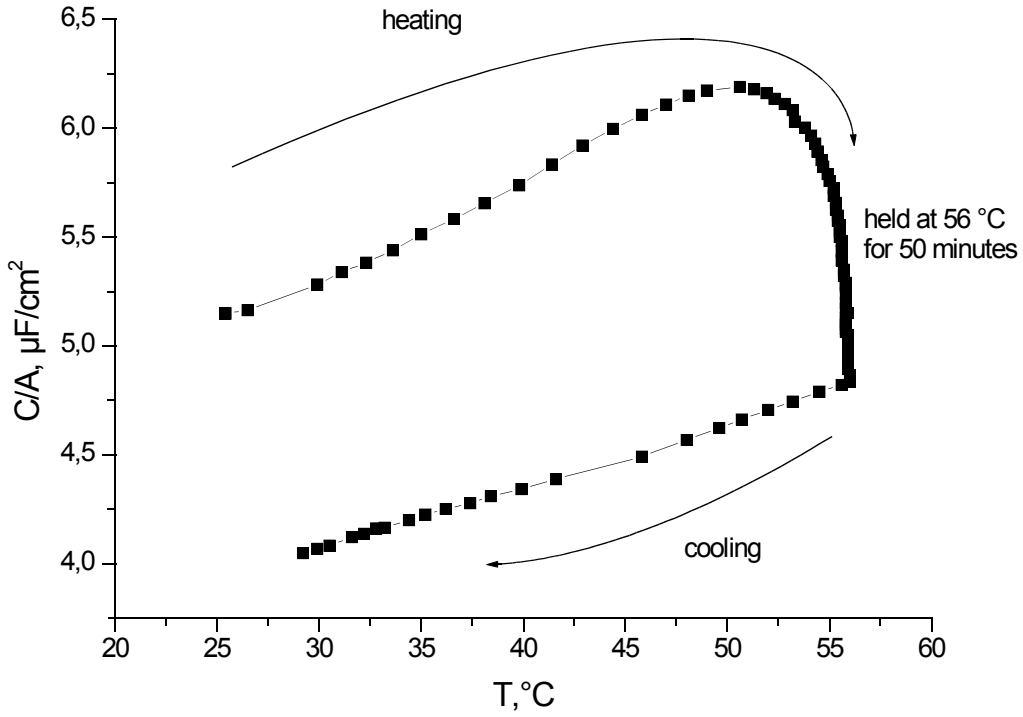


Fig. 4.5. Temperature dependence of the capacitance of a SAM of 16-mercaptohexadecanoic acid heated to 56 °C and held at this temperature for 50 minutes, then cooled down to 28 °C (Au electrode). A nonlinear behavior can be seen.
Electrolyte: 0.1 mol/L HCl, 1 mol/L KCl, pH 1.35

A possible explanation of this nonlinear behavior could be a reorganization of thiols with an elimination of defects in the layer at the same time.

As pointed out in chapter 2.3, the thiol layer is not defect-free. There are several possible types of defects: pinholes, collapse sites, or monolayer domain boundaries [Diao et al., 2001], [Becka and Miller, 1992].

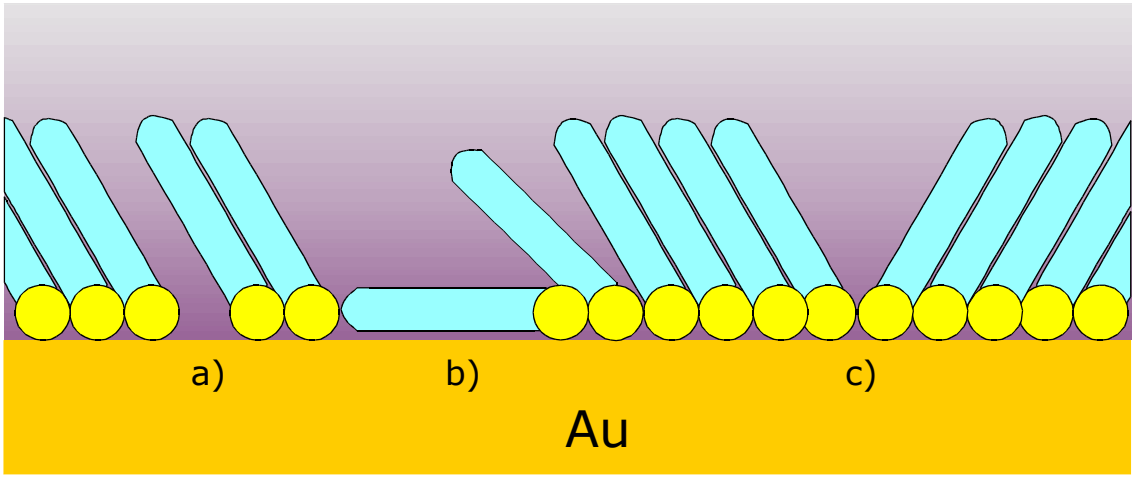


Fig. 4.6. Possible defects of the thiol monolayer. a) pinholes, b) collapse sites c) boundaries of different oriented domains.

Pinholes (Fig. 4.6 a) are defects, where no thiols are adsorbed at the gold surface. However, the SAMs are considered essentially free of pinholes [Diao et al, 1999]. Instead, it is presumed that the thiol molecules near spots without thiols tilt at such a large angle that they in fact cover these defects and form collapsed sites, as shown schematically in (Fig. 4.6 b). Another type of defect is the boundary between two monolayer domains with differing orientation of the surface tilt angle (Fig. 4.6 c). Within these domains the thiol molecules are all directed in the same way. These domain boundaries allow electrolyte ions to reach closer to the electrode, the thickness of the insulating layer is diminished, and therefore the capacitance increases.

Raising the temperature of the electrolyte, into which such an electrode is immersed, and holding it at this higher temperature could lead to a reorientation of these thiol domains caused by a higher

mobility of the molecules, which would lead to larger domains and therefore fewer domain boundaries. The more densely packed insulating layer would then possess a lower capacitance.

Once the reorganization of the thiols has taken place, reheating of the system should not lead to further decreases of the capacitance. To test this, an electrode covered by a SAM of 16-mercaptohexadecanoic acid was heated and cooled twice in electrolyte, which is shown in Fig. 4.7.

While in the first cycle the reorientation takes place and a nonlinear behavior can be seen (black curve), there is only a linear dependence of capacitance on temperature within the second cycle (red curve). The deviation from the linear behavior at the end of the second cooling process is due to drift of the capacitance signal.

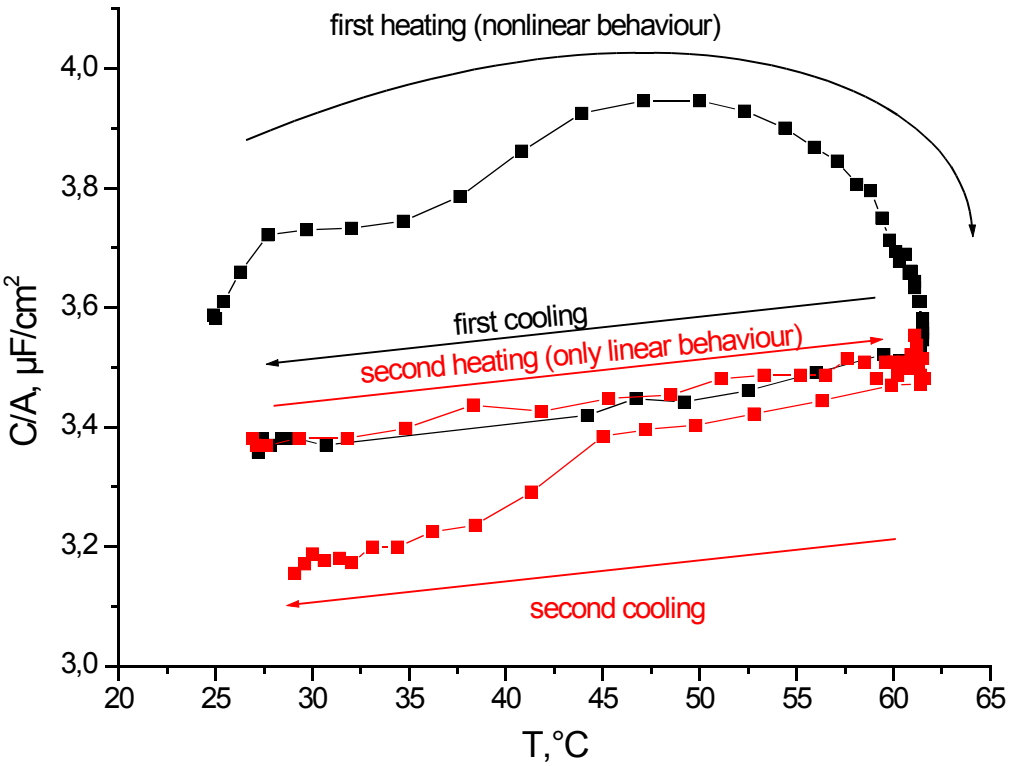


Fig. 4.7. Temperature dependence of the capacitance of a SAM of 16-mercaptohexadecanoic acid heated to 62 °C, held at this temperature for 30 minutes, then cooled (black). After that, the procedure was repeated (red). The curve is nonlinear only for the first cycle.
Electrolyte: 0.1 mol/L HCl, 1 mol/L KCl, pH 1.35

As was shown by these experiments, the effect which leads to a nonlinear decrease of the value of capacitance upon heating is irreversible. Furthermore, electrodes heated as described above, afterwards taken out of the solution, and blown dry in a stream of nitrogen could be placed into another electrolyte but still displayed the lower value of capacitance (data not shown). Also, capacitance changes due to changes of pH or ionic strength upon possible solvent evaporation were negligible.

So, a possibility was found to pretreat the electrodes in order to eliminate defects in the thiol layer but also a predictable behavior of the capacitance of the layer upon heating, a linear dependence, was reached. However, it should be noted, that Bensebaa et al. who studied the effects of heating thiol monolayers on gold *in-vacuo* by infrared spectroscopy, found a non reversible disorder of the molecules upon heating the layer above a temperature of about 80 °C [Bensebaa et al., 1998].

Since the hybridization of DNA is carried out at lower temperatures, this temperature range is sufficient for most applications of the genosensor.

In the last section, the effects of temperature on the capacitance of a SAM of thiols were studied. In the following, the capacitive effect due to nonspecific adsorption at electrodes pretreated by heating is discussed.

As described in chapter 2, it is necessary for the interface preparation of genosensors to immobilize the DNA-oligomers of interest at the surface of the sensor. In many cases, this happens by a chemical reaction between modified DNA-oligomers and molecules with special tail groups mediated by a coupling reagent, for example, amino-modified oligomers are coupled via EDC (1-ethyl-3-(3-dimethylamino-propyl)carbodiimide to carboxy-modified surfaces.

But, in addition to this specific coupling reaction, nonspecific adsorption of the oligomers to the surface (especially on gold) takes place (cf. chapter 2.3). For the interface preparation of a capacitance-based genosensor, the magnitude of this nonspecific adsorption and its effects on the capacitance of a thiol monolayer is an important value.

The magnitude of nonspecific adsorption was tested by measuring the capacitance of gold electrodes coated with alkanethiol monolayers before and after addition of amino-modified DNA-oligomers without any

addition of coupling reagents. Thereby, no specific coupling could happen and resulting capacitance changes would be only due to effects of nonspecific adsorption.

Electrodes which were not pretreated by heating before the addition of oligomers showed a relatively large capacitance decrease caused by nonspecific adsorption of oligomers. In Fig. 4.8, 5 µmol/L of amino-modified DNA-oligomers (24-mer) were added to the electrolyte in which a gold electrode covered by a layer of 16-mercapto-hexadecanoic acid was immersed, causing a very large capacitance decrease (in the range of 6%).

An explanation of this effect could be the still existing defects in the alkanethiol monolayer, like collapse sites or domain boundaries (Fig. 4.6). If, in this situation, DNA-oligomers are added, the capacitance decrease should be very large because of two synergistic effects. The first one is the decrease of capacitance due to formation of an additional layer of molecules (all of them being nonspecifically adsorbed) as shown in Fig. 2.7, p. 15. The second effect is the decrease of capacitance due to the coverage of some of the defects by these adsorbed molecules, resulting in the layer getting more isolated against influences of the electrolyte and therefore the capacitance further decreases.

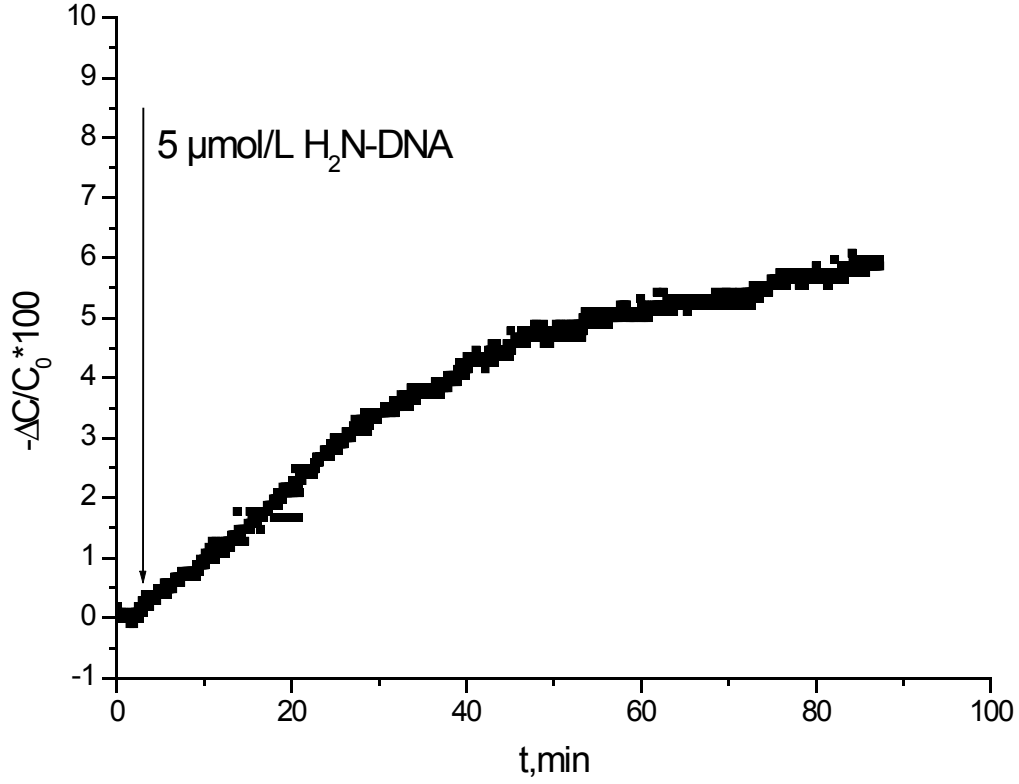


Fig. 4.8. Capacitance change due to nonspecific adsorption of 5 µmol/L of amino-modified DNA-oligomers (24-mers) on a SAM of 16-mercaptohexadecanoic acid without heating before addition of DNA (Au electrode).
Electrolyte: 0.1 mol/L HCl, 1 mol/L KCl, pH 1.35

In contrast, the decrease in capacitance should not be so high for electrodes pretreated by heating than for those used without pretreatment. The part of the decrease of capacitance due to the formation of an additional layer should still be present, whereas the part due to the coverage of defects by adsorbed molecules should be smaller because there exist less defects which could be covered. To test this, gold electrodes covered with 16-mercaptohexadecanoic acid were pretreated twice by heating to 60 °C in electrolyte for one hour and

cooled to room temperature. Then they were immersed in another sample of the identical electrolyte and a similar amount of amino-modified DNA-oligomers (3 µmol/L) was added (Fig. 4.9).

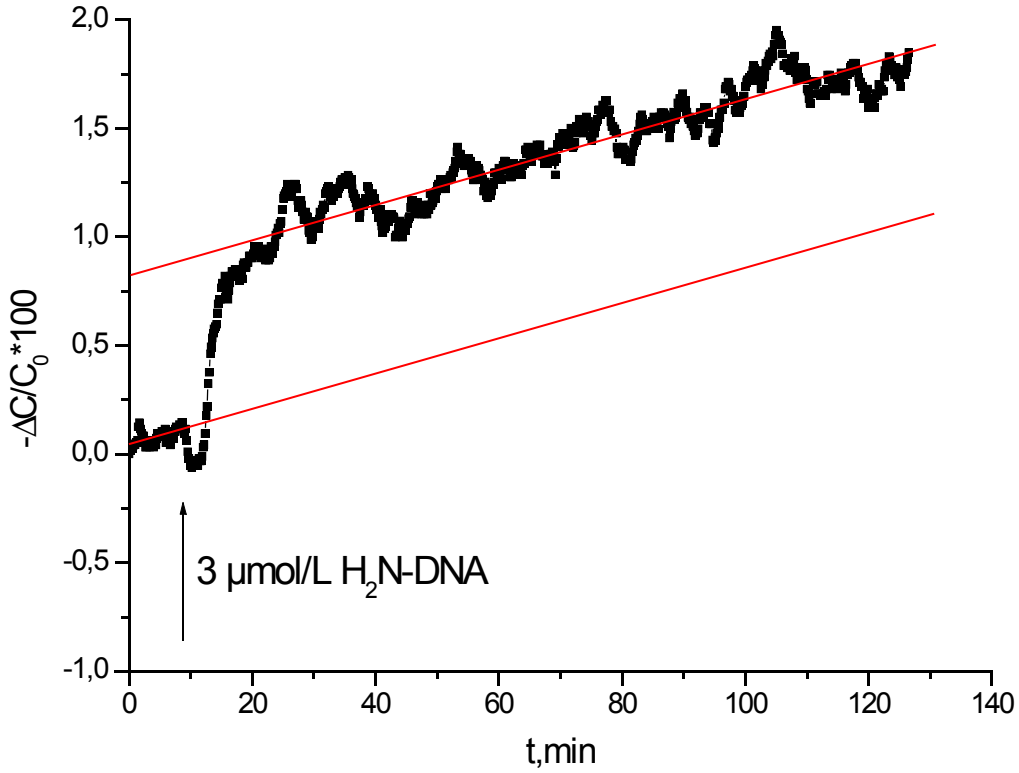


Fig. 4.9. Capacitance change due to nonspecific adsorption of 3 µmol/L of amino-modified DNA-oligomers (24-mers) on a SAM of 16-mercaptohexadecanoic acid heated twice to 60 °C before addition of amino-modified DNA (Au electrode). A drift of the capacitance signal before and after addition of DNA can be seen (red lines).
Electrolyte: 0.1 mol/L HCl, 1 mol/L KCl, pH 1.35

The capacitance change due to nonspecific adsorption of DNA was about eight times less than for electrodes which were not pretreated by heating. Although the added amount of amino-modified DNA-oligomers was a little less than it was for the experiment in Fig. 4.8 (3 vs. 5 µmol/L total concentration of amino-modified DNA-oligomers in

the measuring chamber), the total amount of nonspecifically adsorbed DNA should be nearly the same in both cases.

However, a dramatic difference in capacitance change could be detected. Therefore it can be supposed, that the capacitive responses of the electrodes vary because of a different amount of defects in the layer.

In conclusion, it can be said that a pretreating of gold electrodes covered by a SAM of thiols by heat, does not lead to a decrease of nonspecifically adsorbed molecules but to a diminution of defects in the layer. With less defects, the coverage and shielding effect of nonspecifically adsorbed molecules against the influences of the electrolyte is less and therefore a smaller change in capacitance is detected.

4.2. TEMPERATURE DEPENDENCE OF THIOL LAYERS ON PALLADIUM

In the last section the temperature effects on the capacitance of SAMs of alkanethiols on gold were tested. As shown in chapter 1, SAMs also form on other metals, e.g. palladium.

The advantage of palladium for analytical purposes would be that the stability of monolayers of thiols with a shorter chain length is considered to be higher on palladium than on gold [Riepl, 2000].

Thiols with a shorter chain length possess a reduced thickness of the insulating layer. This should lead to a higher relative capacitance change upon reaction of analyte and target molecule according to eq. 2.1 (p. 6) and eq. 2.2 (p. 13). Therefore, it would be promising to use these short chain thiols for the preparation of electrochemical biosensors. In the following, the influence of temperature on self-assembled thiol monolayers at palladium electrodes are tested.

Thiol monolayers on palladium electrodes used for this work were not as densely packed as on gold electrodes because the palladium electrodes could not be cleaned in the same way as the gold electrodes and therefore impurities remained at the surface.

For this reason, the starting value of capacitance of these layers was generally higher than for monolayers on gold. Furthermore, it was shown that the phase angles between applied voltage and resulting current deviated from 90 °C for monolayers on the palladium electrodes used here than for those on gold electrodes [Riepl, 2000]. This also indicates that there are more defects in the alkanethiol layer on palladium than on gold electrodes.

As for gold electrodes, the dependence of the capacitance signal of SAMs on temperature was tested for palladium electrodes.

Temperature effects for monolayers on palladium were similar to those for monolayers on gold. A palladium electrode covered by a SAM of 11-mercaptoundecanoic acid (a thiol with a shorter chain length) was heated to 68 °C in electrolyte and the capacitance of the monolayer was measured.

As seen for gold electrodes in Fig. 4.5, a nonlinear behavior was also detected for palladium electrodes and the capacitance values after heating were lower than before (Fig. 4.10). Similarly to thiol layers on gold, only a linear dependence of the capacitance on temperature resulted after a second cycle of heating and cooling for thiol layers on palladium electrodes (data not shown).

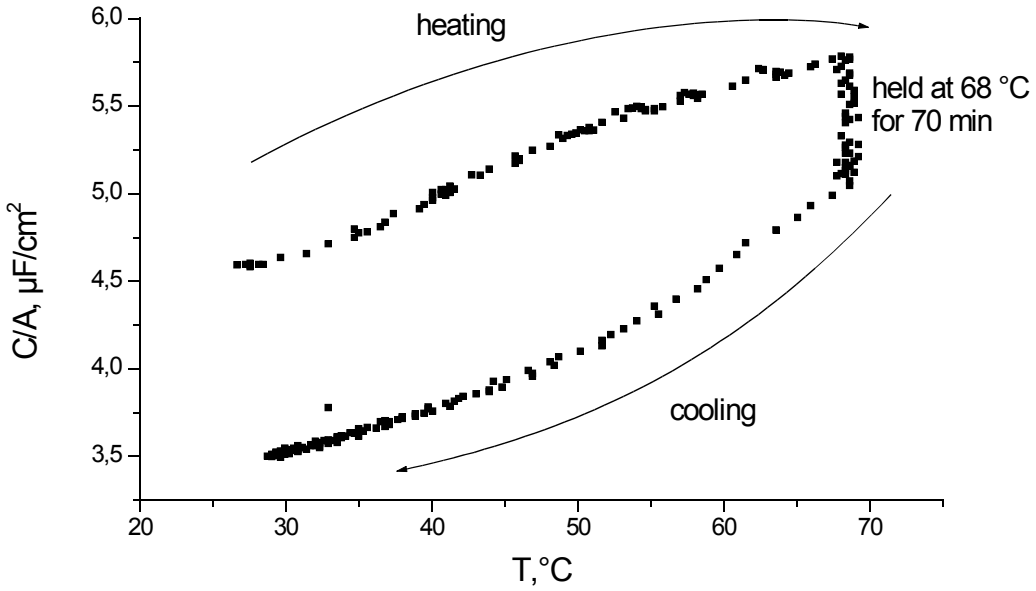


Fig. 4.10. Temperature dependence of the capacitance of a SAM of 11-mercaptoundecanoic acid on a palladium electrode heated to 68 °C, and held at this temperature for 70 minutes, then cooled down to 28 °C. The dependence shows nonlinear behavior.
Buffer: 0.5 mmol/L Na_2HPO_4, 10 mmol/L KCl, pH 4

So, defects in the monolayers after self assembly of the thiols can be diminished by heating of the palladium electrode in electrolyte. According to the results in chapter 4.1, an addition of DNA-oligomers to the measuring chamber into which a palladium electrode is immersed in electrolyte, should lead to a smaller capacitive response for pretreated electrodes than for those which were not heated before the addition. This is tested in the experiments of Fig. 4.11 and Fig. 4.12. A palladium electrode covered by a SAM of 11-mercaptoundecanoic acid without pretreatment by heating was used in the first experiment (Fig. 4.11). The addition of 12 µmol/L of amino-modified DNA-oligomers to the electrolyte in which the electrode was immersed resulted in a strong capacitance decrease of about 10%.

Like for gold electrodes, the defects of the thiol layer on palladium are supposed to be partly covered by added oligomers and the accessibility of electrolyte molecules to the palladium electrode is hindered. Also, nonspecifically adsorbed molecules are thought to build up an additional layer at the electrode/electrolyte interface and to lead to a smaller total capacitance. Again, there are two synergistic effects leading to a very large decrease in capacitance.

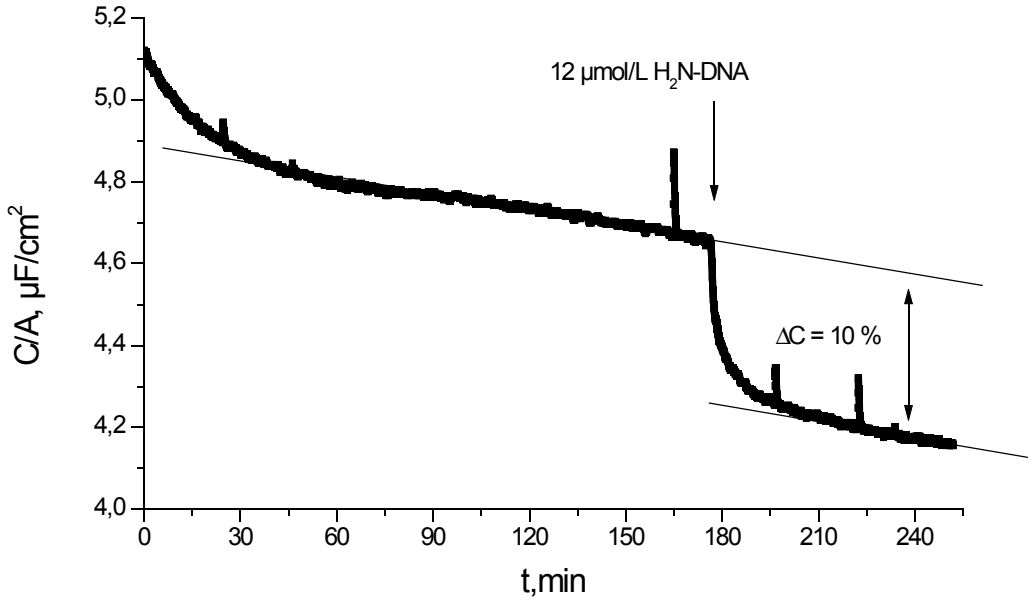


Fig. 4.11. Capacitance change due to nonspecific adsorption of 12 µmol/L of amino-modified DNA-oligomers (24-mer) at a SAM of 11-mercaptoundecanoic acid on a palladium without heating before addition.
Buffer: 0.5 mmol/L Na_2HPO_4, 10 mmol/L KCl, pH 4

As in case of gold electrodes, the decrease of capacitance of pretreated palladium electrodes due to nonspecific adsorption of DNA-oligomers is expected to be much smaller. This was tested in the experiment in Fig. 4.12. The same kind of electrode as in Fig. 4.11 was used: a palladium electrode covered by a monolayer of 11-mercapto-

undecanoic acid was immersed in another sample of the identical electrolyte and heated to 70 °C prior to an addition of 12 µmol/L of amino-modified DNA-oligomers.

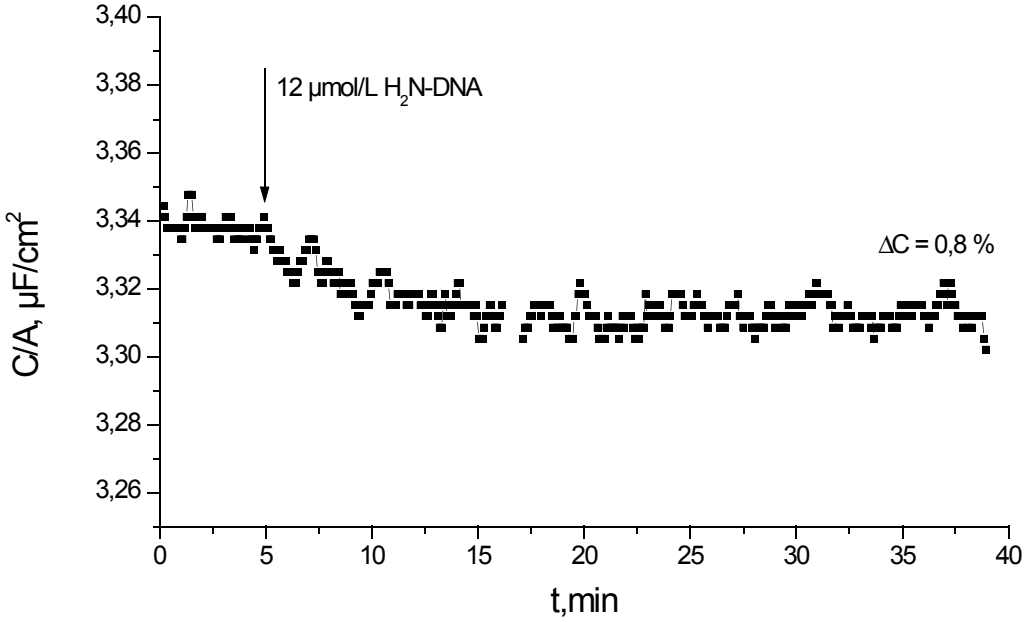


Fig. 4.12. Capacitance change due to nonspecific adsorption of 12 µmol/L of amino-modified DNA-oligomers (24-mer) on a SAM of 11-mercaptoundecanoic acid on palladium electrodes heated to 70 °C prior to addition.
Buffer: 0.5 mmol/L Na_2HPO_4, 10 mmol/L KCl, pH 4

Fig. 4.12 shows, after addition of the same amount of amino-modified DNA-oligomers, a more than ten times smaller capacitance change due to nonspecific adsorption for the pretreated palladium electrode in contrast to the unheated one. Again, as in the experiments with gold electrodes in chapter 4.1, no coupling reagent was added to the electrolyte. Therefore, only nonspecific adsorption could be the reason for the detected capacitance changes. The difference in capacitance changes for the palladium electrodes is even larger than for the gold electrodes. This may be caused by the existence of more

defects in the thiol monolayer on palladium due to the absence of a proper cleaning procedure. Compared to the capacitance change of heated gold electrodes (Fig. 4.9, p.46), the value is a little smaller, but in the same range (0.8% vs. 1% change of capacitance).

In this chapter, a predictable reaction of the capacitance of alkanethiol layers on gold or palladium was achieved. This makes alkanethiol layers on those substrates a useful basic layer for the interface preparation of genosensors, where, in the analytical step, higher temperatures are needed in order to improve the selectivity of the sensor.

Furthermore, it was shown that for gold electrodes as well as for palladium electrodes the capacitance of electrodes – that were not pretreated by heating – decreases dramatically upon addition of DNA-oligomers and their nonspecific adsorption at the electrode surface.

These capacitance decreases happen for two reasons: firstly, there is the build-up of a layer of molecules in addition to the already existing thiol layer. Secondly, the nonspecifically adsorbed molecules cover a part of the defects in the layer, leading to a diminished accessibility of the electrode surface for electrolyte molecules.

These defects in the monolayer can be partly eliminated by heating (and cooling) the electrode prior to adding the DNA molecules, which will result in a considerably smaller capacitance decrease.

Heated palladium electrodes covered by a monolayer of short chain thiols displayed a little smaller relative capacitance change than heated gold electrodes covered by a monolayer of long chain thiols upon nonspecific adsorption of DNA molecules. Although a promising alternative to gold electrodes, palladium electrodes were not further used in this thesis as due to the lack of a proper cleaning procedure, influences of impurities at the electrode surface could not be excluded.

The amount of nonspecifically adsorbed DNA-oligomers on electrodes pretreated by a heating procedure is definitely not smaller than of those not heated. What is smaller, though, is the capacitive response, which varies according to the elimination of defects in the monolayer. However, for the application in biosensors it is extremely important to reduce the amount of nonspecifically adsorbed molecules in order to minimize the number of false positive results. The following chapter will focus on finding an effective method to reduce the amount of DNA molecules that adsorb nonspecifically on alkanethiol covered gold surfaces.

5. PREVENTION OF NONSPECIFIC ADSORPTION OF DNA-OLIGOMERS

The working principle of genosensors (biosensors with ssDNA as receptors) is the detection of the hybridization process between the immobilized DNA strand and the target strand which is present in a fluid sample (cf. chapter 1).

As shown in chapter 4, one major problem working with DNA-oligomers in combination with self-assembled monolayers (SAMs) of carboxy-modified thiols on gold (or palladium) is the large nonspecific adsorption of these DNA-oligomers to the surface. The pretreatment by heating and the concomitant optimization of the alkanethiol monolayer only diminished the capacitive response caused by nonspecific adsorption; nonspecific adsorption itself could not be eliminated.

Nonspecifically adsorbed DNA molecules are not only unwanted during immobilization of the amino-modified strands to the surface but can also be the reason for alterations of the results during analysis.

This is due to the fact that target molecules, too, can adsorb nonspecifically ((b) in Fig 5.1), which would lead to an unwanted capacitive response of the sensor, since only hybridization is expected to give a signal change: (a) in Fig 5.1.

If nonspecifically adsorbed receptors (remaining from the immobilization step) also take part in the analytical step (hybridization), the results cannot be quantified since some of the attached DNA molecules may not be fully accessible for hybridization: (c) Fig 5.1.

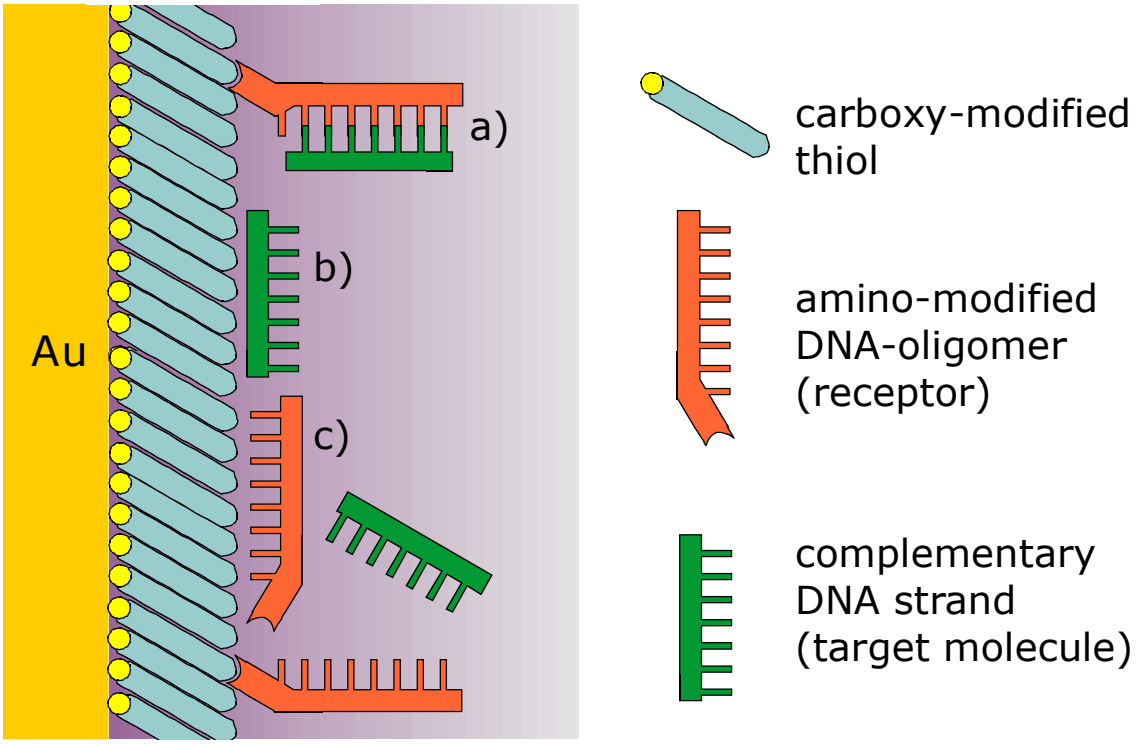


Fig 5.1. Analysis by hybridization (a) and reasons for false positive results. (b): due to nonspecific adsorption of target molecules. (c): if nonspecifically adsorbed receptor molecules take part in the analysis, some of them may not be accessible for hybridization, therefore quantification would be difficult.

Both reasons ((b) and (c) in Fig 5.1) for alterations in the measured signal would be weakened if there was a way to prevent DNA-oligomers from adsorbing nonspecifically at the surface of sensor chips.

In the following chapter (5.1), the influence of the existence of different tail groups at the alkanethiols on nonspecific adsorption will be tested (chapter 5.1). A possibility will then be presented to lessen nonspecific adsorption of DNA-oligomers without influencing the possibility to immobilize them specifically (chapter 5.2).

5.1. UNIFORM MONOLAYERS

Fig. 5.2 explains the actual structure of the solid substrate-solution interface of the sensor chip during immobilization of DNA-receptors at a SAM of carboxy-modified thiols.

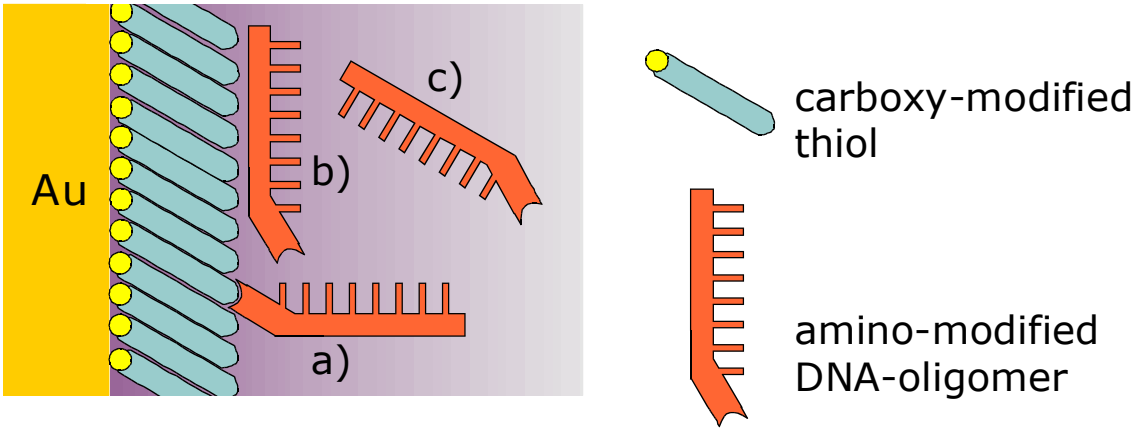


Fig. 5.2. Solid substrate-solution interface of a sensor chip for genosensors during immobilization. A gold surface is covered by a SAM of carboxy-modified thiols. Immobilization (a) of the amino-modified DNA-oligomers as well as nonspecific adsorption (b) occurs. There are still some DNA molecules in solution (c).

The metal surface (gold or palladium) is covered by a SAM of carboxy-modified thiols. Amino-modified DNA-oligomers and a coupling reagent are added to the electrolyte. The amino groups of the DNA-oligomers react with the carboxy groups of the monolayer (a). Some of the DNA molecules adsorb nonspecifically at the surface (b), while some of them are still in solution (c).

What is important for the use of capacitance-based biosensors is the magnitude of the capacitive response caused by nonspecific adsorption. To prove this point on a gold electrode covered with a SAM of carboxy-modified thiols, amino-modified DNA-oligomers were added in the absence of a coupling reagent. Consequently, the resulting

capacitive response should be solely due to nonspecific adsorption. Fig. 5.3 shows the result of such an experiment. A small amount (total concentration in the measuring chamber: 5 µmol/L) of amino-modified DNA-oligomers was added to the electrolyte, in which the gold electrode – covered by a monolayer of 16-mercaptohexadecanoic acid – was immersed. The result was a dramatic change of capacitance (ΔC = 10%). If no (nonspecific) adsorption had happened, the value of capacitance should have remained stable at the value of about 2.4 µF/cm^2 (Fig. 5.3).

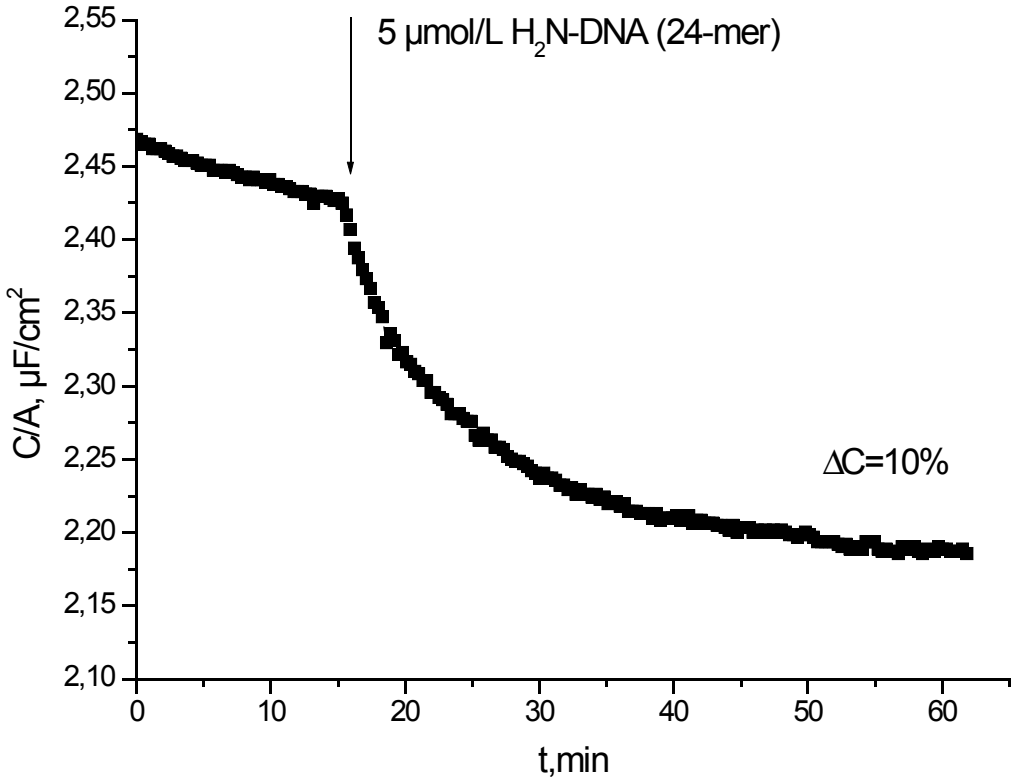


Fig. 5.3. Capacitance change of a gold electrode covered by a SAM of 16-mercaptohexadecanoic acid due to nonspecific adsorption of amino-modified DNA-oligomers (24-mers).
Total concentration in the measuring chamber: 5 µmol/L.
Buffer: 0.5 mmol/L Na_2HPO_4, 10 mmol/L KCl, pH 4.

A possible solution to this problem of nonspecific adsorption of DNA molecules can be found in the literature (e.g. [Tombelli et al., 2000], [Hermanson, 1995]).

[Tombelli et al., 2000] try to detect DNA hybridization by means of a quartz crystal microbalance (QCM). QCM is a mass sensor and any molecule able to bind to the surface is a possible interferent. Therefore nonspecific adsorption is also a problem for QCM measurements. As a blocking layer to prevent nonspecific adsorption, dextran (Fig. 5.4) is used (personal communication S. Tombelli, Florence University).

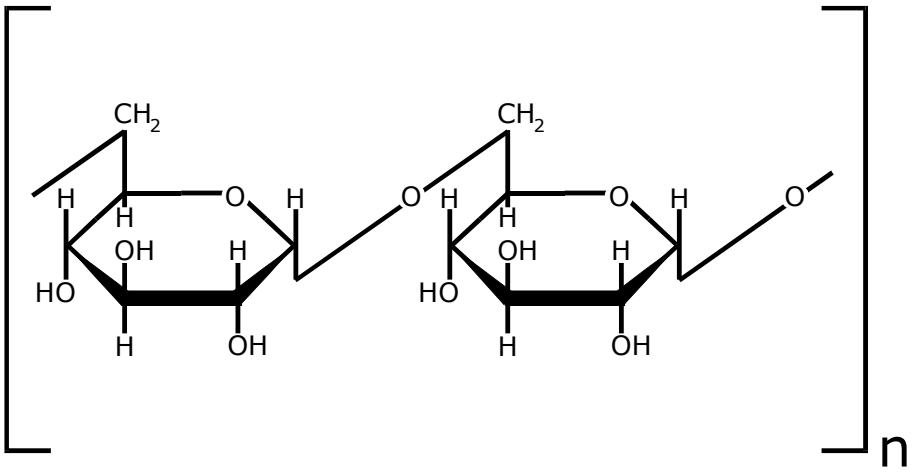


Fig. 5.4. Isomaltose (α-1,6) repeating unit of dextran polymer chains, after [Hermanson, 1995] and [Voet, 1994].

For capacitive measurements this approach would not be very useful because of the dextran molecules possessing a large volume. An additional layer of these blocking molecules between the gold/thiol layer and the immobilized DNA receptor would shift the detection event further away from the surface. As shown in chapter 2, the capacitance of the layer on the metal electrode is reciprocal to the layer thickness (see eq. 2.1, p. 6). Fig. 5.5 shows that the change of capacitance (ΔC) decreases when the change of layer thickness (Δd), for example by a reaction between receptor and target molecule, happens further away

from the surface, even if the change of layer thickness is the same for both cases.

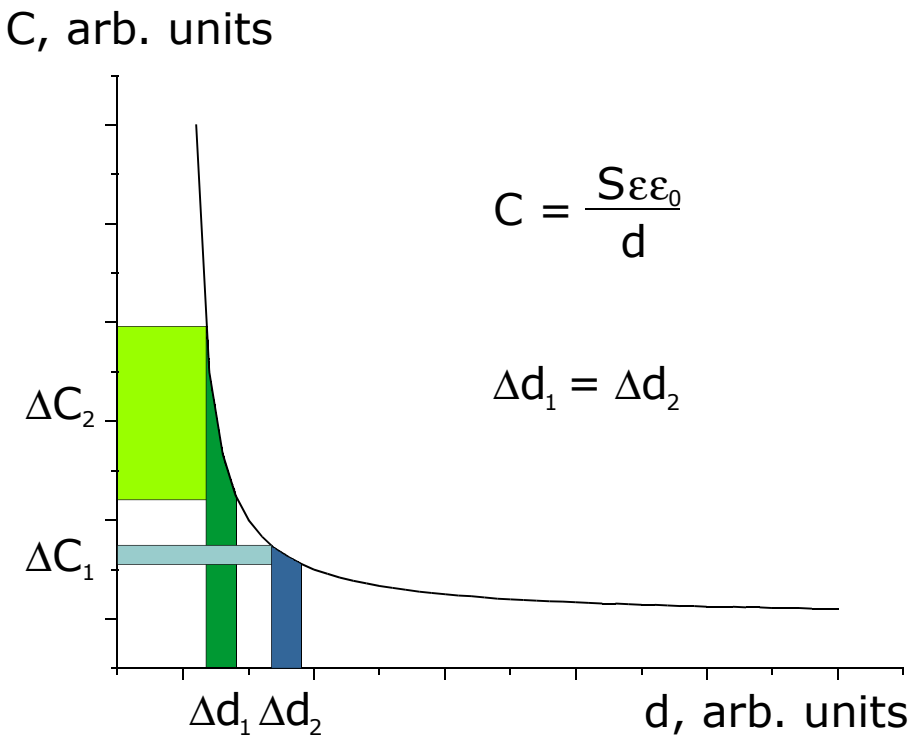


Fig. 5.5. Change of capacitance upon change of layer thickness in dependence on the distance to the electrode surface. The same change in layer thickness (Δd) results in a decreased change in capacitance (ΔC) if it happens farer away from the surface.

Therefore it is not useful to insert a thick layer of polymers in order to prevent nonspecific adsorption because this would result in a lower relative capacitance change during analysis.

Dextran (Fig. 5.4) is very rich in hydroxy groups. If the hydroxy groups prevent nonspecific adsorption of DNA-oligomers, then hydroxy-modified thiols should also display this effect. Also [Levicky et al., 1998] proposed to use hydroxy-modified thiols in order to diminish nonspecific adsorption of DNA, which would be favorable for capacitance-based measurements because nonspecific adsorption of DNA would be reduced while the insulating layer would remain thin.

In order to examine this, a gold electrode was covered by 11-mercaptoundecanol bearing solely hydroxy groups as tail groups. According to the proposition mentioned above, no or only very small capacitance changes are expected after addition of DNA.

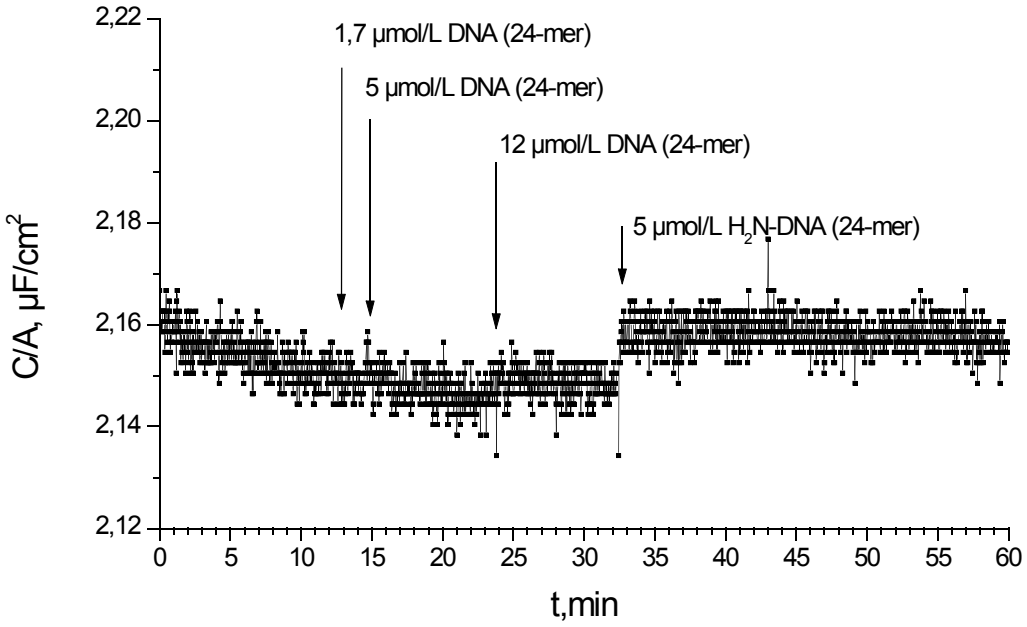


Fig. 5.6. Capacitance change of a SAM of 11-mercaptoundecanol due to addition of DNA-oligomers (24-mers), unmodified and modified by amino groups.
Electrolyte: 5 mmol/L Na_2HPO_4, 10 mmol/L KCl, pH 4.

Fig. 5.6 shows the capacitive effect after addition of DNA-oligomers to this electode. It is evident that neither the addition of unmodified DNA-oligomers nor of amino-modified DNA-oligomers triggers a change of capacitance. The capacitance remains at a value of about 2.15 to 2.16 µF/cm^2 with only a small drift noticeable.

As there is no capacitance change after addition of DNA-oligomers to the electrolyte into which a gold electrode covered by a SAM of hydroxy-modified thiols is immersed, the hydroxy groups of the thiols

evidently prevent nonspecific adsorption of DNA. Consequently (with regard to nonspecific adsorption) hydroxy-modified thiols would be a perfect basic layer for the preparation of genosensors.

The only downside of using hydroxylated thiols is that immobilization (via EDC) of amino-modified oligomers to these thiols is not possible because chemical conjugates may be formed using EDC only if one of the molecules contains a primary amine and the other a carboxy group [Hermanson, 1995]. While the amine is present with the amino-modified DNA-oligomers, there are no carboxyl groups present when hydroxy-modified thiols are used to form the monolayer at the surface, which is why a coupling via EDC is not possible.

5.2. MIXED SELF-ASSEMBLED MONOLAYERS

The advantages of both of the thiol tail groups – immobilization of amino-modified oligomers via EDC to carboxy groups as well as prevention of nonspecific adsorption of DNA by using hydroxy groups – shall now be integrated. For that purpose an alkanethiol layer with a mixture of tail groups on the thiol/electrolyte interface is prepared. This can be achieved during the interface preparation procedure (cf. chapter 3) by coadsorption of thiols modified with different tail groups (carboxy groups and hydroxy groups) at the gold surface.

Gold electrodes were immersed in chloroform bearing mixtures of the two species of alkanethiols in different ratios and thiols were deposited via self assembly at the electrodes. Consequently, the influence of the ratio of the mixture on the electrodes´ capacitance was examined. For this purpose, electrodes prepared with different ratios of carboxy-modified thiols and hydroxy-modified thiols were immersed in electrolyte and their capacitance was determined after reaching a stable value (starting value).

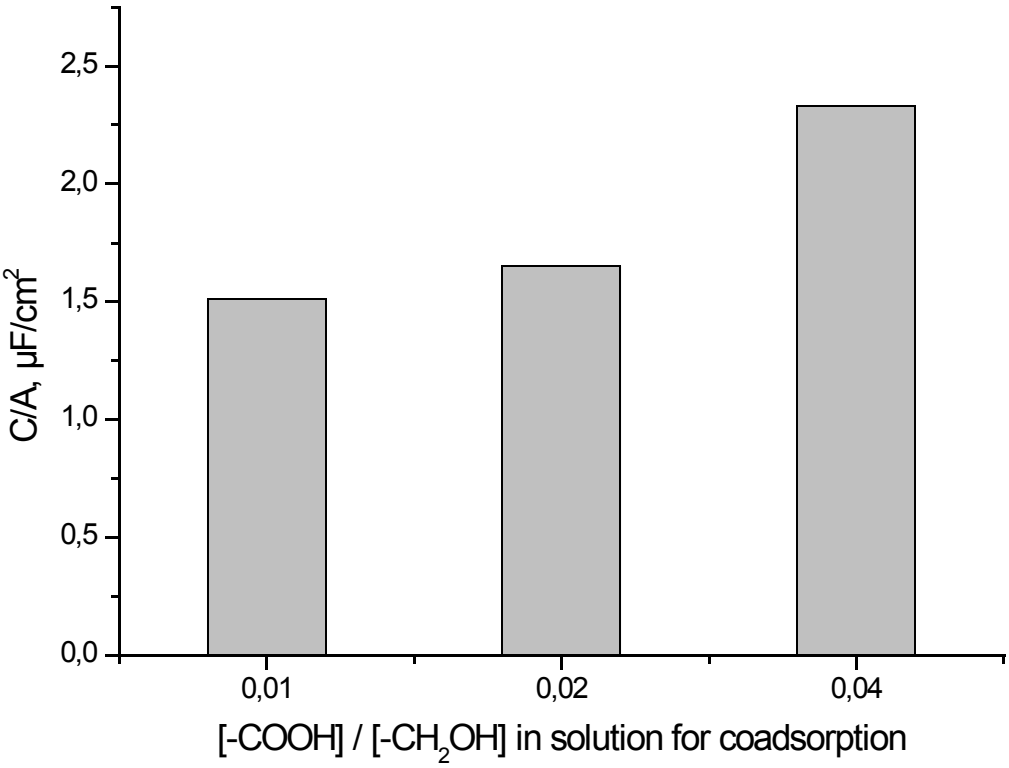


Fig. 5.7. Values of capacitance of SAMs of thiols (C16) after stabilization in electrolyte. Thiols with different ratios of tail groups dissolved in chloroform were used for coadsorption (carboxy groups/hydroxy groups = 1 : 100, left, 1 : 50, middle, and 1 : 25, right).
Electrolyte (0.5 mmol/L Na_2HPO_4, 10 mmol/L KCl, pH 4)

The initial capacitances for SAMs composed of mixtures of thiols with carboxy and hydroxy tail groups are slightly different according to the ratio of tail groups in the solution used for coadsorption (Fig. 5.7). The values for SAMs composed of only one species of thiols varied from about 1.3 µF/cm^2 for 16-mercaptohexadecanol to about 2.4 to 3 µF/cm^2 for 16-mercaptohexadecanoic acid in 0.5 mmol/L Na_2HPO_4, 10 mmol/L KCl, pH 4. In literature a dependence of the starting values of capacitance and the tail group of the thiol used for self assembly at

the surface of a gold electrode also can be found. Capacitance values in 0.1 mol/L NaF vary from 1.8 μF/cm^2 for monolayers composed of $HS(CH_2)_{11}OH$ to 2.5 to 3.5 μF/cm^2 for those built of $HS(CH_2)_{10}COOH$ [Finklea, 1996]. The differences in starting values for capacitance are thus an indication that there actually are different ratios of thiols with the two tail groups attached to the gold surface. Having attached different mixtures of thiols to the gold surface, the magnitude of nonspecific adsorption at surfaces covered by these mixed SAMs shall be studied. Fig. 5.3 and Fig. 5.6 show quite clearly that the capacitance changes of the monolayers consisting solely of 16-mercapto-hexadecanoic acid and 16-mercaptohexadecanol vary considerably. We shall now examine how the capacitances of mixed monolayers composed of these two kinds of alkanethiols react upon addition of DNA-oligomers.

For the experiment in Fig. 5.8, a gold electrode covered by a mixed SAM was immersed in electrolyte. Thiols with carboxy groups and hydroxy groups in a ratio of COOH : CH_2OH of 1 : 25 in the solution used for coadsorption. Amino-modified DNA-oligomers but again no coupling reagent was added and the capacitance change upon addition was detected.

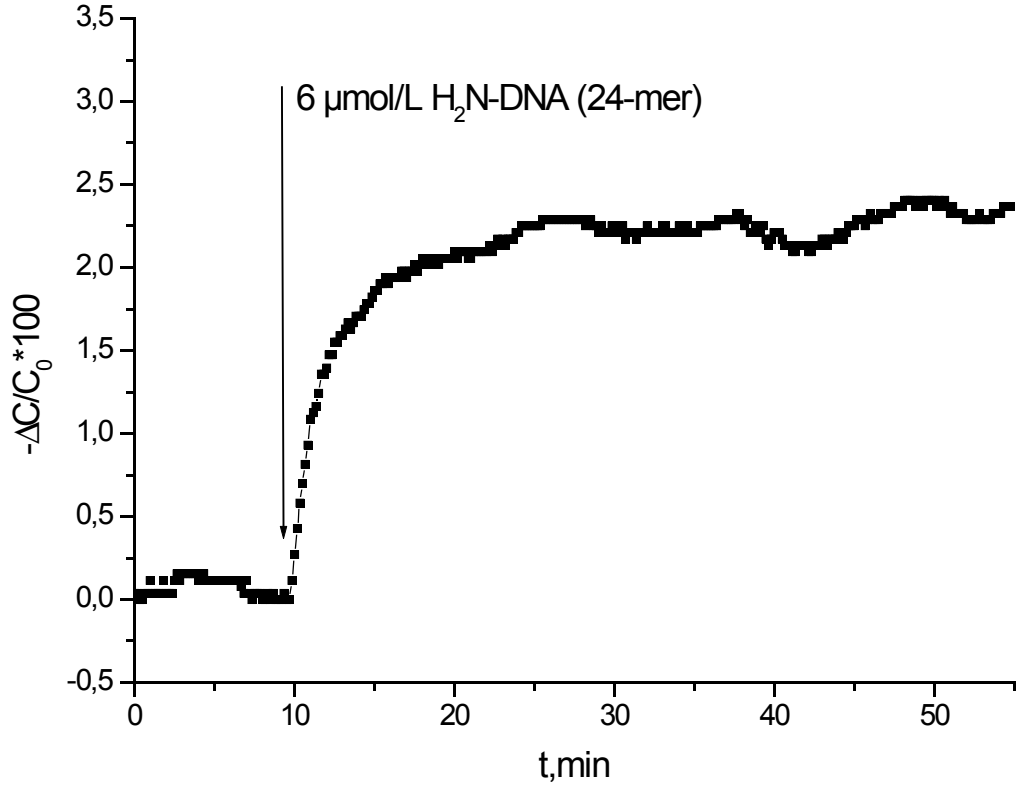


Fig. 5.8. Capacitance change of a mixed SAM coadsorbed from chloroform with of 16-mercaptohexadecanoic acid and 16-mercaptohexadecanol (1 : 25) due to addition of 6 µmol/L (total concentration) of H_2N-DNA-oligomers (24-mers).
Electrolyte: 0.5 mmol/L Na_2HPO_4, 0.5 mol/L NaCl, pH 5.9

Regarding the ratio of alkanethiols used in this experiment, some nonspecific adsorption of DNA-oligomers can still be observed. The capacitance change amounts to 2.5%. However, for electrodes in a similar buffer solution and covered only by a monolayer of carboxy-modified thiols, the addition of nearly the same amount of DNA-oligomers leads to a capacitance decrease of about 10% (Fig. 5.3). It follows that nonspecific adsorption of DNA-oligomers could, to some

extend, be blocked by using this mixed monolayer consisting of 16-mercaptohexadecanoic acid and 16-mercaptohexadecanol.

In many applications of genosensors, large amounts of DNA can be present in the solution. It will be interesting to see if the hydroxy groups will still be able to block nonspecific adsorption if more DNA is added to the electrolyte. This will be tested by the following experiment.

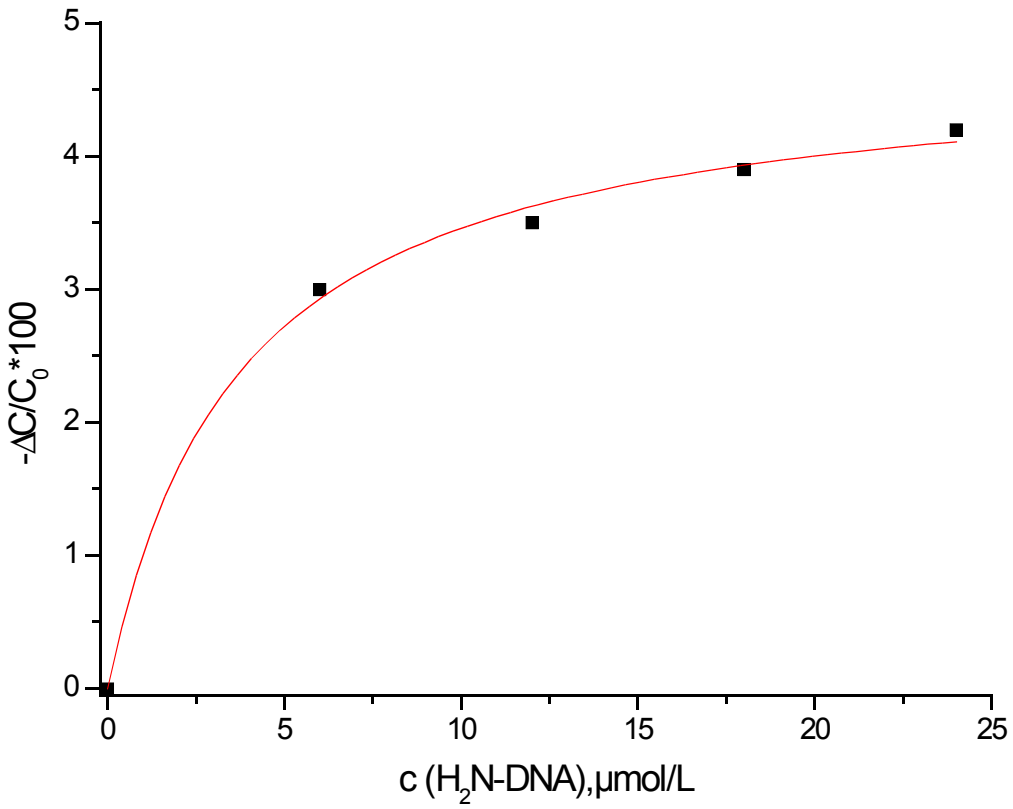


Fig. 5.9. Capacitance change of a mixed SAM prepared from a solution of thiols in chloroform of 16-mercaptohexadecanoic acid and 16-mercaptohexadecanol (1 : 25) due to addition of H_2N-DNA-oligomers (24-mers). The red curve corresponds to a fitted Langmuir adsorption isotherm.
Electrolyte: 0.5 mmol/L Na_2HPO_4, 0.5 mol/L NaCl, pH 5.9

A gold electrode covered by a mixed monolayer prepared from a solution of thiols with a ratio of carboxy groups : hydroxy

groups = 1 : 25 was immersed in electrolyte and several additions of amino-modified DNA-oligomers were performed. Fig. 5.9 shows the relative capacitance changes upon these additions. The red curve corresponds to a fitted Langmuir adsorption isotherm $Kc/(c_{1/2}+c)$ according to a proportional dependence of the capacitance change on the amount of adsorbed molecules [Krause et al., 1996]. A good fit was obtained for $K = 4.7 \pm 0.2$ and $c_{1/2} = 3.7 \pm 0.6$ µmol/L, corresponding to a binding constant of $(2.7 \pm 0.5) \cdot 10^{-6}$ L/mol.

The saturation value K for the capacitance change ΔC of about 4 to 5% derived from fitting shall now be compared to saturation values for monolayers composed of thiols with different ratios of tail groups in the solution used for coadsorption.

Fig. 5.10 gives an overview of the saturation values of the relative capacitance changes after addition of amino-modified DNA-oligomers to electrodes covered by alkanethiol monolayers prepared from solutions of thiols with different ratios of carboxy groups to hydroxy groups. Depending on the ratio, different magnitudes of these saturation values can be observed and are decreasing with a decreasing amount of thiols with carboxy groups present in the solution used for coadsorption.

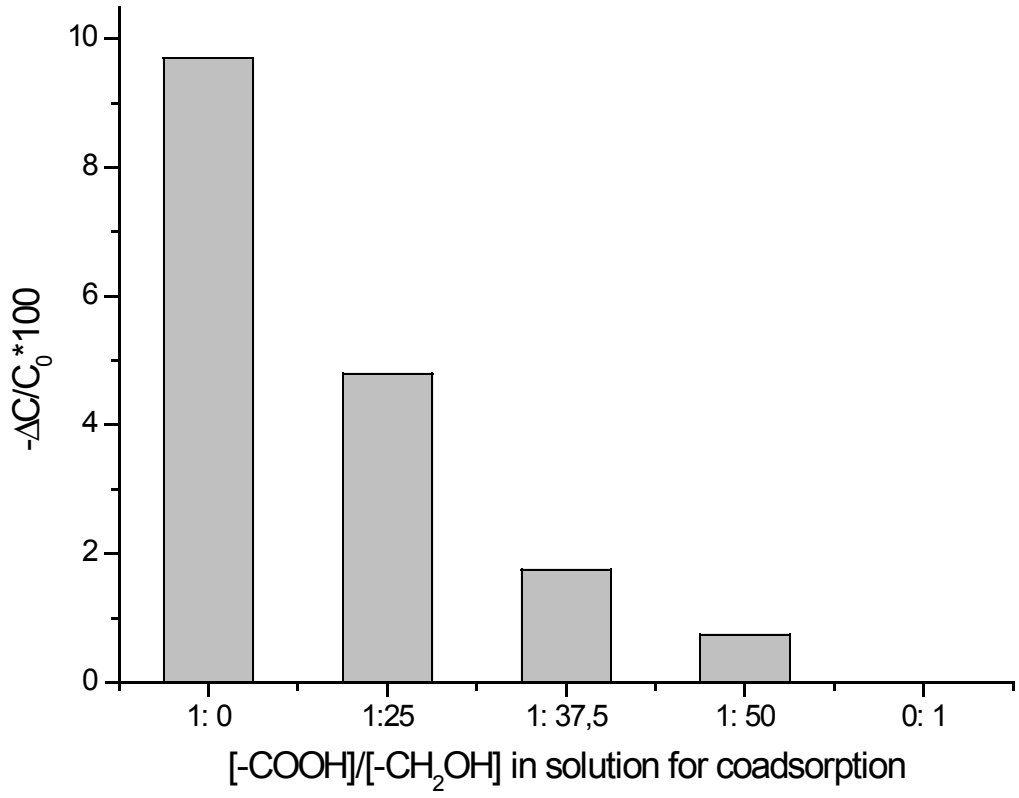


Fig. 5.10. Saturation values (K) of capacitance change derived from Langmuir fitting for addition of H_2N-oligomers (24-mers) of SAMs of thiols (C16) prepared from solutions with different ratios of tail groups (carboxy tail groups/hydroxy tail groups). Electrolyte: 0.5 mmol/L Na_2HPO_4, 10 mmol/L KCl, pH 4.

With regard to the interface preparation of genosensors, not only the diminution of nonspecific adsorption (maximum of hydroxy groups at the surface) has to be taken into account but also an optimum of coupling sites to be able to immobilize the DNA-oligomers via a chemical reaction (maximum of carboxy groups at the surface). These two contradictory requirements have to be integrated in order to find the ideal composition of tail groups at the interface of genosensors. A

theoretical value for this ideal ratio will be calculated in the following section.

5.3. CALCULATION OF THE OPTIMIZATION OF THE RATIO OF TAIL GROUPS

The following sketch shows the situation on the electrode after immobilization of DNA-oligomers at a mixed SAM with the ideal ratio of carboxy groups to hydroxy groups (Fig. 5.11).

Optimal space between the oligomers for hybridization is provided, but nonspecific adsorption of DNA-oligomers – either during their immobilization or during analysis – is prevented.

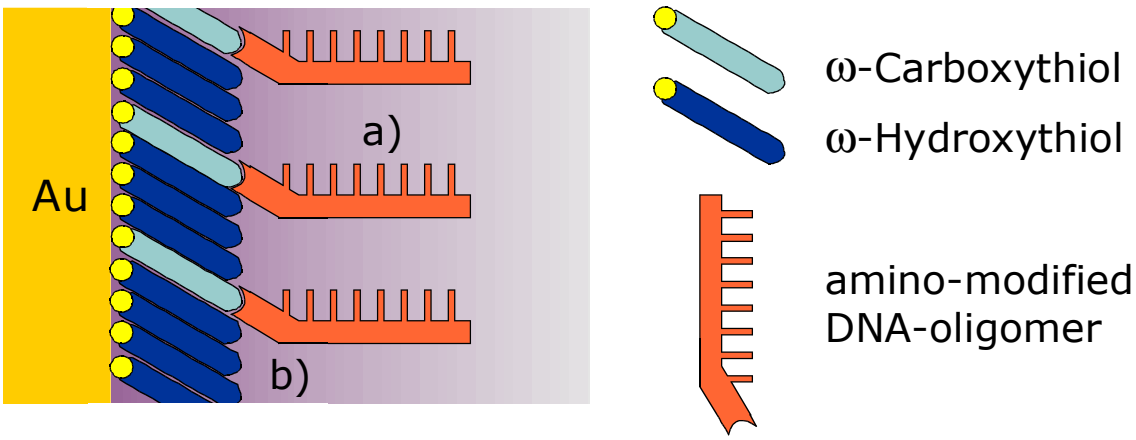


Fig. 5.11. A gold electrode covered by a mixed SAM of alkanethiols with carboxy groups and hydroxy groups as tail groups after immobilization of DNA-oligomers (a). The ratio of carboxy groups to hydroxy groups is such that there is optimal space between the oligomers for DNA hybridization. Nonspecific adsorption of DNA is prevented for the most part (b).

In order to find the ideal ratio of carboxy groups and hydroxy groups, the varying cross sections of alkanethiols and oligomers were taken into account. The area covered by an alkanethiol molecule is

assumed in literature to be 18 $Å^2$ [Finklea, 1996]. The area of the cross section of dsDNA can be calculated (via its radius of 10 Å [Voet, 1994]) to be 314 $Å^2$. Then the ratio of cross sections arising from this data is 17 : 1. Therefore, a SAM of thiols with a ratio of carboxy groups to hydroxy groups of 1 : 17 at the surface would be provide a maximum of binding sites for the chemical attachment of amino-modified DNA-oligomers and still lessen nonspecific adsorption.

According to literature, the ratio of the concentration of the two components in a mixed monolayer is, in general, not the same as in the solution used for coadsorption [Bain et al., 1989b]. The study showed that for methyl terminated thiols and thiols with polar tail groups the adsorption of one species is preferred, depending on the hydrophilic properties of the solvent. In alkanes, the adsorption of the thiols with the polar tail groups is strongly favored over the methyl terminated thiols. Hydroxy groups and carboxy groups are more similar in polarity than either of them compared with methyl groups. Therefore the ratio of the mixture in chloroform will be nearly the same as in the monolayer at the surface with a slight preference of the carboxy terminated thiols. Assuming that the carboxy-modified thiols are distributed homogeneously in the mixed SAM [Bain et al., 1989b], the ratio of areas of dsDNA and alkanethiol will then come close to the ratio of thiols with carboxy and hydroxy tail groups in the solution used for coadsorption.

The area of the cross section of one single alkanethiol molecule is about 20 times smaller than that of one DNA-molecule in double helix configuration. Taking into account the considerations above, a ratio for carboxy groups to hydroxy groups of 1 : 25 should be optimal to lower nonspecific adsorption of DNA-oligomers as well as to offer enough carboxy groups to perform the coupling reaction via EDC.

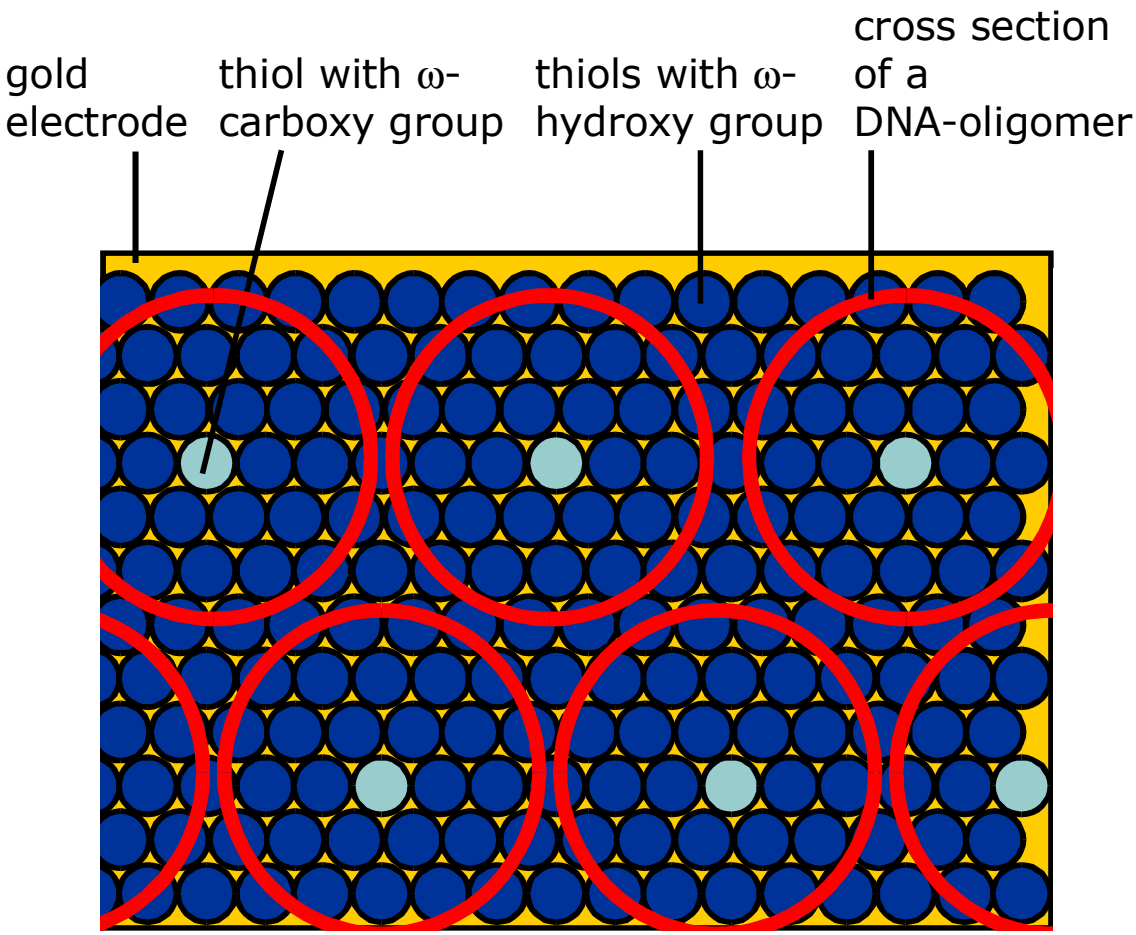


Fig. 5.12. Ratio of cross sections of alkanethiol and dsDNA molecules. For the immobilization combined with maximum prevention of nonspecific adsorption of DNA at the surface, a ratio of carboxy groups to hydroxy groups of 1 : 25 would be ideal.

Fig. 5.12 explains this situation. The small light blue circles designate the alkanethiols with carboxy groups as tail groups, the small dark blue circles the alkanethiols with hydroxy groups as tail groups. The large red circles signify the cross section of DNA in double helix configuration.

In this chapter, we were able to design a system, which will be able to reduce nonspecific adsorption with the formation of self-assembled

mixed monolayers of hydroxy-modified thiols and carboxy-modified thiols. In addition, we were able – by means of a theoretical calculation – to determine an ideal ratio of tail groups for performing immobilization. Consequently we were able to integrate both requirements.

6. THE COUPLING REAGENT EDC

In the previous two chapters, two sources of possible limitations concerning capacitance-based genosensors have been diminished. Chapter 4 was related to defects in the thiol monolayer and their effect on capacitive measurements. This problem could be solved by pretreating the electrodes with heat. Chapter 5 explained that the amount of nonspecifically adsorbed DNA molecules could be diminished by insertion of thiols with hydroxy groups into the SAM of carboxy-modified thiols.

With regard to the interface preparation of biosensors – and genosensors in particular – a third problem has to be tackled, namely optimization of the immobilization conditions for the attachment of receptors (in the case of genosensors, the DNA-oligomers) to the solid support of the sensor chip.

We shall now examine immobilization procedures using the coupling reagent 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC).

At first, a simple test for the stability of EDC in aqueous solution will be presented and the results will be used to explore the influence of various electrolyte components as well as the influence of pH on the stability of EDC. Knowledge of either the stability or the loss of EDC in aqueous solutions will result in the application of appropriate immobilization conditions.

6.1. DEPENDENCE OF ABSORPTION CHARACTERISTICS OF EDC ON EDC CONCENTRATION

Water-soluble carbodiimides, especially 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide (EDC) (Fig. 6.1), are often used as zero-

length cross-linkers in organic chemistry, peptide synthesis, or for immobilization of different biological molecules on solid support [Hermanson, 1995], [Rich and Singh, 1979], [Wendlberger, 1974]. The conjugation reaction itself is described in chapter 2, Fig. 2.8.

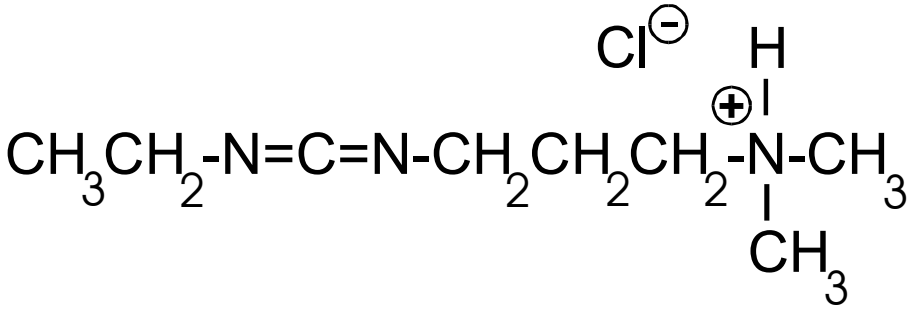


Fig. 6.1. EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride)

However, carbodiimides are very unstable: they can easily react with nucleophiles including sulfhydryl [Carraway and Triplett, 1970] or amino [Kurzer and Douraghi-Zadeh, 1967] groups, with tyrosine [Carraway and Koshland, 1968], and other compounds [Kurzer and Douraghi-Zadeh, 1967]. However, the stability of those carbodi-imides in water lasts only for a short time [Kurzer and Douraghi-Zadeh, 1967]. An application of carbodiimide techniques to new objects demands optimization of the reaction conditions that take possible side reactions of this component into account. Therefore, a simple test for carbodiimide stability is required.

A method was described in literature to test the stability of water-soluble carbodiimides [Gilles et al., 1990]. The effect of phosphate containing compounds, glycine methyl ester, amines, halides, and pH was studied. However, the assay used is nondirect and extremely time consuming: it is based on the formation of colored products depending on the reaction of carbodiimides with dimethylbarbituric acid.

In the subsequent paragraph, an alternative and very simple technique is described: the on-line monitoring of carbodiimide concentration based on UV-absorbance of this component.

Freshly prepared aqueous solutions of EDC show a maximum absorbance at 213/214 nm (Fig. 6.2). By way of comparison, the peaks of absorbance for different cycloalkylcarbodiimides in heptane are in the same range – between 212 and 218 nm [Behringer and Meier, 1957].

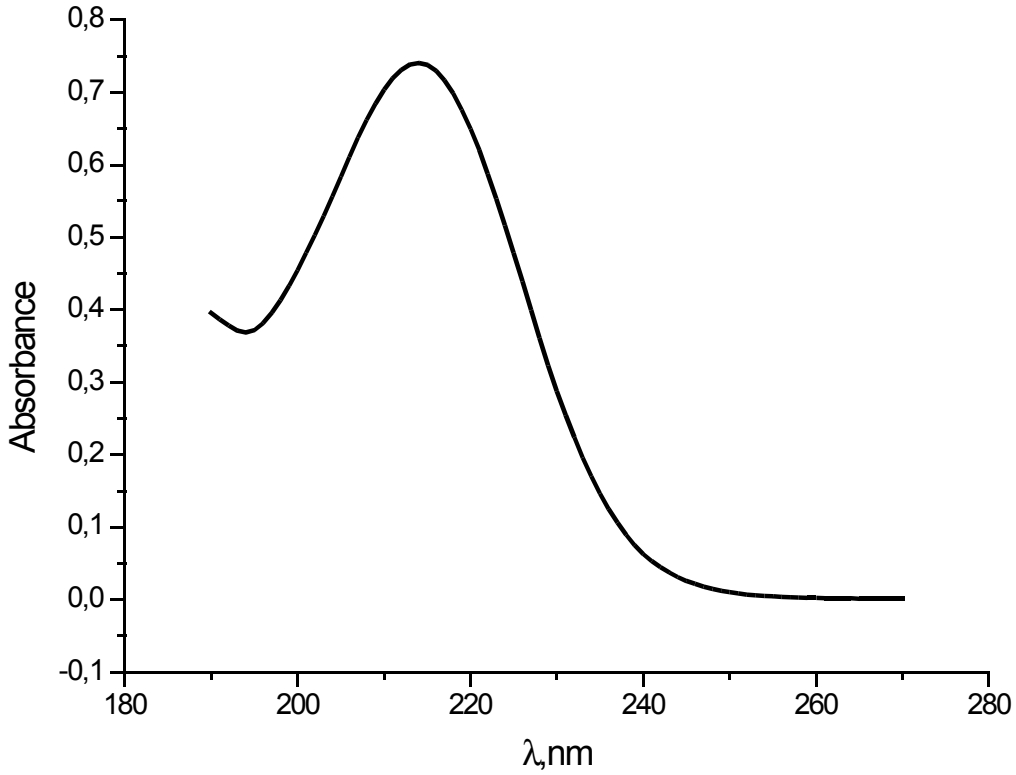


Fig. 6.2. Absorbance of 1 mmol/L EDC in water, pH 5.8.

In order to use UV-monitoring of EDC for a quantitative determination of this component, it is necessary to give a linear dependence of absorbance (*A*) on the concentration (*c*) of this component present in the solution and for the Lambert-Beer law (eq. 6.1) to be valid.

(6.1) $$A = d \sum_{i=1}^{n} \epsilon_i c_i ,$$

with *d* being the length that is passed by the beam of monochromatic light, and ϵ_i and c_i the molar extinction and concentration of each substance present [Hesse et al., 1995].

In Fig. 6.3, the absorbance of EDC at 214 nm is plotted vs. EDC concentration. No deviation from the Lambert-Beer law can be observed in the studied concentration range (5 µmol/L to 5 mmol/L).

The molar extinction at 214 nm can be calculated from this dependence as $\epsilon_{214} = 6.3 \times 10^3$ L mol^{-1} cm^{-1}.

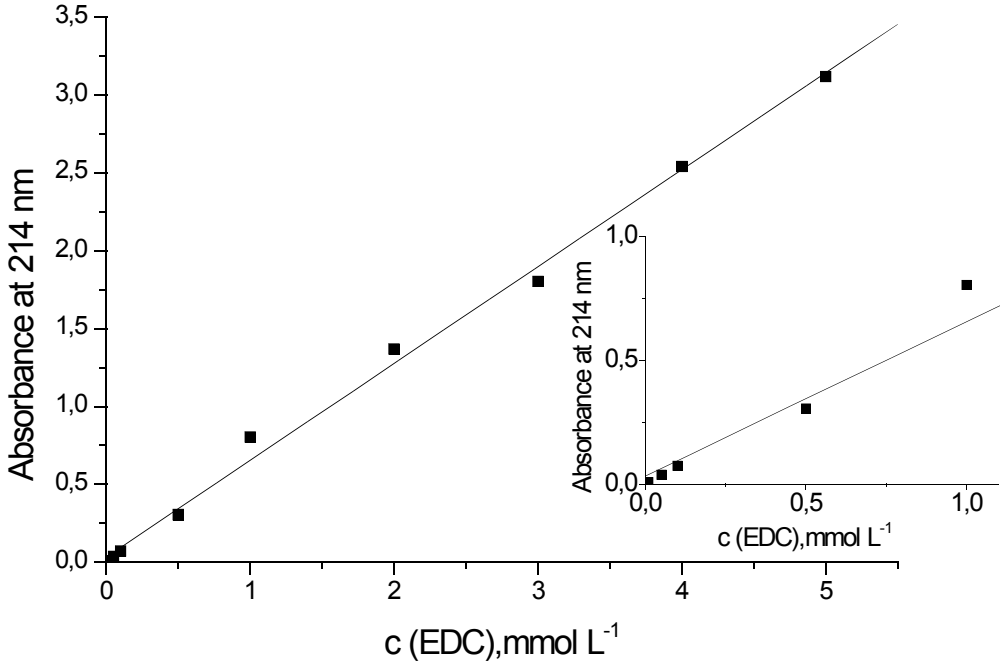


Fig. 6.3. Concentration dependence of the absorbance of EDC at 214 nm in water, pH 5.8. The line corresponds to a linear fit (r = 0.99809).

Therefore, UV-monitoring provides a simple way to study the kinetics of the reaction of EDC with various components present in the solution, an issue which will be discussed in the following section.

6.2. INTERACTION OF EDC WITH BUFFER COMPONENTS

An interaction of EDC with a carboxylic acid, for example, acetate, results in the formation of an urea by-product (cf. chapter 2.4) and simultaneously leads to the disappearance of the absorbance at 214 nm. This is shown in Fig. 6.4, where the peak at 214 nm (curve 1, black) gets smaller and vanishes within 15 minutes of time at pH 4 (curves 2, red, and 3, blue).

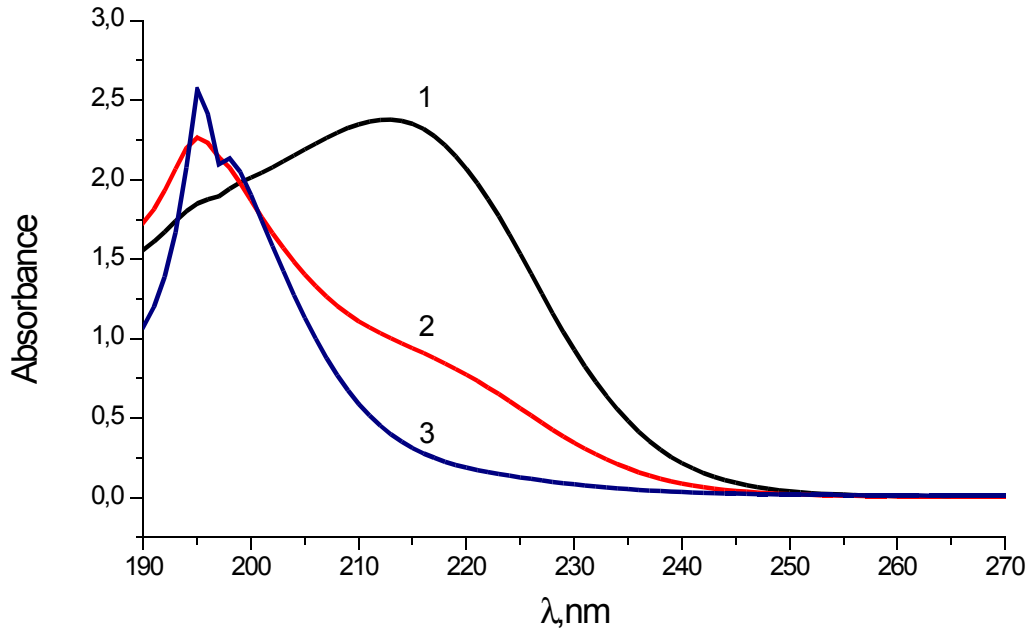


Fig. 6.4. Absorbance spectrum of EDC in acetate buffer at pH 4 (curve 1, black) and its changes during reaction progress for 5 min (curve 2, red) and 15 min (curve 3, blue).

In order to obtain more information about these changes of absorbance spectra with time, kinetic runs were performed. EDC was added to solutions of buffer components and its absorbance at 214 nm was monitored on a scale of half an hour.

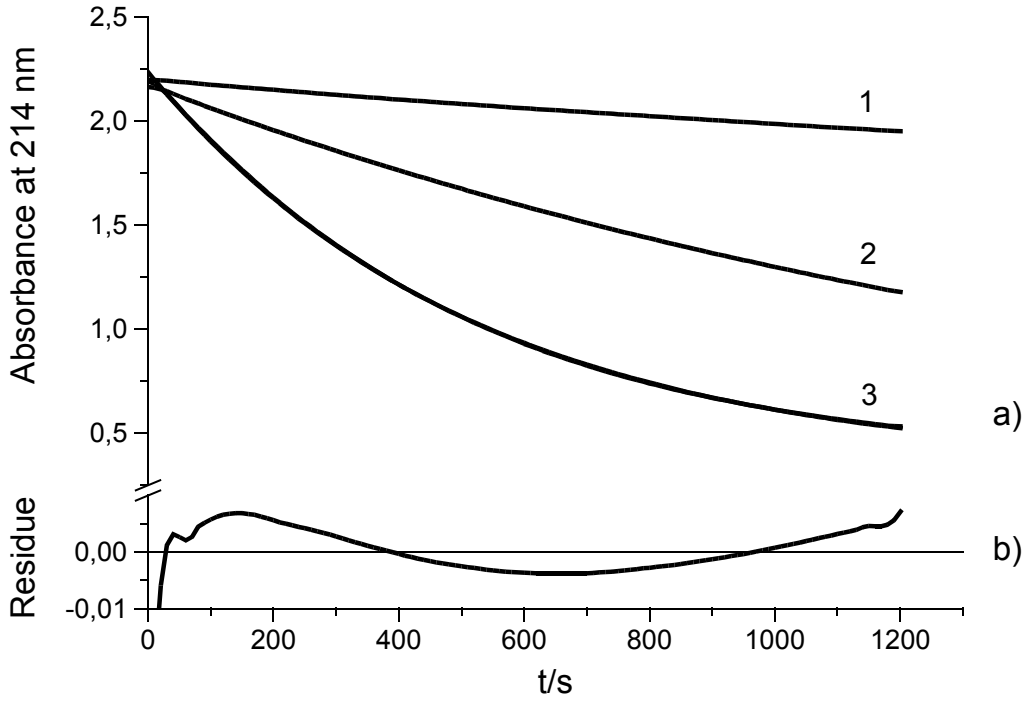


Fig. 6.5. Decrease of EDC absorbance at 214 nm after dissolving in aqueous solutions of acetate (a). Concentrations of acetate: 1 mmol/L (curve 1), 5 mmol/L (curve 2), 15 mmol/L (curve 3), each at pH 4. The monoexponential fitting of curve 3 and its deviation from the experimental data are shown (b).

The changes of absorbance were tested for different concentrations of buffer components. Typical kinetic curves measured in the presence of, for instance, acetate (1 mmol/L, 5 mmol/L, and 15 mmol/L) are shown in Fig. 6.5 a), curves 1-3. Similar curves were obtained for other substances studied, including pure water.

The kinetics of the decomposition of EDC was found to be monoexponential; there were only small variations of the fitted monoexponential curve from the experimentally found values. This can be seen in Fig. 6.5 b), giving the deviation of the two curves. Based on this monoexponential dependence, the rate of EDC decomposition can be calculated and the resulting values can serve as a means to compare the influences of different buffer components, as well as pH, on EDC.

Buffer components, as well as pH, affect the kinetics of the decomposition of EDC. Fig. 6.6 shows the effect of pH on the rate of EDC hydrolysis in pure water (only HCl or NaOH were added to adjust pH). The results found during the work for this thesis are compared in Fig. 6.6 with experimental data from [Gilles et al., 1990] and [Williams and Ibrahim, 1981], obtained by applying other methods.

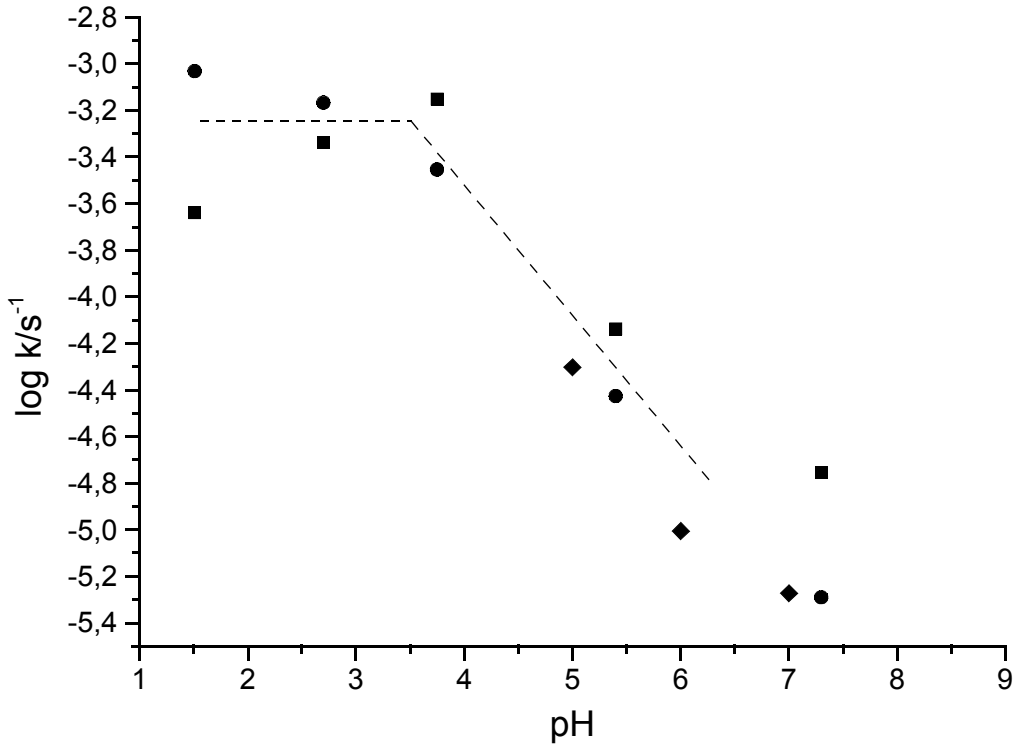


Fig. 6.6. Dependence of the rate constant of EDC decomposition on pH: (■) own data, (◆) data from [Gilles et al., 1990], (●) data from [Williams and Ibrahim, 1981]. The dashed line corresponds to the simple model based on the assumption that the pK of EDC is at 3.5 and only its protonated form takes part in the reaction (cf. Appendix B, p. 123)

The dependence of the rate of EDC hydrolysis is very strong for a pH ranging between 3.5 and 7, as opposed to the range below pH 3.5 where there is nearly no dependence of the rate on pH.

The dashed line corresponds to the simple model which is based on the assumption that the pK of EDC is at 3.5 and only its protonated form is involved in the reaction (for a calculation cf. Appendix B, p. 123). The latter statement was also suggested in [Williams and Ibrahim, 1981].

The UV-monitoring of EDC (and the following comparison of the values for the rates of decomposition) can easily be applied to study effects of additives typically present in biological samples (buffers, surfactants, etc.) on EDC stability. Some examples are presented in Fig. 6.7 and Table 6.1.

The carboxylic acids tested here were found to have the strongest effect on EDC decomposition: 50 mmol/L of citrate at pH 4 leads to an approximately 1000-fold shorter lifetime of EDC in aqueous solution compared to pure water. The decomposing properties of carboxylic acids depend on the number of carboxyl groups per molecule: citrate possessing three carboxyl groups has an effect about 30 times higher than acetate with only one carboxyl group. Some decomposing effects were observed for sulfonic acids (MOPS or HEPES), or TRIS, but the absence of a linear concentration dependence suggests other reaction mechanisms.

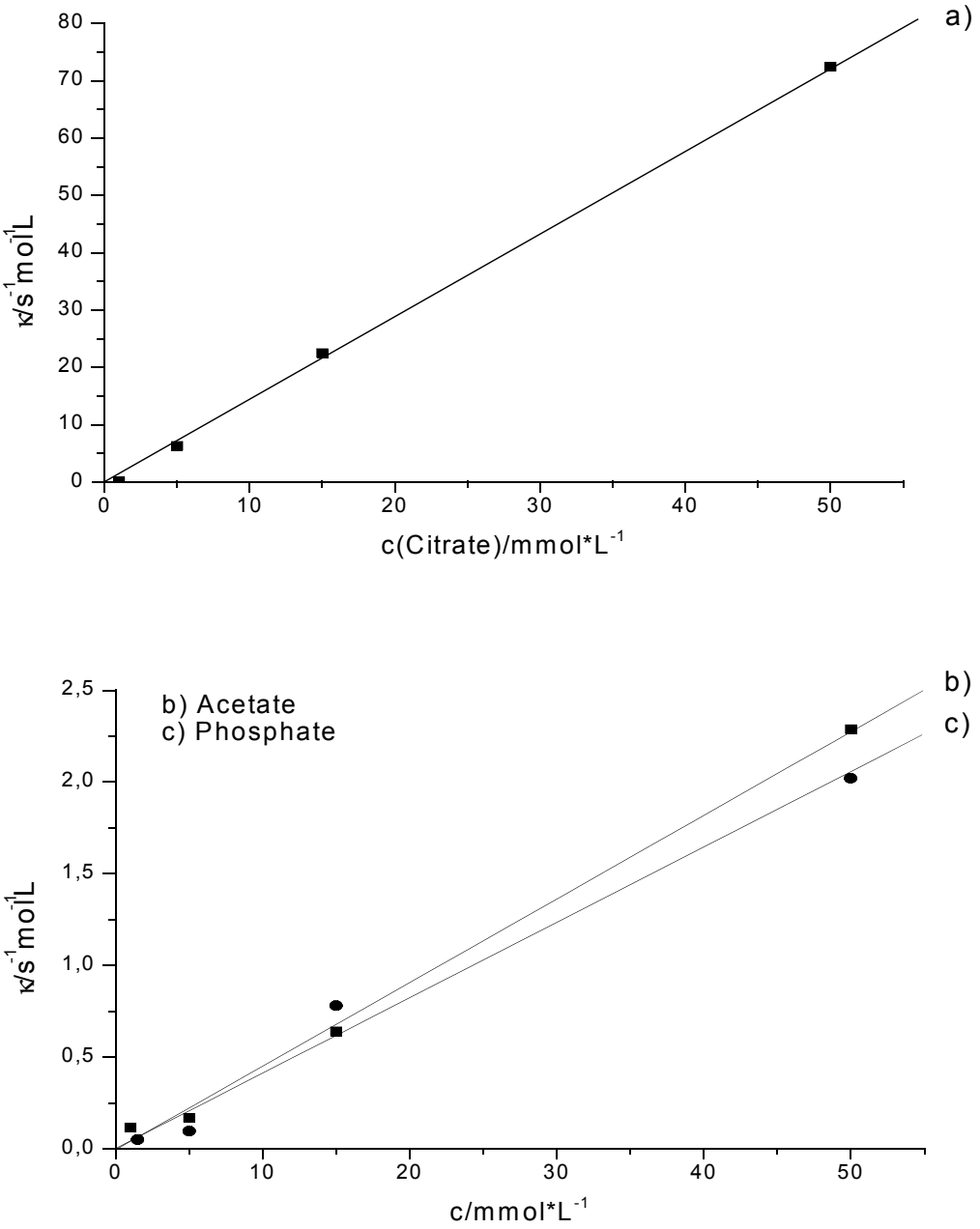


Fig. 6.7. Rate of EDC concentration decrease normalized to initial EDC concentration. The reaction has been performed in aqueous solution in the presence of different concentrations of citrate (a), acetate (b), and phosphate (c) at pH 4. The initial EDC concentration was 1 and 3 mmol/L.

Table 6.1. Rate of EDC reaction in aqueous solutions of different components [a]

	k (s^{-1}), EDC at 3 mmol/L concentration			
Component	15 mmol/L pH 4	50 mmol/L pH 4	50 mmol/L pH 5.5	50 mmol/L pH 7
Sodium acetate	0.002	$6.9 \cdot 10^{-3} \pm 4 \cdot 10^{-4}$	$8 \cdot 10^{-4} \pm 2 \cdot 10^{-4}$	$8.6 \cdot 10^{-4} \pm 5 \cdot 10^{-5}$
Citrate	0.07 ± 0.02	0.22 ± 0.05	0.043 ± 0.002	—
Disodium hydrogen phosphate[b]	$8 \cdot 10^{-4} \pm 1 \cdot 10^{-4}$	$2.0 \cdot 10^{-3} \pm 2 \cdot 10^{-4}$	$4.29 \cdot 10^{-4} \pm 2 \cdot 10^{-6}$	$3 \cdot 10^{-7} \pm 1 \cdot 10^{-7}$
TRIS	$5 \cdot 10^{-3} \pm 3 \cdot 10^{-3}$	$5 \cdot 10^{-3} \pm 4 \cdot 10^{-3}$	$3.3 \cdot 10^{-3} \pm 9 \cdot 10^{-4}$	—
MOPS	$4 \cdot 10^{-3} \pm 1 \cdot 10^{-3}$	$3 \cdot 10^{-3} \pm 2 \cdot 10^{-3}$	$3.2 \cdot 10^{-3} \pm 1 \cdot 10^{-4}$	—
HEPES	$1.9 \cdot 10^{-3} \pm 4 \cdot 10^{-4}$	$2 \cdot 10^{-3} \pm 1 \cdot 10^{-3}$	—	$4 \cdot 10^{-3} \pm 2 \cdot 10^{-3}$
SDS	$2.9 \cdot 10^{-4} \pm 3 \cdot 10^{-5}$	—	—	$2.7 \cdot 10^{-3} \pm 2 \cdot 10^{-4}$

[a] —: no data.

[b] EDC at 1 mmol/L concentration for 15 mmol/L and 50 mmol/L pH 4.

An increase in the catalytic activity of carboxylic acids at acidic pH confirms that these conditions are most favorable for reaction of EDC with carboxyl groups. The optimum pH for the whole process, however, is a compromise between this reaction of *O*-acylisourea formation and the following reaction with nucleophiles, such as primary amines. With regard to the immobilization of proteins, the empirically determined

optimum pH is usually close to their isoelectric point or slightly more acidic [Mirsky et al., 1997], [Seigel et al., 1997].

With regard to amino-modified DNA-oligomers this ideal pH will be determined in the following chapter using radiolabeled DNA molecules. The influence of ionic strength will also be examined. Eventually, the immobilization conditions that were found to be ideal for the immobilization of DNA-oligomers will be compared to the immobilization conditions used by two companies for the preparation of their sensor chips.

7. OPTIMIZATION OF DNA-OLIGOMER IMMOBILIZATION

In the previous chapter, the effect of pH and electrolyte components on the stability of EDC was studied. As mentioned before, a successful immobilization depends on influences of pH and other reaction conditions, like ionic strength and on substances inherent in the immobilization reaction, especially the receptor molecules which have to be immobilized at the surface.

In the case of DNA-oligomers as receptor molecules, it seems very reasonable that pH and ionic strength, influence the immobilization of DNA-oligomers because these parameters determine charge and solubility of the DNA molecules [Voet, 1994].

To test the influence of pH and ionic strength an independent method to capacitive measurements was selected, which used radiolabeled DNA-oligomers and scintillation counting (cf. chapter 3.2). This is a very sensitive approach, another advantage is, that immobilization or adsorption of DNA-oligomers can be measured even if the layer of immobilized or adsorbed molecules is not very densely packed or the layer that is formed is not sufficiently insulating to be detected by capacitive measurements (cf. chapter 2.3).

Using radiolabeled DNA-oligomers offers the possibility to test not only their immobilization to the surface but also to evaluate the success of different washing procedures, which help to remove nonspecifically adsorbed molecules. Even if only a small number of molecules remain on the surface (after suboptimal immobilization and washing) they can be quantitatively detected unlike with capacitive measurements. This is the reason why, for this application, the use of radiolabeled DNA-

oligomers and consequent scintillation counting seemed the more promising method than capacitive measurements.

For the scintillation counting experiments with immobilized radiolabeled DNA-oligomers, another kind of substrate was used for building up the self-assembled monolayer (SAM) of thiols. The design of the electrodes shown in Fig. 3.1 was not suitable for this kind of measurement. The immobilization was performed at open potential, therefore it was not necessary to provide an electric contact. On the other hand, the area covered by gold had to be much higher because of a high nonspecific adsorption of DNA not only to the area covered by gold, but also to the rest of the sample surface.

With regard to the electrodes described in Fig. 3.1, only 8% of the total area is covered by gold. In this design, scintillation counts caused by specific immobilization of radiolabeled DNA-oligomers were totally hidden in the background counts caused by molecules nonspecifically adsorbed at the silicon surface. Therefore, a design had to be selected to provide a larger area of gold.

The design of the plates used for the scintillation counting of immobilized, radiolabeled DNA-oligomers is shown in Fig. 3.3 where 43% of the total area consists of gold.

7.1. NONSPECIFICALLY ADSORBED DNA-OLIGOMERS

The influence of different immobilization conditions (different pH, ionic strength) on the magnitude of nonspecific adsorption were studied adding amino-modified DNA-oligomers to the solution in which the square silicon wafer samples (Fig. 3.3), covered by a SAM of 16-mercaptohexadecanoic acid, were immersed. The tail groups present at the surface were only carboxy groups. This "worst case scenario" could lead, according to chapter 5, to a very high amount of

nonspecifically adsorbed DNA-oligomers. Under these conditions, the efficiency of washing procedures could be thoroughly tested.

For each of the immobilization conditions under study (different pH, ionic strength), two of the wafer samples were incubated for 90 minutes with EDC and radiolabeled, amino-modified DNA-oligomers, and one sample without EDC, but the same amount of DNA-oligomers. After that, all samples were rinsed with water and the scintillation counts were determined (as described in chapter 3.2). The mean values of the counts for the samples with EDC were taken and plotted in Fig. 7.1 as gray columns. The difference between the single measurements and the mean value is designated by an error bar.

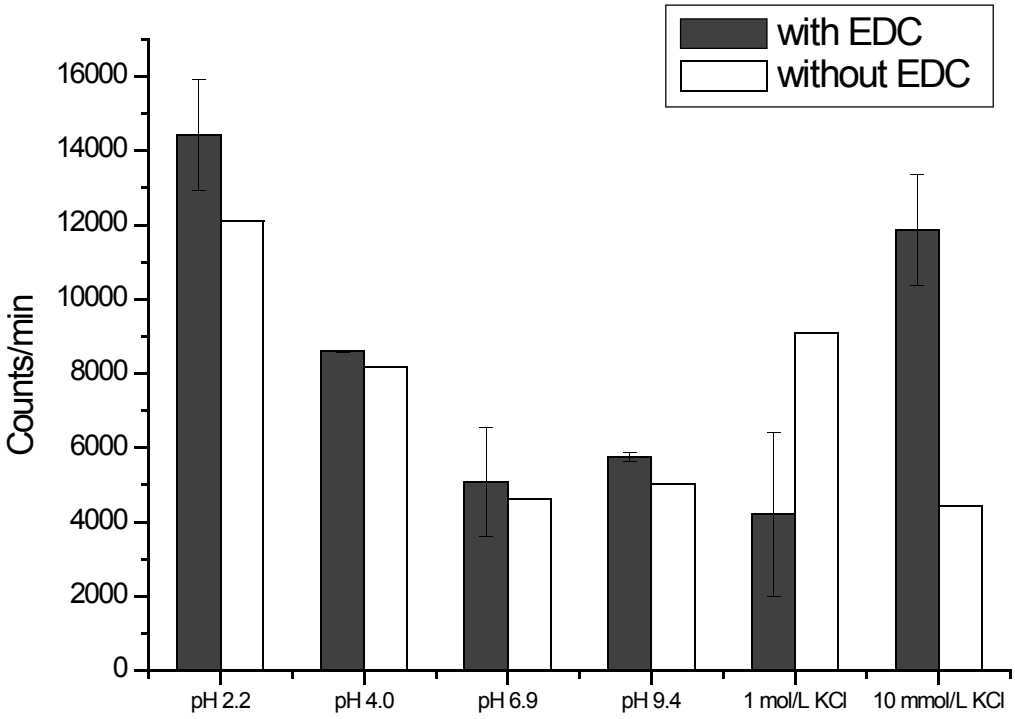


Fig. 7.1. Counts per minute for electrodes covered by a SAM of 16-mercaptohexadecanoic acid after addition of radiolabeled, amino-modified oligomers with 400 mmol/L of EDC (gray) and without EDC (white). After 90 minutes of incubation the electrodes were rinsed with water and the scintillation counts were determined. For the experiments where EDC was added, the mean value of two determinations was taken.

As can be seen in Fig. 7.1, the scintillation counts vary with pH (the first four pairs of columns in Fig. 7.1). The largest values arise from strong acidic pH. At neutral pH, a minimum of scintillation counts can be observed, whereas the values increase again with more basic pH.

However, the scintillation counts are only a little smaller for the control samples (without EDC, white columns) compared to the corresponding samples with EDC (gray columns). That means that the largest amount of the DNA-oligomers present on the chip is not

chemically immobilized but nonspecifically adsorbed at the surface of the sample.

For the two different ionic strength conditions (the last two pairs of columns) the values for the samples with and without EDC are quite different. In the case of 1 mol/l KCl the value for the control sample is even higher than that for the samples with EDC. However, the variation of the counts here is very large as can be observed for the two identical samples (large error bar). In the case of 10 mmol/L KCl, the value for the control sample is obviously smaller than that for the sample with EDC. Hence, nonspecific adsorption of DNA-oligomers here is smaller than for the samples without addition of salts (the pH samples). A reason for this smaller nonspecific adsorption could be the ionic strength, where the DNA molecules could be more soluble.

But for either condition to be varied, pH and ionic strength, it can be stated that the amount of nonspecifically adsorbed DNA-oligomers is very high. In order to evaluate in which of the different conditions the immobilization process is most successful, it is necessary to remove this amount of oligomers, which requires a suitable washing procedure.

7.2. WASHING PROCEDURES

There are two possibilities used widely in biology to remove nonspecifically adsorbed DNA-oligomers from the samples. One of them is to wash with 0.2 mol/L NaOH, the other is a washing procedure with 0.1 mol/L NaCl (personal communication Dr. Werner Deininger, Institute of Biochemistry, Genetics, and Microbiology, Regensburg University). The solubility of DNA molecules is higher under these conditions and some adsorbed molecules should be washed from the surface [Voet, 1994].

The washing procedure with 0.2 mol/L NaOH was tested in the following experiment. After the first scintillation measurements (Fig. 7.1), the samples were deposited separately in 0.2 mol/L NaOH and shaken for 30 minutes. The samples were then rinsed with water and the values for the scintillation counts determined (as described in chapter 3.2). Data was processed like before, as described for Fig. 7.1. Fig. 7.2 gives the results of the measurements.

After the samples were washed in 0.2 mol/L NaOH, the situation is quite different than after having rinsed them solely with water (Fig. 7.1). While the counts of the control samples (without EDC) vary by an average of about 400 counts per minute (red line), the differences between the samples involving EDC are more obvious. The average of counts for the control samples is equal to 0.6 pmol of DNA-oligomers.

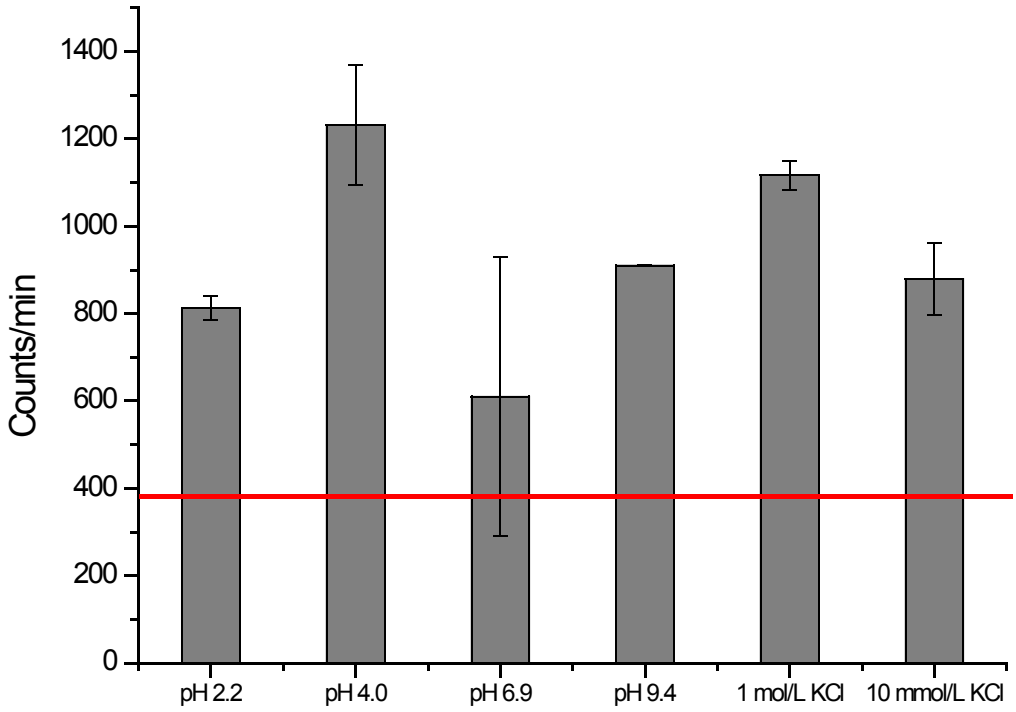


Fig. 7.2. Counts per minute for electrodes covered by a SAM of 16-mercaptohexadecanoic acid after addition of radiolabeled, amino-modified DNA-oligomers with 400 mmol/L of EDC. After 90 minutes of incubation the electrodes were rinsed with water, then washed in 0.2 mol/L NaOH for 30 minutes and rinsed again with water. The scintillation counts were then determined. The mean value of two determinations was taken. The average of the values for the control samples (without EDC) is designated by the red line (380 counts/min, SD = 142).

Regarding the different pH conditions (the first four columns), the maximum scintillation count is at a pH of 4.0. Also the difference for the counts between the samples treated with EDC and the average of counts for the control samples is most obvious. This indicates that regarding this condition nonspecifically adsorbed DNA molecules could be washed away rather effectively with 0.2 mol/L NaOH. For the tested pH range, after washing with 0.2 mol/L NaOH, the immobilization of

amino-modified DNA-oligomers to carboxyl groups on the surface via EDC is most effective at a value of pH 4.

Regarding the samples studied for varying ionic strength (the last two columns) the situation is not as obvious. If the variation (error bars) is taken into consideration, the total values of scintillation counts for both conditions are similar, just as the differences between control samples and samples with EDC involved (of about 650 counts per minute). The values resulting from the samples with EDC for 1 mol/L KCl were slightly higher than the ones for 10 mmol/L KCl, the advantage of the latter condition being that, from the outset, there is less nonspecific adsorption (cf. Fig. 7.1).

However, it should be noted, that by applying 0.2 mol/L NaOH (pH 13.3) as a washing solution the gold-thiol-bond is not totally stable. This was suggested in a diploma thesis: a dependence of the stability of the gold-thiol bond of 16-mercaptohexadecanoic acid on pH was found [Schweiß, 1997]. This effect depends on the chain length of the alkanethiol, which is used for building up the SAM, and on the potential applied. Alkanethiols with shorter chains are not as stable as thiols with longer chains [Riepl, 2000]. It was shown that for SAMs of 16-mercaptohexadecanoic acid (which were used for the experiments in this chapter) a stability for at least 100 minutes at pH 12 and open potential was reached. In order to remove a considerable amount of nonspecifically adsorbed DNA-oligomers, washing with 0.2 mol/L NaOH should take no longer than 30 minutes, with most of the immobilized thiols still remaining at the surface.

As depicted in the last section, the use of 0.2 mol/L NaOH as a washing solution is relatively limited, thus it is interesting to consider other washing techniques.

Another method used in biology, as mentioned above, to elute ssDNA from various surfaces, is to wash with a solution of 0.1 mol/L of

NaCl [Voet, 1994]. Under these conditions the gold-thiol-bond is stable therefore this washing procedure could be a more suitable and promising alternative for genosensors which are based on the stability of the gold-thiol-bond.

In the following section it is tested, whether a washing procedure with 0.1 mol/L NaCl is able to remove the part of the nonspecifically adsorbed DNA-oligomers still remaining at the surface (after washing with 0.2 mol/L NaOH).

The samples which were previously washed with 0.2 mol/L NaOH, were separately deposited in 0.1 mol/L NaCl, shaken for the duration of 10 hours, and rinsed with water. Eventually, the scintillation counts were measured. Fig. 7.3 refers to the subsequently received data, which was treated in the same manner as mentioned earlier.

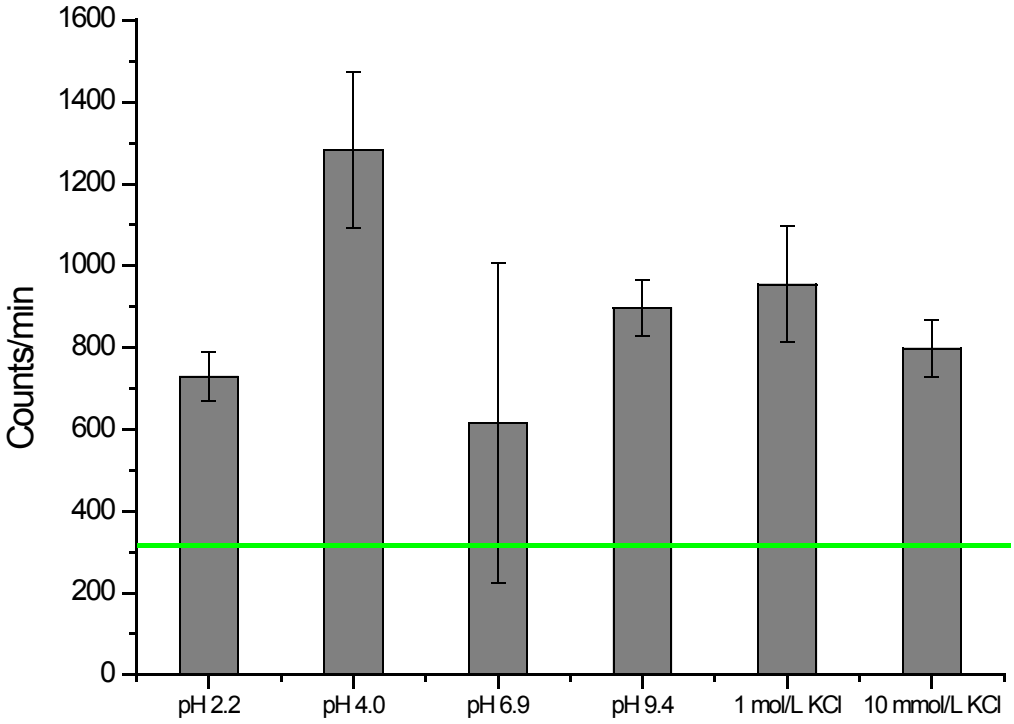


Fig. 7.3. Counts per minute for electrodes covered by a SAM of 16-mercaptohexadecanoic acid after addition of radiolabeled, amino-modified DNA-oligomers with 400 mmol/L of EDC. After 90 minutes of incubation, the electrodes were rinsed with water, then washed in 0.2 mol/L NaOH for 30 minutes and another 10 hours in 0.1 mol/L NaCl. Then the scintillation counts were determined. For the experiments involving EDC, the mean value of two determinations was taken. The average of the control values (without EDC) is designated by the green line (315 counts/min, SD = 124).

The values for the scintillation counts in Fig. 7.2 look very similar to those of Fig. 7.3. The values for the control samples (without EDC) vary by about 300 counts, while the values for the samples including EDC have remained nearly unchanged. This indicates that washing with 0.1 mol/L NaCl is able to further remove some of the nonspecifically adsorbed DNA-oligomers still remaining at the surface, but the effect is not very large. Again, the value of counts for the EDC containing

samples at pH 4 (second column) is the largest of all the pH conditions. The values of counts for the EDC containing samples tested for changes in ionic strength (the last two columns) are nearly identical, meaning that they only differ within the variations of the measured values.

The columns of Fig. 7.2 and Fig. 7.3 are combined in one figure, thus facilitating a comparison of the results of both washing procedures (Fig. 7.4). The dark gray columns refer to scintillation counting of samples washed with 0.2 mol/L NaOH for 30 minutes only, while those on the right refer to samples washed with 0.1 mol/L NaCl for an additional 10 hours.

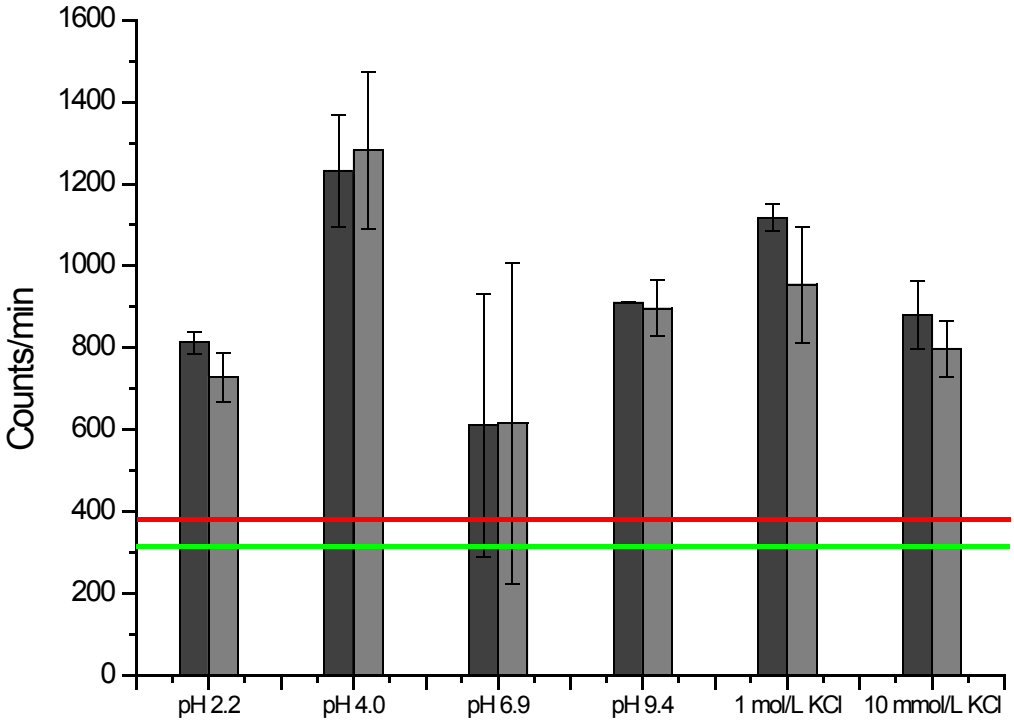


Fig. 7.4. Counts per minute for electrodes covered by a SAM of 16-mercaptohexadecanoic acid after addition of radiolabeled, amino-modified DNA-oligomers with 400 mmol/L of EDC. After 90 minutes of incubation the electrodes were rinsed with water, then washed in 0,2 mol/L NaOH for 30 minutes (dark gray columns) and additionally for 10 hours in 0,1 mol/L NaCl (light gray columns). The scintillation counts were then determined. For the experiments where EDC was added, the mean value of two determinations was taken. The average values of the control samples are designated by the red (NaOH) and green (NaCl) line.

The direct comparison between the results of the two washing procedures shows that the values for the samples with EDC used during the immobilization step are (within their variations) nearly identical. The average of values for the control samples (without EDC during immobilization) is a little smaller for the samples that were additionally

washed with 0.1 mol/L NaCl (green line) than for the for the samples washed with 0.2 mol/L NaOH alone (red line).

One result of the examination in this chapter is that nonspecifically adsorbed DNA molecules are very strongly attached to the samples consisting of a silicon support combined with an area covered by gold and an additional layer of alkanethiols.

Different washing procedures are able to remove large amounts of these molecules but there remains a rest of about 1/20 of the counts resulting from nonspecifically adsorbed DNA.

Furthermore it can be said that washing with 0.1 mol/L NaCl gives only small advantages in removing nonspecifically adsorbed DNA-oligomers after the sample is washed with 0.2 mol/L NaOH, as it takes a very long time (ten hours) to get any small additional effect.

7.3. EFFICIENCY OF IMMOBILIZATION

In the last section, mainly the amounts of nonspecifically adsorbed DNA molecules were considered. In the following, the focus will be more on the efficiency of the immobilization itself under the different conditions of pH and ionic strength.

Therefore the surface coverage by immobilized DNA-oligomers is compared to the ideal value of a completely and densely covered surface: Taking into account the diameter of DNA, a theoretical value of scintillation counts can be determined (the light gray area between 5333 and 5917 counts per minute in Fig. 7.5) that can be expected for an ideal immobilization with densely packed DNA-oligomers. The resulting number of counts in Fig. 7.5 is compared to the counts that derive from the radiolabeled DNA-oligomers after immobilization followed by washing with different solutions. The counts for the control

samples are subtracted from the values for the samples with EDC to take into account only the immobilized part of DNA-oligomers.

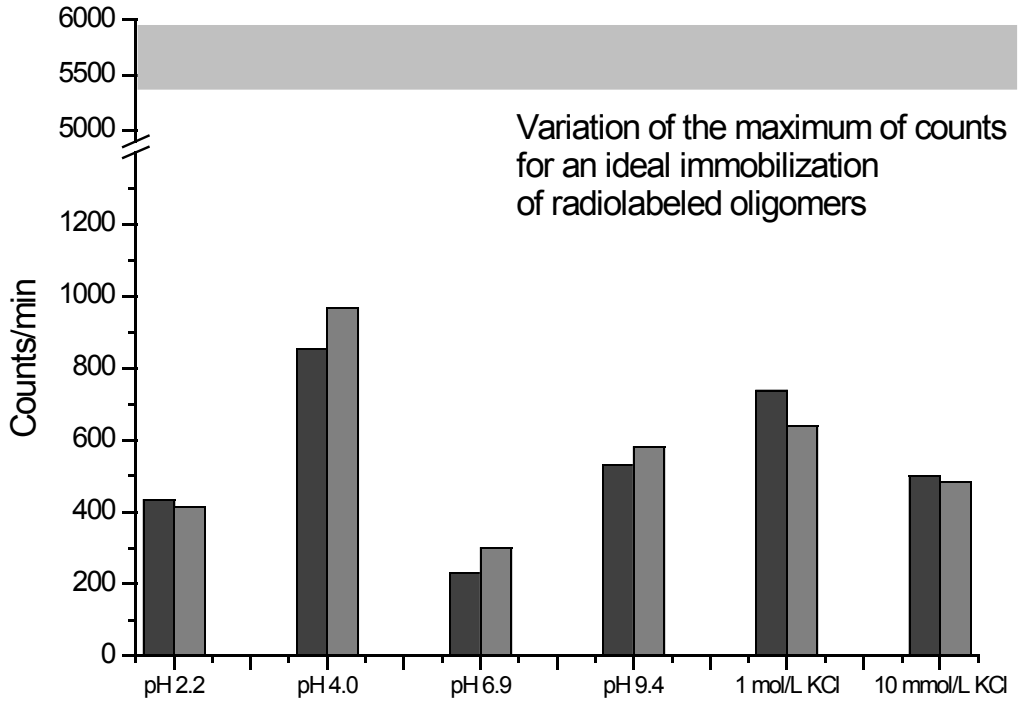


Fig. 7.5. Counts per minute for electrodes covered by a SAM of 16-mercaptohexadecanoic acid after addition of radiolabeled, amino-modified oligomers with 400 mmol/L of EDC and without EDC. The treatment after 90 minutes of incubation was: rinsing the electrodes with water, then washing in 0,2 mol/L NaOH for 30 minutes (dark gray columns) and additionally for 10 hours in 0,1 mol/L NaCl (light gray columns). After washing the scintillation counts were determined and the average values for the control samples were subtracted. The area between 5333 and 5917 counts shows the maximum values for an ideal immobilization of radiolabeled oligomers resulting in a layer of densely packed molecules.

It can be seen in Fig. 7.5, that the values for immobilization at pH 4 are larger than for all of the other immobilization conditions. The rate of immobilized oligomers amounts to about 20% of the ideal, theoretically determined value for the scintillation counts. The values for

immobilization at 10 mmol/L and 1 mol/L KCl are very similar and amount to 13%. However, the ionic strength of 10 mmol/L of KCl is slightly favorable because the amount of nonspecifically adsorbed DNA-oligomers is smaller from the beginning (cf. Fig. 7.1).

There are several reasons imaginable why the yield of the immobilization reaction and therefore the scintillation counting values are not very high.

One reason is that the DNA molecules being negatively charged are repelled by each other. The more DNA is attached to the surface, the larger is the amount of negative charges present and the more difficult is it for further DNA molecules to be coupled to the surface.

A second reason could be that the time for washing with 0.2 mol/L of NaOH was too long. As mentioned in chapter 7.2 (p. 92), the gold-thiol bond is not very stable under these basic conditions and thiols can be washed from the surface. If the thiols with immobilized DNA molecules are affected, then the results of immobilization would be reduced.

Another reason is that the yield of the immobilization reaction itself, is surely not 100%, being a reaction at the surface and not in bulk solution which would render it more effective [Junhui et al., 1997]. Even with the best immobilization technique, the ideal value will not be reached.

The immobilization conditions used in the production of commercially available chips for genosensors are thought to be optimized. It is interesting to compare the results of this study with results from immobilization protocols of two different companies. The comparison of these results is described in the following chapter.

7.4. COMPARISON WITH IMMOBILIZATION PROTOCOLS OF TWO COMPANIES

For the preparation of commercially available DNA biochips, specifically bound DNA molecules are the prerequisite for further investigations. It is therefore interesting to compare our results for immobilization arising from the variation of pH and ionic strength with the immobilization success following the protocols which were made available for the public by two companies.

The protocols of the two companies, herein referred to as company 1 and 2, mainly differ from the conditions described before by the use of S-NHS additionally to EDC. In one of the protocols an external activation of the surface with 0.8 mol/L EDC and 0.2 mol/L S-NHS for 60 minutes is proposed followed by addition of the amino-modified DNA-oligomers, while in the other all reagents including EDC, S-NHS, and H_2N-DNA are added simultaneously to the buffer solution.

The wafer samples for testing the protocols of the companies were prepared as described in chapter 3.2. After 90 minutes of incubation the electrodes were rinsed with water and the values for the scintillation counts were determined and compared to two selected values (the one for pH 4 and the one for 10 mmol/L KCl) originating from the experiment in chapter 7.1 (p. 88).

With reference to Fig. 7.6, the values for the samples (with EDC applied) according to the protocols of the two companies are not very large (dark gray columns of the last two pairs). For company 2 with its external activation, the value is extremely low, as well as the value for the control sample (without EDC, white column). For company 1 the value for the sample with EDC involved is higher, the value for the control sample, however, is remarkably larger than the values for the samples with EDC.

In contrast, the values for the samples with EDC involved, arisen from the experiment of testing different pH and ionic strengths, are much larger (dark gray columns of the first two pairs). The values for the control samples (without EDC, white columns) vary by approximately the same value as for the control sample of company 1.

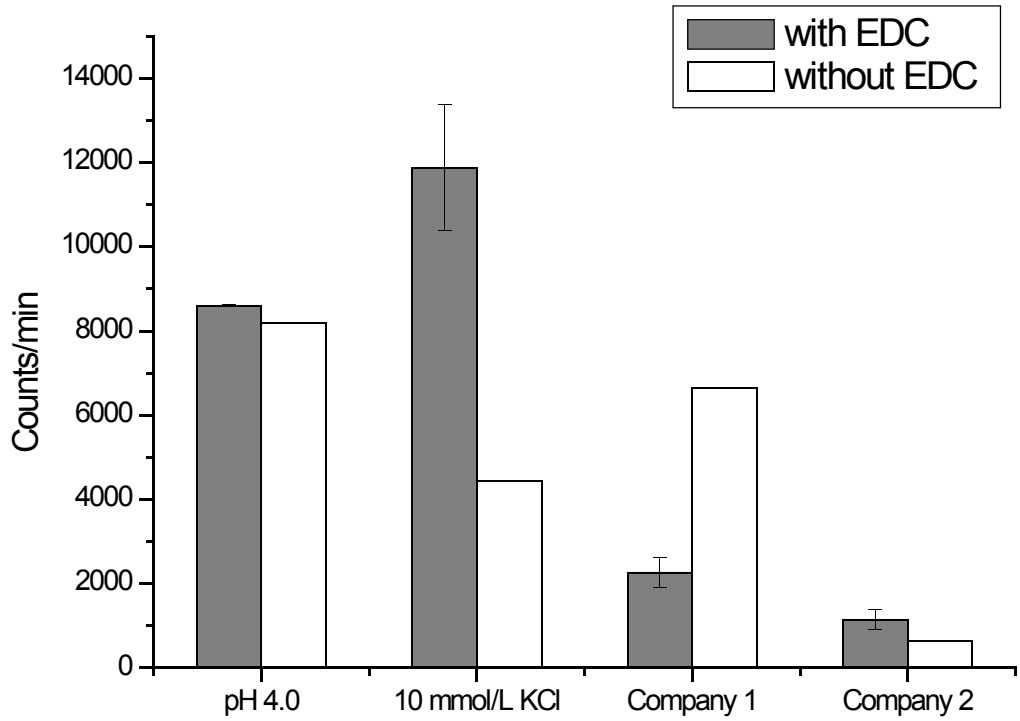


Fig. 7.6. Counts per minute for electrodes covered by a SAM of 16-mercaptohexadecanoic acid after addition of radiolabeled, amino-modified DNA-oligomers. Comparison of the values for pH 4 and 10 mmol/L KCl with the values after immobilization according to the protocols of the two companies. The gray columns correspond to measurements with EDC while the white columns to measurements without EDC. After 90 minutes of incubation the electrodes were rinsed with water and the scintillation counts were determined. For the experiments where EDC was added, the mean value of two determinations was taken.

It can be stated that for the samples prepared according to the protocols of the two companies, the immobilization success from the

beginning is not very high and nonspecific adsorption of DNA-oligomers is obvious, especially for the sample prepared according to the protocol of company 1.

The question now is whether the amount of nonspecifically adsorbed DNA-oligomers can be removed by washing with 0.2 mol/L NaOH and 0.1 mol/L NaCl. Naturally, washing will not enlarge the amount of specifically bound DNA-oligomers.

Washing with 0.2 mol/L NaOH and 0.1 mol/L NaCl was performed as described previously and all characteristics of the washing solutions described in the previous chapter, that affected the samples when various pH and ionic strengths were tested, influenced the samples in the same way when the different commercial immobilization protocols were tested.

Fig. 7.7 shows the scintillation counts after washing with 0.2 mol/L NaOH and 0.1 mol/L NaCl. For the samples prepared according to the two companies, the scintillation counts for all samples (with and without EDC) drop to a value of 1/10 of the starting value permitting a large amount of nonspecifically adsorbed DNA-oligomers to be removed. The remaining DNA molecules which should be specifically bound are quantitatively less than for the samples with DNA-oligomers immobilized in pH 4 or 10 mmol/L KCl and the resulting counts are smaller than the average of counts derived from the control samples (without EDC).

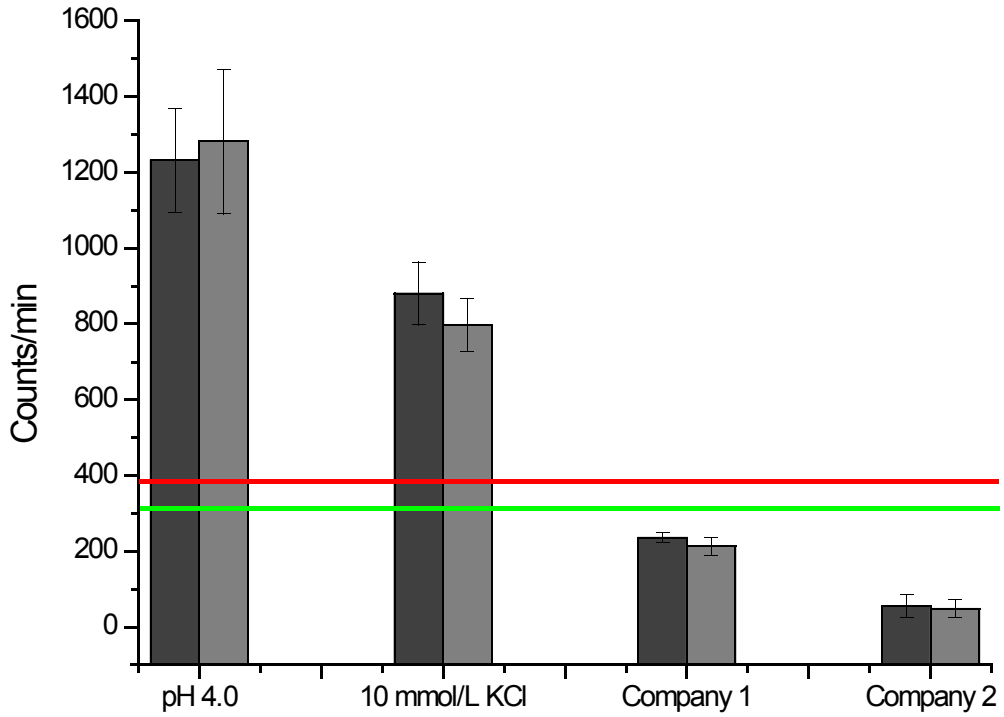


Fig. 7.7. Counts per minute for electrodes covered by a SAM of 16-mercaptohexadecanoic acid after addition of radiolabeled, amino-modified DNA-oligomers. Comparison of the values for pH 4 and 10 mmol/L KCl with the values after immobilization according to the protocols of two companies. After 90 minutes of incubation the electrodes were rinsed with water and washed 30 minutes with 0.2 mol/L NaOH (dark gray columns) or, additionally to the washing with NaOH, were washed 10 h with 0.1 mol/L NaCl (light gray columns). After washing the electrodes were rinsed with water and the scintillation counts were determined. For the experiments where EDC was added, the mean value of two determinations was taken. The red and green line correspond to the average of counts of the control samples (without EDC) after washing with NaOH, and NaOH/NaCl, respectively.

It can be summarized that the conditions for immobilization corresponding to the protocols provided by the two companies give only poor results according to the efficiency of the immobilization reaction. The use of S-NHS seems not to give any additional advantages

regarding the immobilization success. Nonspecific adsorption varies in the same range for the samples according to company 1 as for the other conditions tested, excluding the samples according to company 2. For the samples according to company 2, both values are very low. The method of external activation coupled with a subsequent incubation in a solution with DNA molecules seems not to be very effective.

8. INTEGRATION OF PREVIOUS OPTIMIZATION STEPS

In the previous chapters (4 – 7), single aspects of the whole interface preparation process were optimized. In order to find optimum conditions for the complete procedure of interface preparation for capacitance-based genosensors, it is reasonable to combine the results derived from these chapters mentioned above. The interface preparation requires a basic layer of alkanethiols (a SAM) as free of defects as possible. At this layer amino-modified DNA-oligomers have to be immobilized with high yield. The influence of nonspecific adsorption of DNA-oligomers however, has to be diminished by prevention as well as by subsequent removal of nonspecifically adsorbed molecules.

The ideal conditions (derived from this thesis) for performing an immobilization of amino-modified DNA-oligomers at alkanethiols at a metal surface are as follows:

- The substrate of metal (gold or palladium) should be cleaned thoroughly in order to remove impurities from the surface (cf. chapter 4).
- The metal surface should then be covered by a mixed self-assembled monolayer (mixed SAM) of long chain alkanethiols (e. g., 16 carbon atoms) with a ratio of tail groups of carboxy functionality : hydroxy functionality = 1 : 25. The optimal ratio of carboxy and hydroxy tail groups for the mixed SAM was calculated in chapter 5.
- Once the mixed SAM is attached to the surface, the layer should be pretreated by heating to 75 to 80 °C (at least for 1 hour) in order to reduce the amount of defects (domain boundaries of thiols with different orientation), cf. chapter 4. The pretreatment temperature

should not exceed this temperature range as irreversible changes in the layer occur according to literature [Bensebaa et al., 1998].

- To perform the coupling via EDC, a buffer or electrolyte is necessary which doesnot block EDC in side reactions, in order to spare this coupling reagent for the immobilization itself. Therefore phosphate buffer or buffers like HEPES, MOPS, or TRIS should be chosen (cf. chapter 6). In these buffers EDC possesses a half life of about 3 min at room temperature. If EDC is added in a surplus, the loss of this coupling reagent is negligible.
- The immobilization reaction of tethering DNA to the surface via EDC was empirically found to work best at pH 4 and at an ionic strength of 10 mmol/L KCl for the tested ranges of pH and ionic strength (cf. chapter 7).
- After immobilization, a washing procedure is necessary in order to remove a large amount of the lasting DNA-oligomers being nonspecifically adsorbed, despite of coadsorbed thiols with hydroxy tail groups during preparation of the SAM. Long chain thiols should be used as they are more resistant to the washing procedures than short chain thiols. The washing solution consists of 0.2 mol/L NaOH and the duration of washing should not exceed 15 minutes, to prevent the thiol molecules (coupled with DNA molecules) from desorbing from the surface. Another possibility is a washing procedure with 0.1 mol/L NaCl of at least 12 hours duration (cf. chapter 7).

After this preliminary work listed above, the genosensor is ready for analysis.

This combination of optimum conditions (the immobilization protocol proposed in this thesis), for the complete procedure of interface preparation for capacitance-based genosensors was applied in the following experiment utilizing all optimized conditions for immobilization mentioned above.

After cleaning, a gold electrode was covered by a mixed SAM of 16-mercaptohexadecanoic acid and 16-mercaptohexadecanol in a ratio of 1 : 25. The electrode was pretreated by heating it to 55 °C for 225 minutes, and afterwards cooled down to room temperature. A buffer 0.5 mmol/L Na_2HPO_4, 10 mmol/L KCl, pH 3.8 was used. For immobilization, 160 mmol/L EDC and 7.5 µmol/L amino-modified DNA-oligomers were added and the capacitance was monitored. The capacitance change after addition of amino-modified DNA-oligomers and EDC is presented in Fig. 8.1.

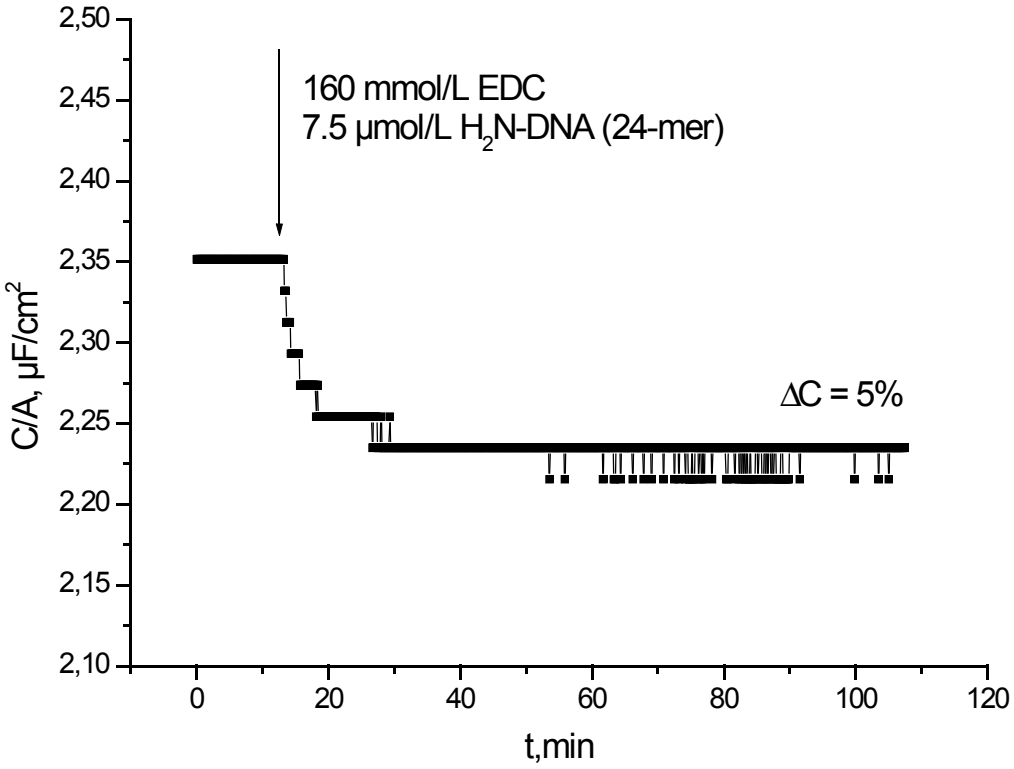


Fig. 8.1. Immobilization of amino-modified DNA-oligomers at a gold surface covered by a mixed SAM of 16-mercaptohexadecanoic acid and 16-mercaptohexadecanol in a ratio of 1 : 25. Prior to addition, the electrode was heated to 55 °C for 225 minutes. The steps in the curve result from poor AD-conversion.
Buffer: 0.5 mmol/L Na_2HPO_4, 10 mmol/L KCl, pH 3.8

As mentioned before (chapter 7), capacitance changes due to nonspecific adsorption cannot be distinguished from capacitance changes due to specific immobilization. Capacitance-based measurements only detect the dielectric thickness of the layer that is formed and as the dielectric properties are the same for specifically immobilized as well as for nonspecifically adsorbed molecules of the same kind, the capacitance will decrease in both cases. For the

experiment in Fig. 8.1, however, the part of specifically immobilized DNA molecules shall now be estimated.

In chapter 5 it was shown, that for mixed SAMs with a ratio of 1 : 25 (not pretreated by heating), an addition of 7.5 µmol/L DNA-oligomers would result in a capacitance decrease of about 3% (cf. Fig. 5.9, p.65). In the experiment of Fig. 8.1 the same amount of DNA-oligomers resulted in a capacitance decrease of about 5%. This difference of 2% in capacitance decrease could be interpreted as the result of a successful immobilization. Adding EDC alone, without DNA-oligomers, results in no changes or only a small drift of the capacitance value (data not shown).

For an application in analysis, these immobilized DNA molecules have to hybridize to target strands and discriminate against noncomplementary DNA oligomers (with mismatches). In the following, a first test in analysis will be shown.

The interface of a gold electrode was prepared according to the immobilization protocol mentioned above and amino-modified DNA molecules were immobilized. After that, the electrode was washed for 10 minutes in 0.2 mol/L NaOH and immersed in a hybridization buffer according to [Sambrook et al., 1989]. The temperature of the buffer was stabilized at 50 °C. Then, noncomplementary DNA-oligomers (with 3 mismatches) were added. After 45 minutes, the same amount of complementary DNA molecules was added (Fig. 8.2).

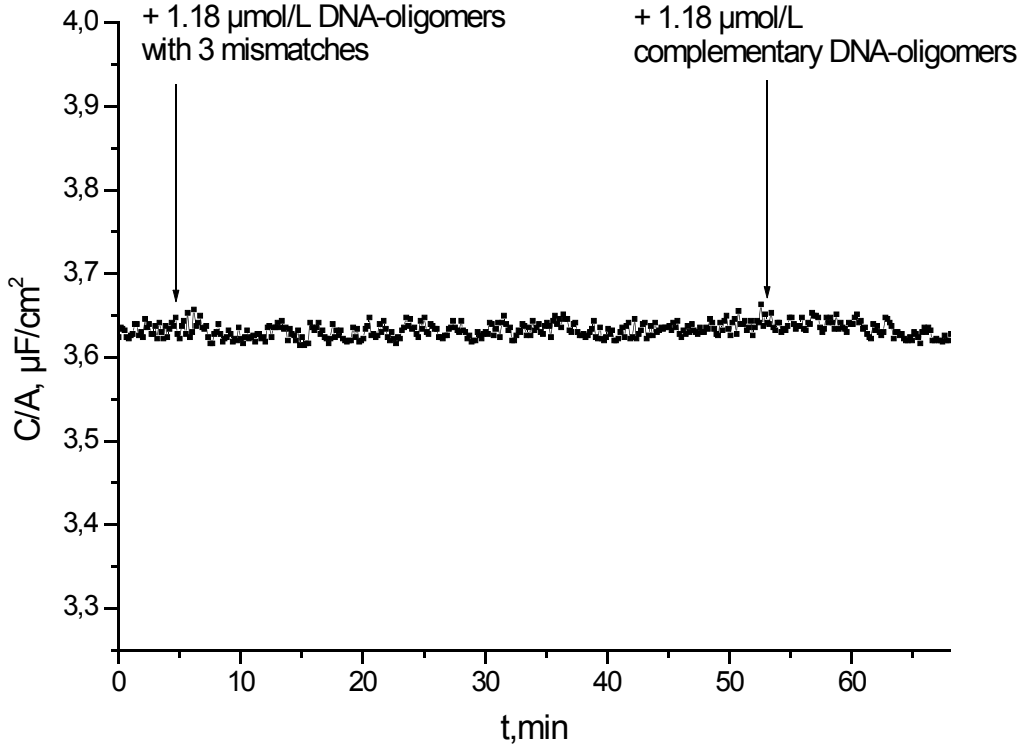


Fig. 8.2. Electrode prepared according to the optimized immobilization protocol. Addition of noncomplementary and complementary DNA-oligomers.
Hybridization buffer: 10 mmol/L TRIS·HCl, 1 mol/L NaCl, 1 mmol/L EDTA, pH 7.4
Temperature: 50 °C

Fig. 8.2 shows the results of the experiment using an immobilization protocol derived from the results of chapters 4 - 7. The first addition, that of noncomplementary DNA-oligomers, is designated by the first arrow in Fig. 8.2. The DNA-oligomers added here had a sequence of nucleobases with 3 mismatches and therefore were not supposed to hybridize to the receptor strand. As a result, no capacitance change due to hybridization was expected. However, if the capacitance was to change, it would only be due to nonspecific

adsorption. Only a very small change of capacitance could be detected therefore nonspecific adsorption during hybridization could be nearly totally blocked. The addition of complementary strands (second arrow in Fig. 8.2) led to a small change of capacitance, only marginally larger than the effect caused by the noncomplementary strands.

One reason for this lack of change in capacitance might be that the capacitive effects caused by hybridization are too small to be detected with this method. If solely hybridization occurs, only one monolayer is formed, in contrast to nonspecific adsorption, where the formation of multilayers is very likely. The dielectric thickness of a layer formed only by the annealing of receptor and target strands will therefore not experience large increases.

A solution to this problem could be a signal amplification. There are several possible methods for signal amplification. Only one possibility shall be briefly discussed in the following, with the use of labeled complementary strands. Complementary strands could be labeled with proteins, or large lipophilic molecules and are allowed to hybridize to the immobilized receptor strands. The use of large additional molecules would lead, according to the considerations above (and in chapter 2), to a larger capacitance decrease. In the analytical step, detection would then occur in the form of a so called competitive assay: The target molecules that are to be detected, replace the labeled strands and a large capacitance increase would occur. Noncomplementary strands would not be able to replace the labeled oligomers and the capacitance would remain stable. So, the signal of capacitance would be amplified and a discrimination between complementary and noncomplementary strands would be guaranteed.

A second reason for the lack of change in capacitance upon hybridization is that the hybridization conditions used here also would have to be optimized (as well as any amplification method). In the

experiment above (Fig. 8.2) hybridization conditions according to literature were used [Sambrook et al., 1989]. The conditions, however were optimized for a reaction in solution and not for hybridization at a surface, necessitating adjustment of the hybridization conditions to the special requirements of sensor chips as well as for the immobilization conditions. Once again the influences of, e.g., buffer components, pH, ionic strength, and temperature would have to be tested in order to optimize the hybridization conditions.

A third reason for the poor performance during detection of the hybridization reaction is that surface reactions in general, lead to lower yields than reactions in bulk solution. This is caused by the fact that the receptors at the two-dimensional surface are not so accessible for the target molecules than receptors in solution (a three-dimensional environment). This problem is additionally aggravated by the fact that the negatively charged target molecules could be repelled by the negative charges of receptor molecules, though this should be repressed by the presence of counterions. It is recognized in literature that the hybridization at a (sensor) surface is not so effective as hybridization in solution, the rate of solid-phase hybridization is dramatically less than the rate for hybridization in solution – the difference being about a tenth to even a hundredth [Junhui et al., 1997].

The problems connected to solid-phase hybridization at sensors in form of chips, electrodes, or crystals will not easily be solved. Therefore it is especially important to thoroughly optimize all other conditions for hybridization, particularly for capacitance-based measurements, although a huge but still promising task for future work.

9. SUMMARY

The task of this interdisciplinary thesis was the optimization of the preparation of interfaces for biosensors. The focus here was on genosensors (biosensors using DNA molecules as recognition units) and a capacitance-based detection method. The interface preparation included several steps: Self assembly of a thiol layer on a metal electrode is followed by a tethering (immobilization) of receptor molecules (here DNA-oligomers) to the thiol molecules via a chemical reaction.

During each step some limitations have been identified, which can disturb the success of the whole procedure and which may lead, for example, to alterations of the measured signal and, in the worst case, to false positive results.

First of all, imperfect self assembly may lead to defects in the thiol layer. Capacitance-based detection methods however, require densely packed alkanethiol layers at the surfaces of electrodes. Therefore, it is necessary to find a method to improve the thiol layer on metal surfaces (gold or palladium). An appropriate method to achieve such an improvement has been suggested in chapter 4, utilizing a heating procedure as an electrode pretreatment (for gold: cf. chapter 4.1, for palladium cf. chapter 4.2). After pretreating the electrodes, the capacitive response to nonspecific adsorption was smaller due to the elimination of defects in the monolayers. For gold, the reduction of capacitance change due to addition of DNA molecules was about eight times (without pretreatment: 6%, with pretreatment: 0.75%), for palladium about ten times (without pretreatment: 10%, with pretreatment: 0.8%).

It is important, especially for genosensors based on capacitance measurements, to know the influence of temperature on the electrode covered by an alkanethiol layer. This is the case as many of applications of genosensors require higher temperatures. In chapter 4, the temperature effects on the capacitance of thiol layers were studied. It could be shown that in a first heating and cooling cycle the capacitance of the thiol layer shows nonlinear behavior. However, in further heating and cooling cycles, the capacitance increases and decreases linearly with temperature, displaying a predictable reaction.

The quality of a (DNA-)biosensor depends on the efficiency of the immobilization technique. However, it is not easy to accomplish perfect immobilization of the receptor molecules. Properties of the solution in which the immobilization is performed influence the coupling reagent itself as well as the receptors. In order to achieve a good coupling of the biological substances to the inorganic support covered by thiols, two optimization procedures were applied.

The first is the prevention of an unnecessary loss of coupling reagent as coupling reagent that is lost in unwanted side reactions is not available to catalyze the desired reaction. The concentration of EDC (used here as a coupling reagent) was monitored (cf. chapter 6) and the effects of electrolyte components on EDC concentration and its loss were determined. Buffer components with carboxylate groups as well as a low pH led to a strong increase of the rate of EDC decomposition. Furthermore those electrolyte components could be identified which had only small influences on the rate of EDC hydrolyzation. These include MOPS, TRIS, and HEPES.

The second optimization procedure applies to the immobilization of DNA-oligomers at the self-assembled monolayer of thiols at gold electrodes. In order to increase the yield of chemically attached DNA molecules, a study of immobilization conditions and washing procedures

to remove nonspecifically adsorbed DNA molecules was performed in chapter 7. To test these immobilization conditions and washing procedures, an alternative detection method was applied, based on scintillation counting. From these experiments it could be stated that immobilization with EDC works best at a pH of 4 and an ionic strength of 10 mmol/L KCl. The yield of the reaction amounts to 13 - 20% of the ideal value. These results were compared to the results performing immobilization according to the protocols (suggesting the additional use of S-NHS) of two companies producing genosensors. It is demonstrated that the commercial protocols led to even lower yields (2 - 4%).

Aside from the optimization of immobilization mentioned above, nonspecific, physical adsorption of DNA at the surface of biosensors was diminished. Nonspecific adsorption of DNA not only can occur during immobilization but also during the actual analysis and leads to alterations of the measurements. Especially label-free methods like capacitance-based methods are sensitive to this. In capacitance-based methods, the dielectric thickness of specifically immobilized molecules and of nonspecifically adsorbed ones is detected. However, the response due to nonspecifically adsorbed molecules would lead to a misinterpretation of the results. In chapter 5, a way was found to minimize the amount of nonspecifically adsorbed molecules by building up a mixed self-assembled monolayer (mixed SAM) of alkanethiols with different tail groups. One kind of tail groups (the carboxy groups) provide the possibility of a coupling between thiol and DNA-oligomer. The other kind (the hydroxy groups) prevent nonspecific adsorption of DNA molecules. The optimized ratio of these tail groups was calculated to be 1 : 25 for carboxy groups : hydroxy groups.

The results of chapters 4 to 7 then were integrated in chapter 8 and the optimized conditions for the surface preparation of genosensors in combination with a capacitance-based detection method was

proposed. A positive effect on prevention of nonspecific adsorption as well as on the success of immobilization could be shown.

A first test in the analytical step (the hybridization) again showed the prevention of nonspecific adsorption of DNA molecules however, the success of hybridization was only very small with a low signal to noise ratio. Several reasons for this as well as possible solutions to this problem were given and the necessity for further optimization of the conditions for hybridization at the sensor surface was pointed out.

Hopefully, this thesis will contribute to a better understanding of the processes during interface preparation and the optimized immobilization protocol derived therein will help prepare efficient genosensors, especially for capacitance-based measuring methods.

10. ZUSAMMENFASSUNG

Ziel dieser interdisziplinären Arbeit war eine Optimierung der Grenzflächenpräparation für Biosensoren. Dabei lag der Schwerpunkt auf DNA-Biosensoren, d.h. auf Biosensoren, bei denen DNA Moleküle als Erkennungseinheit dienen, sowie auf einer elektrochemischen Messmethode, die die kapazitiven Eigenschaften von Biomolekülen ausnützt.

Bei der Grenzflächenpräparation erfolgte in mehreren Schritten. Zunächst wurde auf eine Metallelektrode (Gold oder Palladium) eine selbst organisierte Monoschicht von Alkylthiolen aufgebracht, an welche im Anschluss entsprechende Rezeptormoleküle (in diesem Fall DNA-Oligomere) über eine chemische Reaktion gekoppelt wurden. Letzteren Vorgang bezeichnet man als Immobilisierung.

Sowohl bei der Selbstorganisation der Alkylthiole als auch bei der Immobilisierung konnten Schwierigkeiten identifiziert werden, die einem erfolgreichen Aufbau von Biosensoren entgegen stehen, da sie zu einer unerwünschten Veränderung des gemessenen Signales und schlimmstenfalls zu falsch positiven Ergebnissen führen.

Hierbei seien zunächst Defekte in der Alkylthiolschicht selbst genannt, welche sich während des Prozesses der Selbstorganisation ergeben können. Da aber gerade elektrochemische Messmethoden im allgemeinen und die kapazitive Messmethode im speziellen defektfreie Monoschichten benötigen, erschien es erforderlich, eine Methode zur Verbesserung von bereits an die Metallelektrode angelagerten Alkylthiolschichten zu entwickeln. Die in Kapitel 4 vorgestellte Methode beruht auf der Hitzebehandlung der Elektroden und damit auch der Alkylthiolschicht. Nach einer derartigen Elektrodenvorbehandlung konnten Kapazitätsänderungen, die auf eine unspezifische Anlagerung

von DNA-Molekülen zurückzuführen sind deutlich verringert werden. Bei Goldelektroden ergab sich eine Reduktion von etwa acht Prozent, während sich dieser Wert für Palladiumelektroden auf ca. zehn Prozent belief.

Viele Anwendungen von DNA-Biosensoren arbeiten bei deutlich höheren Temperaturen, um die Sensitivität der Detektion zu verbessern. Daher ist es gerade bei DNA-Sensorsystemen wichtig, den Einfluss der Temperatur auf den Messaufbau zu kennen. In Kapitel 4 wurden daher Kapazitätsänderungen untersucht, welche durch eine Erhöhung der Temperatur hervorgerufen werden. Ein Ergebnis dieser Untersuchung war, dass bei einem ersten Erhitzen und anschließenden Abkühlen der mit Alkylthiol beschichteten Elektrode nichtlineare Kapazitätsänderungen auftreten. Bei weiteren Temperaturzyklen jedoch ist eine lineare und damit vorhersagbare Abhängigkeit der Kapazität von der Temperatur zu erkennen.

Die Qualität eines jeden (DNA-)Biosensors ist von einer möglichst erfolgreichen Ankopplung der Rezeptormoleküle an die Sensor-oberfläche abhängig, was sich bei einer Kopplung aus wässriger Lösung allerdings als schwierig erwies. Sowohl die zu koppelnden Moleküle als auch das zu verwendende Kopplungsreagenz wurden durch Eigenschaften der Lösung wie z.B. pH-Wert oder Ionenstärke beeinflusst. Daher wurden auf zwei Bereichen Optimierungen durchgeführt. Zunächst wurden oben genannte Einflüsse auf die Konzentration des hier verwendeten wasserlöslichen Carbodiimids (EDC) mittels einer UV-spektrometrischen Methode untersucht. Durch eine geeignete Wahl der Puffer- oder Lösungsbedingungen kann verhindert werden, dass Kopplungsreagenz in Nebenreaktionen zu schnell verbraucht wird und damit der eigentlichen Reaktion nicht mehr zur Verfügung steht. Es stellte sich heraus, das Puffersubstanzen mit Carboxylgruppen sowie generell ein niedriger pH-Wert die Zerfallsrate

von EDC erhöhen. Es konnten aber auch Puffersubstanzen gefunden werden, die einen deutlich geringeren Einfluss auf die Zerfallsrate haben wie etwa MOPS, TRIS oder HEPES.

Ein weiterer Optimierungsschritt betraf die Immobilisierungsbedingungen, die während der Kopplung von DNA-Oligomeren an die Alkylthiole in der Monoschicht vorherrschen. Um die Ausbeute dieser Immobilisierungsreaktion zu erhöhen, wurden verschiedene Immobilisierungsbedingungen getestet. Außerdem wurden zwei Möglichkeiten untersucht, eventuell unspezifisch angelagerte DNA-Moleküle wieder von der Oberfläche zu entfernen. Zum Test dieser Vorgehensweise wurde ein alternatives und von der kapazitiven Messmethode unabhängiges Verfahren verwendet. Hierzu wurden DNA-Moleküle mit radioaktivem Phosphat markiert und mit Hilfe eines Szintillationszählers detektiert. Es ergab sich im Rahmen der untersuchten Messbereiche für EDC optimale Immobilisierungsbedingungen von einem pH-Wert von 4 sowie einer Ionenstärke von 10 mmol/L KCl. Die Ausbeute betrug hier etwa 13 - 20%. Diese Ausbeuten wurden mit denen verglichen, welche nach den Arbeitsempfehlungen (die sich unter anderem durch den Einsatz von S-NHS unterscheiden) zweier Firmen, welche Biosensoren auf Goldsubstraten herstellen und vertreiben, erhalten wurden. Es konnte festgestellt werden, dass die Ausbeuten hier erheblich niedriger waren, nämlich 2 - 4%.

Aber nicht nur die Immobilisierungsbedingungen konnten optimiert werden, sondern es wurde auch eine Methode zur Verringerung rein physikalischer und daher unspezifischer Adsorption von DNA aufgezeigt. Unspezifische Adsorption kann sowohl während der Immobilisierung der Rezeptormoleküle als auch zum Zeitpunkt der eigentlichen Analyse stattfinden, was bei markerfreien Messverfahren wie der kapazitiven Methode in besonderem Maße störend ist. Da bei der kapazitiven

Messmethode die gemessene Größe die Dicke der dielektrischen Schicht ist und diese für unspezifisch adsorbierte sowie spezifisch immobilisierte Moleküle einer Art nicht zu unterscheiden ist, kann dies zu falsch positiven Ergebnissen führen.

In Kapitel 5 wurde ein Weg gefunden, unspezifische Adsorption zu verringern, indem eine Monoschicht aus einem Gemisch von zwei Arten von Alkylthiolen mit verschiedenen terminalen Gruppen aufgebracht wurde. Während Alkylthiole mit Carboxygruppen eine Kopplung der DNA-Moleküle mit Hilfe von EDC ermöglichen, vermindern Alkylthiole mit Hydroxygruppen deren unspezifische Adsorption. Ein ideales molares Verhältnis beider Arten von Alkylthiolen wurde zu 1 : 25 [-COOH] : [-CH_2OH] in der Beschichtungslösung ermittelt.

Die Ergebnisse aus den Kapiteln 4 - 7 wurden in Kapitel 8 integriert und die optimalen Bedingungen zur Oberflächenpräparation eines DNA-Biosensors in Kombination mit der kapazitiven Messmethode zur Anwendung gebracht. Es konnten sowohl eine Verbesserung des Immobilisierungerfolges als auch eine Verringerung unspezifischer Adsorption gezeigt werden. Ein erster Versuch mit diesem so hergestellten DNA-Biosensor zur Hybridisierung zeigte, dass auch während der Analyse unspezifische Adsorption vermindert werden konnte, dennoch war der auf eine spezifische Bindung mit dem Gegenstrang zufückzuführende Effekt gering. Es konnten Gründe für diese geringe Ausbeute angeführt werden und der Bedarf an weiteren, die Hybridisierung an der Sensoroberfläche betreffenden, Optimierungs-schritten aufgezeigt werden.

Möge diese Arbeit zu einem besseren Verständnis der Abläufe während der Oberflächenpräparation beitragen und mögen die hier aufgestellten optimierten Immobilisierungsbedingungen helfen, effiziente DNA-Biosensoren (im speziellen für die kapazitive Mess-methode) zu erstellen.

APPENDIX A: LIST OF ABBREVIATIONS

dsDNA	Double stranded deoxyribonucleic acid
ssDNA	Single stranded deoxyribonucleic acid
H_2N-DNA	amino-modified DNA-oligomer
A	Adenine
C	Cytosine
G	Guanine
T	Thymine
TdT	Terminal deoxyribonucleotidyl transferase
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
S-NHS	*N*-Hydroxysulfosuccinimide
EDTA	Ethylene diamine tetraacetic acid
HEPES	N-(2-hydroxyethyl)piperazine-N´-(2-ethane-sulfonic acid
MOPS	3-(N-morpholino)propanesulfonic acid
Na_2HPO_4	Disodium hydrogen phosphate
SDS	Sodium dodecyl sulfate
Tris	Tris(hydroxymethyl)amminopropane
SAM	self-assembled monolayer
C	Capacitance
ΔC	Change in capacitance
R	Resistance
Z	Impedance
φ	Phase angle
d	Layer thickness
Δd	Change in layer thickness
S	Surface area
A	Absorbance
ε_r	Relative dielectric constant
ε_0	Permittivity of free space

ϵ	Molar extinction
QCM	Quartz crystal microbalance
Ag/AgCl	Silver/Silver chloride
Si/SiO_2	Silicon/Silicon dioxide
k_d	Dissociation constant
SD	Standard deviation
AD	Analog-digital

APPENDIX B: CALCULATION OF HYDROLYZATION RATE

Calculation of a simple model for the dependence of the hydrolyzation rate of EDC on pH (only its protonated form takes part in the reaction, cf. chapter 6)

$$\text{EDC-H} \underset{}{\overset{k_d}{\rightleftharpoons}} \text{EDC} + H^+$$

$$k_d = \frac{[\text{EDC}]\cdot[H^+]}{[\text{EDC-H}]}, \text{ with } [\text{EDC-H}] + [\text{EDC}] = c(\text{EDC}) \Rightarrow$$

$$k_d = \frac{(c(\text{EDC})-[\text{EDC-H}])\cdot[H^+]}{[\text{EDC-H}]}$$

$$k_d\cdot[\text{EDC-H}] = c(\text{EDC})\cdot[H^+]-[\text{EDC-H}]\cdot[H^+]$$

$$[\text{EDC-H}] = \frac{c(\text{EDC})\cdot[H^+]}{k_d+[H^+]}$$

Assumption: Only the protonated form of EDC (EDC-H) takes part in the reaction. Then, the reaction rate v is proportional to [EDC-H].

$$v \sim [\text{EDC-H}] = \frac{c(\text{EDC})\cdot[H^+]}{k_d+[H^+]} = \frac{c(\text{EDC})\cdot 10^{-pH}}{k_d+10^{-pH}} = \frac{c(\text{EDC})}{k_d\cdot 10^{pH}+1}$$

$$\text{with } k_d = 10^{-pk_d} \Rightarrow \quad v \sim [\text{EDC-H}] = \frac{c(\text{EDC})}{10^{pH-pk_d}+1}$$

$$\lg\left(\frac{[\text{EDC-H}]}{\text{mol/L}}\right) = \lg\left(\frac{c(\text{EDC})}{\text{mol/L}}\right) - \lg(10^{pH-pk_d}+1)$$

for pH >> pk_d: $\lg(\frac{[EDC\text{-}H]}{mol/L}) = \lg(\frac{c(EDC)}{mol/L}) - \lg(10^{pH-pk_d})$

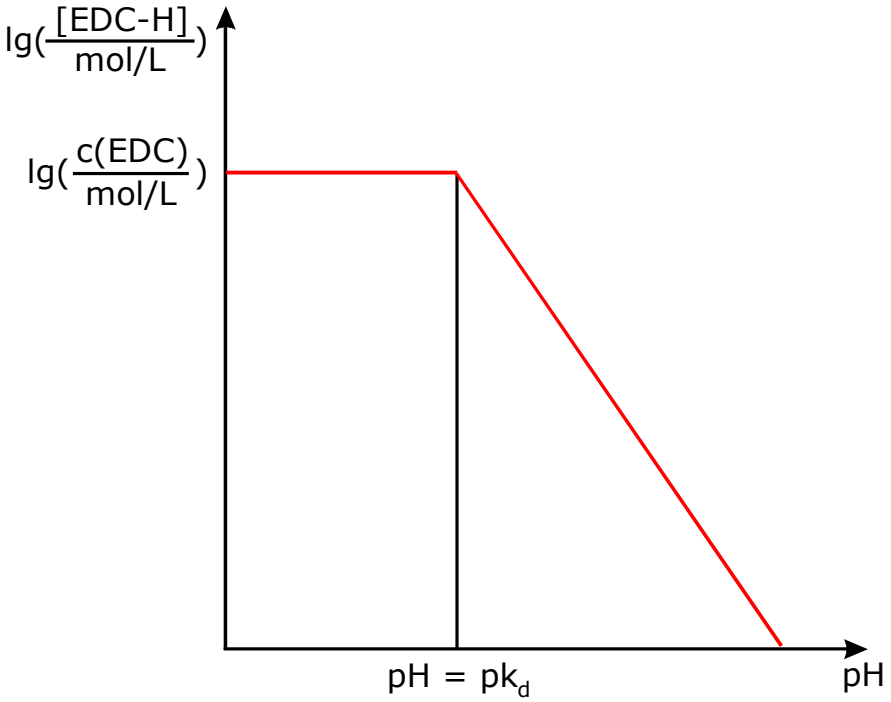
$$= \lg(\frac{c(EDC)}{mol/L}) - (pH-pk_d)$$

for pH < pk_d: $\lg(\frac{[EDC\text{-}H]}{mol/L}) = \lg(\frac{c(EDC)}{mol/L})$

Point of intersection:

$$\left.\begin{aligned} y &= \lg(\frac{c(EDC)}{mol/L}) \\ y &= \lg(\frac{c(EDC)}{mol/L}) - (pH-pk_d) \end{aligned}\right\} \quad \lg(\frac{c(EDC)}{mol/L}) = \lg(\frac{c(EDC)}{mol/L}) - (pH-pk_d) \Rightarrow$$

$$pH = pk_d$$



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LIST OF PUBLICATIONS AND PRESENTATIONS

Diploma Thesis

Tieftemperaturspektroskopie an Bacteriochlorophyll *a*: Fluoreszenzlinien-Einengung und Lochbrennspektroskopie (1997), Regensburg University

Publications

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Wrobel, N., Riepl, M., Mirsky, V. M., and Wolfbeis, O. S. (1999). Capacitive biosensors: signal amplification and preparation of sensor arrays. ABI Summerschool, September 02-04, Neuchatel, Switzerland