Hybridization of surface-tethered oligonucleotide brushes

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Summary of thesis

Part 1. Introduction

In this chapter the chemical structures of DNA and oligonucleotides as building materials for subsequent use in biosensor application are described. The general concepts of modern DNA-based biosensors are presented and the commonly used strategies of immobilization of biomolecules on surfaces are briefly depicted. This chapter also introduces the scope of the thesis and the motivation of the research and chosen methodological strategy.

Part 2. Preparation and Characterization of Substrates for Oligonucleotide Immobilization

Stable, predictable and irreversible immobilization of oligonucleotides on a surface is a crucial step in the development of DNA-based biosensors. Unfortunately, physical chemistry characterizations at each stage of the sample preparation are still missing. In this chapter the experimental approaches for covalent immobilization of oligonucleotides onto silicon surfaces are described. The existing methods of surface silanization and glutaraldehyde coupling were optimized for preparation of homogeneous layers covalently bound to silicon surfaces. The obtained results showed that the oligonucleotides bind to glutaraldehyde-modified substrates with preservation of their functional properties.

Part 3. Optimal Hybridization Efficiency upon Immobilization of Oligonucleotide Double Helices

For the first time, the covalent immobilization of oligonucleotides double helices on surfaces prior to sequential denaturation and rehybridization was proven to lead to optimal hybridization efficiency. Two indirect methods were used for monitoring these processes in situ: the quartz crystal microbalance with dissipation monitoring (QCM-D) and the wavelength interrogated optical sensor (WIOS, Bright Reader®). Both techniques led to the result that with this immobilization approach one could reach nearly 100% hybridization efficiency. Moreover, having applied the existing theory of polymer

adsorption on surfaces to the surface tethering of nucleotide sequences, we demonstrated that for single stranded sequences the coil conformation prevails over the stretched one.

Part 4. A Microcontact Printing Approach to the Immobilization of Oligonucleotide Brushes

The microcontact printing of oligonucleotide double helices on surfaces enables their surface tethering into brushes in patterned structures, which further can be evidenced by efficient and selective sequential dehybridization and rehybridization. This concept was proved via exchange of the fluorescently labeled complementary sequence of the surface tethered nucleotide sequence. Hybridization with complementary sequences labeled with fluorescent tags emitting at different wavelength enabled to assess the sequential de-hybridization and re-hybridization cycle via fluorescence microscopy.

Part 5. General Conclusions and Outlook

The obtained results are summarized. Further investigations and potential applications are suggested.

1. Introduction

As the unique molecular recognition properties of DNA were discovered, new branches of science such as genetic engineering and DNA-based nanotechnologies appeared. The DNA double helix was identified by Watson and Crick in 1953 as being the most likely structure of DNA.[1] Later on this structure has been confirmed and currently its identification appears as one of the most significant discoveries of modern science. The DNA was revealed as the carrier of the genetic information in living organisms. At present, half a century later, the DNA and its sequences, named oligonucleotides, are used as a pivotal structural material in the development of modern biosensors and chips. The possibility to handle the process of hybridization and its identification in vitro led to the design of DNA biosensor or microarray structures, which now are widely used in medical diagnostics for the identification of genetic diseases, detection of warfare agents and forensic diagnostics. The working principles of DNA-based biosensors and microarrays are grounded on the immobilization of single strand DNA or oligonucleotides on a surface and recognition of their complementary sequences, loaded from the analyte solution, via the hybridization process. The surface tethered sequences might further be used as templates to organize macromolecules onto the sensor surfaces through DNA directed immobilization, DDI.

1.1 Structure of DNA

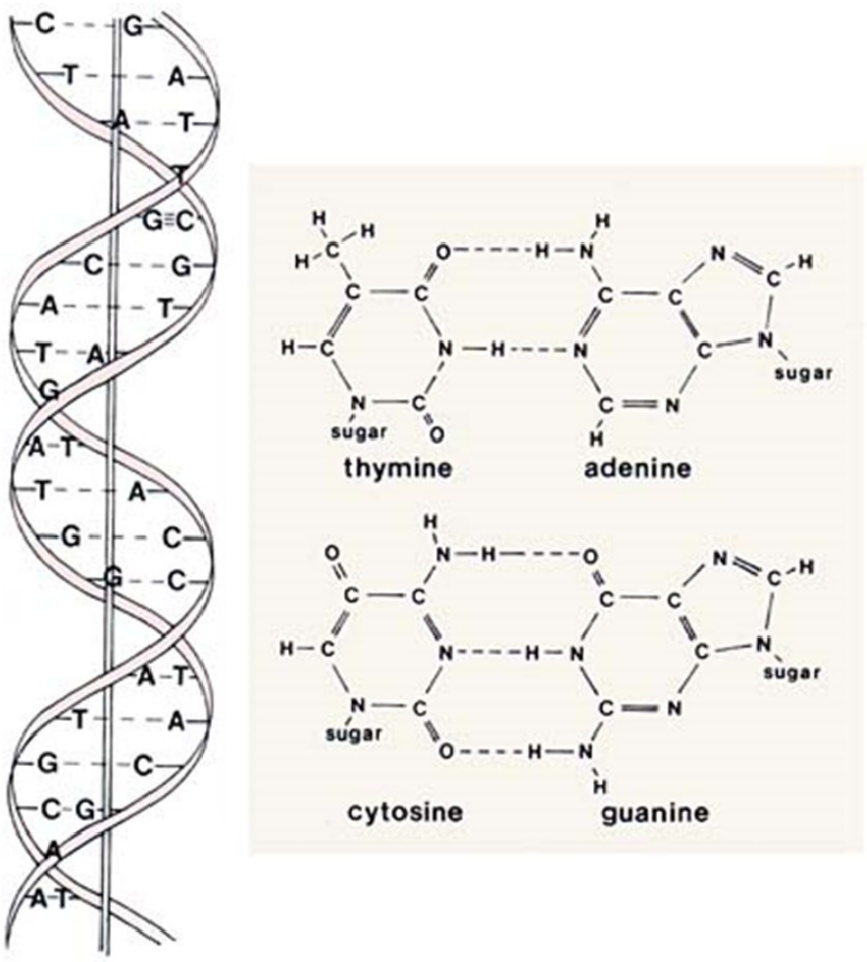

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Figure 1. DNA: (abbr. for deoxyribonucleic acid) the double-stranded, helical molecule that codes the genetic information. Each repeating unit, or nucleotide, is composed of a deoxyribose (a sugar), a phosphate group, and a purine (adenine or guanine) or a pyrimidine (thymine or cytosine) base

From a chemical point of view the DNA chain is a biopolymer composed of an alternating sequence of repetitive units called nucleotides, constituted of a sugar, a phosphate group and a purine or pyrimidine base. There are four types of nucleotides, which are named adenosine (A), cytidine (C), guanosine (G) and thymidine (T). The nucleotides consist of a nucleobase (nitrogenous base: Cytosine, Guanine, Adenine, and Thymine, respectively), a five-carbon sugar (2'-deoxyribose) and a phosphate group.

These four types of nucleobases are subdivided into 2 classes: the purine bases (adenine and guanine) and the pyrimidines (thymine and cytosine). The four bases are complementary, meaning that, when the DNA double helix is assembled, the nucleobases can form the hydrogen bonds between the purine bases or between pyrimidine bases. In other words, cytosine can only pair with guanine and adenine can only pair with thymine. Within the DNA double helix, A forms two hydrogen bonds with T on the opposite strand, and G forms three hydrogen bonds with C on the opposite strand. According to this principle the two strands of DNA wrap around each other and create a right-handed double helix – the structure in which the DNA macromolecules mainly exist in the living organisms.

Some of the prime features of this structure can be summarized here:

- The two polynucleotide chains run in opposite directions. The bases are perpendicular to the axis of symmetry.
- The sugar-phosphate backbone is on the outside; the bases are in the middle. The bases are stacked one on top of the other. Stacking enables the stability.
- The phosphate groups give rise to a large, uniform negative charge, due to the presence of oxygen atoms on the phosphates, which are polar and negatively charged, the bases being hydrophobic.
- There is a wide (major) and a narrow (minor) groove between the backbones on opposite strands.

The right-handed double helix is held together only by H-bonds and hydrophobic interactions between the bases. Heat, acidic or basic conditions, or chaotropic agents can cleave these weak, non-covalent interactions. And thus the DNA strands can be separated. This process is called DNA melting, denaturation or dehybridization. The denaturation conditions are dependent on the proportion of AT and GC base pairs and on the sequence of the bases. In general, double stranded DNA molecules containing a higher proportion of GC bonds are more stable than DNA molecules with a higher proportion of AT bonds. But in contrary to the popular belief, this is not due to the extra hydrogen bond between GC base pairs but rather the contribution of stacking interactions (hydrogen bonding merely provides specificity of the pairing, not stability).[2]

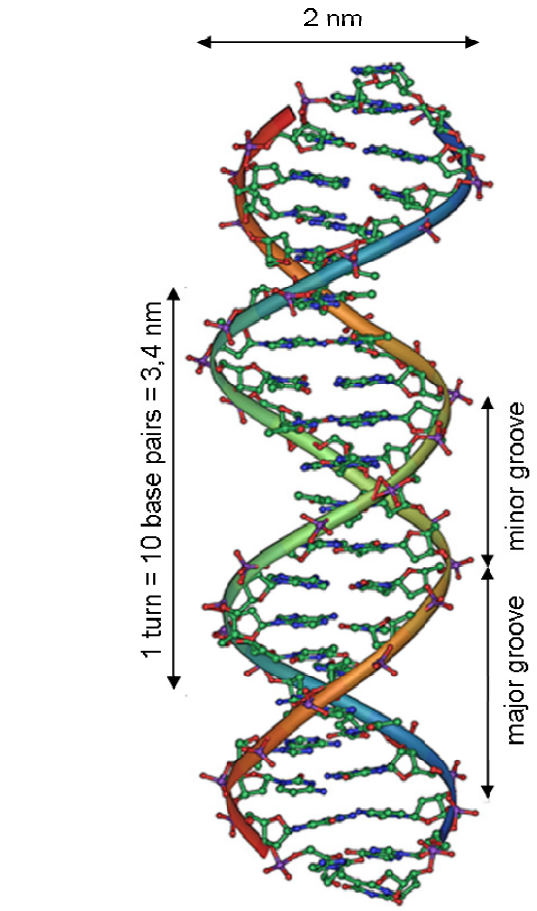

Figure 2. The structure of a part of a DNA double helix

The radius of double stranded DNA (dsDNA) is 9.5 Å and thus its cross sectional area is 284 Å^2, the length of one nucleotide unit is 3.4 Å (0.34 nm).[3] For single stranded DNA (ssDNA) the monomer size is 6 Å[4, 5]. The ssDNA is more stretched than the dsDNA due to its conformation. dsDNA can be described as a rod, whereas ssDNA as a semi-flexible chain.[3] The major groove is 22 Å wide and the minor groove is 12 Å wide.[6]

Based on these macromolecular properties, DNA became a very promising tool for the creation of well-organized high-density arrays and nanostructured sensing devices.

1.2 DNA-based microarrays and biosensors, lab-on-a-chip

In recent years DNA-based assays became a very wide used tool for modern laboratory diagnostic. This system is usually based on the immobilization of single stranded DNA, the probe sequence, onto the sensor surface and on the subsequent recognition of the complementary sequence, the target one, through the specific hybridization process. Different methods can be used to detect the hybridization process onto the surface such as optical, electrochemical, mass-sensitive measurements. All types of DNA-based tests are divided in tree major classes: DNA biosensors (also called genosensors), DNA microarrays (also called gene chips, DNA chips or biochips) and miniaturized DNA analyzers ("Lab-on-a-chip"). On DNA microarrays the DNA targets are physisorbed on the substrate, whereas on oligonucleotide chips short oligonucleotide targets are chemically bound to the surface via their terminal groups.

1.2.1 Biosensors

The common concept of biosensors was described by S. P. J. Higson as "a chemical sensing device in which a biologically derived recognition entity is coupled to a transducer, to allow the quantitative development of some complex biochemical parameters".[7] The standard biosensors combine two parts – bioelement and sensing. The bioelement, may be an enzyme, a nucleic acid (DNA or RNA), an antibody, a cell or a tissue, which should specifically recognize and react with an analyte, and eventually have the possibility to return to its initial stage without significant decrease of its activity. The sensing part should detect the biochemical events and transduce it in an electrical signal. An ideal biosensor must possess some beneficial features: it must be highly specific for the analysis purpose, be stable under normal storage condition and show precise reproducible results over a large number of assays.

In most types of biosensors, fluorescent or radioactive labeling is required in order to record a signal arising from surface events. However the procedure of labeling can influence the molecular interactions by affecting a binding site that can lead to a

mistaken signal. The “ideal” biosensor is therefore preferentially based on a label-free platform.

To create the optimal biosensor or biochip structure the mechanism of hybridization and dehybridization onto the surface must be fully controlled at each stage of the development process.

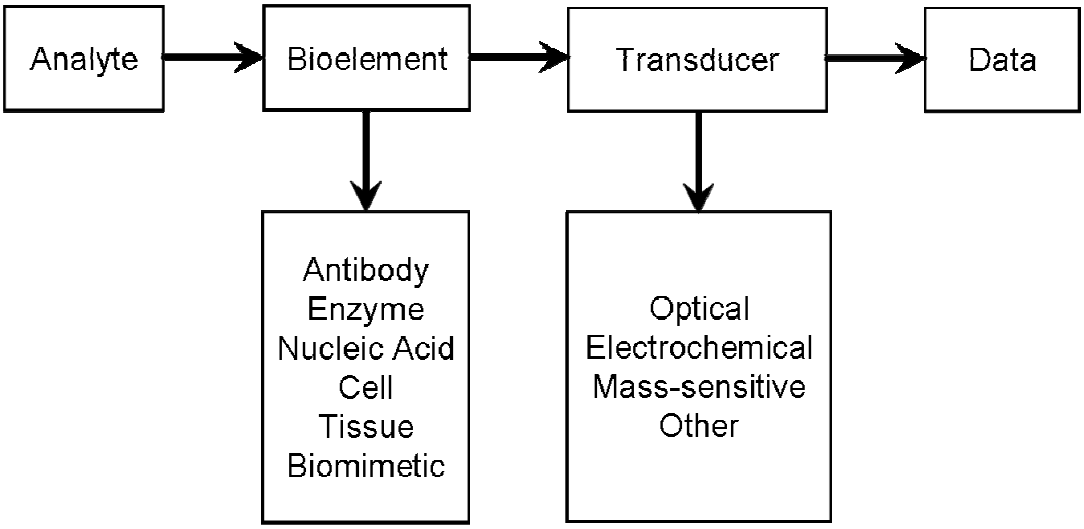

Figure 3. A schematic representation of biosensors

Optical biosensors

In this largest type of biosensors the real measured physical values are the changes of properties of light. Optical biosensors can be created on the base of fluorescence, chemiluminescence, optical diffraction, surface plasmon resonance or wave guiding, which register the spectroscopic properties of light such as propagation or amplitude, polarization, energy, electron transfer to excitation energy levels, time or phase shifts, and refractive index changes.

Fluorescence detection

Visualization of DNA hybridization can be perfectly done, when the target DNA sequence is labeled with a fluorophore. There exist different types of fluorescent dyes in the all range of excitation wavelengths for nucleic acid investigations. Some of them are

mostly specific to either single or double stranded nucleotides. Also the conjugation of fluorescent dye with the target sequences can be used for visualization of the specificity of DNA hybridization. This approach is widely used in the DNA microarray technologies because this technique provides the significant signal amplification and is well suited to small analyte concentrations. Bioaffinity sensors, labeled with fluorophores, have been used to detect DNA hybridization and single-nucleotide polymorphisms.[8] For example, molecular beacon, a DNA hairpin structure, is labeled with both a fluorophore and a quencher.[9] In the absence of target DNA, the hairpin structure is closed and due to the close proximity of the fluorophore and the quencher, fluorescence is turned-off. However, in the presence of a complementary DNA strand, the hairpin secondary structure is destroyed and the fluorophore is active.

Surface-Enhanced Raman Scattering Spectroscopy (SERS)

This optical detection technique measures the Raman scattering spectra of the target biomolecules immobilized on rough metal surfaces. It is based on the Raman effect - a phenomenon observed in the scattering of light passing through a material, whereby the propagating wave changes of frequency and phase. The vibrational frequencies of the molecular bonds reveal the optical fingerprint of chemicals and biomolecules immobilized onto the surfaces. This label-free optical sensing technique thus enables to assess the attachment of molecules on the sensing device.

The detection of oligonucleotides or DNA can be performed by SERS because the nucleotide bases show well-distinguished SERS peaks while the sugar or phosphate groups on the backbone have little interference.[10] However the direct detection of DNA sequences at low concentration with SERS is still evasive[11] because of strong dependences of the obtained spectra on the sequence of the bases[12] and the non significant difference between the Raman spectra of the target nucleic acid and its complementary base since the structural compounds are the same. Nevertheless DNA molecules can still be labeled with a Raman scattering dye, such as cresyl fast violet[13], Rhodamine B[14], Rhodamine 6G and others[15].

Chemiluminescent detection

Chemiluminescence is based on the oxidation of luminol or its derivatives over the course of the reaction with reactive oxygen species or catalyzed by biomolecules. The

oxidation of luminol leads to the formation of an aminophtalate ions in their excited state, which then emit light and return to the ground state. The electrochemical oxidation of luminol is the second most efficient way of triggering the reaction, after the one biocatalysed by horseradish-peroxidase. The main interest in the electro-chemiluminescence of luminol in biochemical and clinical analysis is the possibility of coupling this light-emitting reaction with enzyme-catalysed reactions generating hydrogen peroxide.[16] Therefore several methods use chemiluminescence to design DNA-sensitive sensors, all of them based on specific labeling of DNA with dye molecules or enzymes, catalyzing the oxidation of luminol.[17,18,19,20,21]

Surface Plasmon Resonance (SPR) detection

SPR detection is used to determine the change of refractive index at the surface upon surface modifications. The intensity of the reflected light, incident on a metal-coated prism at a particular angle, is minimal due to internal reflection. But the variation of this intensity is very sensitive to the absorbance of the biomolecules onto this metal surface. Changes of the intensity of the reflected light is proportional to the mass of molecules adsorbed onto the surface.[22] To detect DNA hybridization the probe molecule should be immobilized onto the sensor surface, and when the hybridization occurs the target sequence also binds to the surface, what leads to an increase of mass and refractive index. SPR is a label-free technique, but its drawback is the low sensitivity when investigating molecules of small molecular weight, such as short chains of nucleotides, and to the low grafting densities of immobilized molecules. In these cases the SPR can be combined with fluorescent tagging. The technique is then named Surface Plasmon field-enhanced Fluorescent Spectroscopy (SPFS).[23]

Optical wave-guiding detection

The discovery by Lukosz and his colleagues[24] in the 1980's that waveguides with high refractive indices change their light-guiding characteristics upon changes of their environment provided the basis for the extensive use of planar optical waveguides to design chemical and biochemical sensors.[25] The evanescent field can penetrate in the cover layer up to 200 nm. This evanescent field can alter its properties when the refractive index is changing at the interface, for example, when molecules adsorb onto the waveguide surface forming a thin adlayer with a specific refractive index. This

sensing principle can be used to monitor the binding of chemical or biological molecule onto the surface sensor with high sensitivity. The planar waveguide technology developed by Zeptosens enables the detection of adsorbed molecules down to the picomolar level.[26] The system, based on the monitoring of refractive index changes, is suitable for measuring affinity constants and kinetics, which makes it very convenient for DNA hybridization studies.[27]

Electrochemical biosensors

In many chemical reactions electrons or ions can be produced or consumed, that can cause the change of the electrical properties of the solution. This can be sensed by measurements of the electrical parameters such as conductivity/resistivity, amperometric or potentiometric parameters. This type of biosensors is mainly used for DNA investigations and for measuring glucose concentrations. The electrochemical detection of DNA hybridization is based on the monitoring of current under controlled potential conditions. The hybridization event can be detected as the increase of current generated by a redox indicator, which recognizes the formed DNA double helix, or as the changes of electrochemical parameters, such as conductivity and capacitance, induced by hybridization.[28] For electrochemical detection of DNA hybridization the target sequences can be labeled also with enzymes such as horseradish-peroxidase.[29] In recent years also label-free methods for electrochemical detection of DNA hybridization were developed, where the changes of intrinsic electroactivity of DNA can be measured through the monitoring of guanine oxidation.[30]

Mass-sensitive sensors

The piezoelectric properties of quartz crystals underlie the principle of mass-sensitive devices. Through the application of an electric signal of specific frequency the crystals oscillate at their resonance frequency, which shifts under changing of crystal mass due to mass adsorption. A quartz crystal microbalance (QCM) technique, which represents this kind of biosensors, has been chosen for investigations of DNA hybridization due to the high sensitivity of this method and the possibility of real-time measurements.[31, 32]

Another type of mass-sensing devices is microcantilever sensors, based on the surface stress effect.[33, 34] A microcantilever is a device that can act as a physical, chemical or biological sensor by detecting changes of the cantilever bending or vibrational frequency.[35] Molecules adsorbed on a microcantilever cause vibrational frequency changes and deflection of the microcantilever.[36]

Thermal biosensors

Thermal biosensors are based on the fundamental properties of chemical and also biological reactions, which is production or absorption of heat. This can change the temperature of the reaction media, which is registered by a sensitive thermal sensor. Knowing the total heat changes, which are proportional to molar enthalpy, the calculation of the reaction yield can be done.

1.2.2 DNA microarrays

Microarrays are the set of the immobilized probe molecules onto the sensor surface. DNA microarrays allows investigating the hybridization reaction between the target solution of DNA of interest and thousands of the probe nucleotide sequences bonded onto the sensor surface, which represent gene polymorphism. This construction results in high densities up to billion of experimental spots in a cm^2. The creation of these high-density microarrays involves the immobilization of DNA onto the surface or synthesis of nucleotide sequences from the surface. The first strategy of the DNA microarray technology was developed at Stanford University.[37] There the probe DNA (500-5000 bases long) is immobilized onto a solid surface such as glass using robot spotting and exposed to a set of targets either separately or in a mixture. This method is "traditionally" called DNA microarray. Affymetrix, Inc. developed the second one, “historically” called DNA chips. There the photolithographically fabricated arrays of oligonucleotides (20-80-mer) or peptide nucleic acid (PNA) probes are synthesized either in situ (on-chip) or by conventional synthesis followed by on-chip immobilization. Many companies are manufacturing oligonucleotide-based chips using alternative in-situ synthesis or deposition technologies.

The fluorescence imaging (scanner-based methods and microscopy) is the most commonly used technique to analyze and quantify the fluorescently labeled DNA patterns. However, there remain some imprecisions in the conception of DNA microarrays such as the "halo" or "donut" (or "doughnut") effects, which are often observed through fluorescence imaging of a microarray. "Doughnut" shaped hybridization patterns on DNA microarrays are mainly allocated to spotting or drying artifacts. This can be also associated with a non-brush conformation of oligonucleotides, immobilized onto the surface, that hinder the hybridization with complementary target sequences.

1.2.3 "Lab-on-a-chip"

Lab-on-a-chip (LOC) devices integrate and scale down laboratory functions and processes to a miniaturized chip format to enable transportation of the laboratory to the sample source.[28, 38] LOC systems have several common features such as microfluidic systems and sensing detectors. Microfluidic systems provide fluid flow in small channels using flow control devices (channels, pumps, mixers and valves). Sensing detectors, usually optical or electrochemical sensors are integrated into the chip. One ultrasensitive system was reported to quantify nucleic acid traces using confocal fluorescence spectroscopy and a microfluidic reactor for molecular confinement in an ultra small volume down to fL.[39]

1.3 Surface Immobilization

The immobilization of probe biomolecules onto the sensor surface plays the crucial role in the overall performance of biosensors, microarrays or chips. The immobilization procedure should maintain the native conformation of the biomolecules immobilized on the surfaces. There are tree main types of immobilization methods: adsorption, covalent immobilization and specific affinity interaction (such as streptavidin-biotin interaction).

1. Adsorption

Adsorption is based on forces of non-covalent nature. For example, negatively charged groups on the DNA chains will interact with positive charges onto chitosan or gelatin modified surfaces.[40] For better immobilization of DNA molecules the surfaces is usually pretreated to increase the hydrophobicity and roughness.

2. Covalent immobilization

To stay available for subsequent hybridization DNA molecules should be covalently linked to the surface via their terminal groups. Several methods for covalent immobilization of DNA or oligonucleotides onto surfaces have been described in the literature.

Chemisorption

The thiol-gold interaction is the most wildly used for immobilization of thiol-modified DNA probes onto gold surfaces.

Specific interaction between modified DNA and functionalized surfaces

For example, amino-modified DNA or oligonucleotides can be covalently immobilized on epoxide-, chloromethyl- or aldehyde-modified surfaces directly, to -COOH-modified surface via carbodiimide or –OH-modified surface via cyanobromide. SH-modified DNA can be immobilized to maleimide-modified surface.

3. Affinity interaction

Streptavidin-biotin interaction

Biotin, also known as vitamin H, is a small molecule – part of the common human metabolism, which has a high affinity to bind to avidin or streptavidin. Streptavidin is a tetrameric protein, isolated from the bacterium *Streptomyces avidinii.* Each molecule of

streptavidin can bind four biotin molecules. The streptavidin-biotin system has one of the largest free energies of association for non-covalent binding between a protein and a small ligand in aqueous solution. The complexes are also extremely stable over a wide range of temperature and pH. The immobilization of biotinylated oligonucleotides onto avidin- or streptavidin-modified surface is a widely used method in the development of biosensors.[41-44]

Antibody-antigen interaction

Antibodies are immune system-related proteins called immunoglobulins. Antibodies are complex biomolecules (up to hundred amino acids), which have a specific binding capability for a structure called antigen. Antibody and antigen represent the highly specific key-lock non-covalent interaction.

1.4 Scope of the thesis

The recent yet fast advances in the development of DNA technologies and DNA-based biosensors demand a fully comprehensive identification of the mechanism of immobilization and hybridization of oligonucleotides on the sensor surfaces. Development of an "ideal" biosensor means the creation of a platform with best usability and reproducibility of the outcomes. The theoretical description of the hybridization mechanism on surfaces is still under development and the practical experimental investigations, supporting or disproving these theories, are still needed.

The aim of this research work is the development of an experimental approach to pave the route of the theoretical description of the hybridization mechanism on surfaces in order to advance technologies based on DNA hybridization. Therefore, we have undertaken a physical-chemistry approach to the characterization of the covalent immobilization of oligonucleotides onto surfaces, further quantification of their grafting density and hybridization efficiency. In order to counter molecular crowding, which mainly hinders the hybridization mechanism on surfaces, we immobilized double helices prior to de-hybridization and re-hybridization. We demonstrated that up to 100% hybridization efficiency could be reached due to the suitable grafting density achieved upon immobilization of the double helix prior to de-hybridization.

Strategy

The most widely known method of immobilization of biomolecules onto surfaces was used as an experimental approach to prepare oligonucleotide brushes.[45] Amine groups were introduced on the surface by reaction of the OH-activated silicon surface with 3-aminopropyltriethoxysilane (APTES). The use of glutaraldehyde as crosslinker between the amine groups bound onto the surface and amino-modified oligonucleotides should provide the covalent linkage between oligonucleotides and the surface. Complementary oligonucleotides were hybridized in solution prior to their immobilization onto a glutaraldehyde modified silicon surface. But with this method we faced difficulties of stability of the immobilized oligonucleotides. The optimization of the experimental conditions for covalent immobilization to solve issues of binding stability was the aim of the first part of the project. In order to achieve this, the silicon wafers

were chosen as a well-characterized platform with various possibilities of chemical modification. Each step of the surface modification was controlled by AFM, FTIR, ellipsometry and wettability measurements.

When the efficiency of the immobilization was proved at each step of the sample preparation, the same immobilization procedure was applied to sensor surfaces to evaluate the grafting density of each molecules involved in the immobilization process and eventually the efficiency of hybridization of the surface tethered nucleotide sequences. For these experiments two sensing platforms were used: the quartz crystal microbalance with dissipation monitoring (QCM-D) and the wavelength interrogated optical sensor (WIOS, Bright Reader®). The immobilization of solution-hybridized oligonucleotides was introduced as the method to achieve the optimal grafting density leading to maximal hybridization efficiency.

The next step was to use a microcontact printing approach for the immobilization of oligonucleotide brushes onto the glutaraldehyde-modified surface. To prove the specificity of the interaction of the amino-modified oligonucleotides with the glutaraldehyde pretreated surfaces the solution hybridized oligonucleotides were printed with a PDMS mask, and successfully dehybridized and rehybridized. The fluorescently labeled complementary oligonucleotide sequences were used for visualization of the microstructure of the surface and to follow the sequential dehybridization and rehybridization processes with complementary sequences, labeled with fluorescent dies emitting at different wavelengths.

Contribution to the field

The present research introduces an experimental approach to the covalent immobilization of the oligonucleotide brushes onto surfaces and the subsequent hybridization with optimal reaction yield. The evaluation of the grafting density of immobilized oligonucleotide brushes and their hybridization efficiency was performed. The experimental approach described in this thesis allows achieving the optimal grafting density to reach almost 100% hybridization efficiency of surface-immobilized oligonucleotides. This approach can be suggested with confidence to advance the development of DNA-based biosensors.

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Part 2. Preparation and Characterization of Substrates for Oligonucleotide Immobilization

2.1. Introduction

The controlled attachment of biomolecules to solid supports is a crucial step in most of biotechnological applications such as the design of microchips or biosensors. Several publications describe the immobilization of oligonucleotides on various substrates with preservation of the functional properties for subsequent hybridization.[1-7] Since the covalent immobilization provides a stable, predictable and irreversible attachment of oligonucleotides on a surface, we have chosen this surface tethering over others, such as physisorption or thiol-gold interaction.

We used the immobilization procedure, described by Y. Jiang and coworkers.[8] Glutaraldehyde was used as a crosslinker between amino-modified oligonucleotides and the amine function revealed on the surface. Unfortunately fluorescence microscopy of the glutaraldehyde film shows defects such as holes due to either delamination or dewetting (Figure 1). This film-like structure showed that the covalent immobilization of glutaraldehyde onto the silicon surface modified with 3-aminopropyltriethoxysilane (APTES) did not take place efficiently.

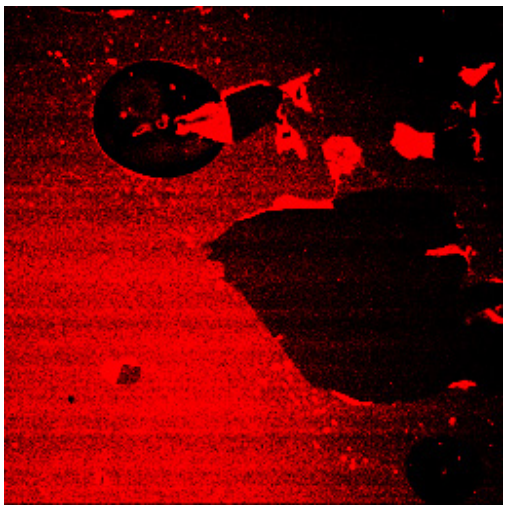

Figure 1. Fluorescence imaging of the autofluorescent glutaraldehyde subsequent to reaction with the APTES-modified surface

These results motivated the exploration of the appropriate reaction conditions for the sequential immobilization of APTES, glutaraldehyde and the oligonucleotides on the surface for subsequent studies of the hybridization mechanism on surface.

In this chapter a complete description and characterization of the immobilization of nucleotide sequences onto a substratum are presented. The surface characterization methods such as atomic force microscopy (AFM), Fourier-transform infrared spectroscopy (FTIR), laser scanning microscopy (LSM), contact angle measurements and ellipsometry were combined to optimize, stepwise, the immobilization of amino-modified nucleotide sequences onto silicon wafers.

2.2. Materials and methods

The amino-modified oligonucleotide sequence amino-C3-5`-AGAGAGAGAGGGAGAGAGAGAGGG-3` and its fully complementary sequence were purchased from Operon Biotechnologies GmbH, Germany.

3-Aminopropyltriethoxysilane (APTES), anhydrous toluene 99%, glutaraldehyde 25% grade II solutions were purchased from Sigma-Aldrich, Switzerland.

Silicon wafers single side polished, orientation N/Phos <100>, 525 ± 25 μm thickness were purchased from Si-Mat Silicon Material, Landsberg am Lech, Germany.

Milli-Q purified water (18 MΩ) was used for all samples preparation.

All rinsing solutions were filtered through 0,45 μm filter membrane (Millipore).

Fourier-transform infrared spectroscopy (FTIR)

FTIR spectra were performed with a FTIR-8400S spectrophotometer, Shimadzu. All spectra of either the reference or any samples were recorded after 128 scans with a resolution of 2 cm^{-1}.

Contact angle

The contact angle measurements were performed using the sessile drop technique with a KRUSS G1 contact angle meter (Krüss GmbH, Germany). Doubly distilled water (pH=5,5, 1-2 μL) was dropped for analysis. The contact angle values were taken as

average of the contact angles (right and left) of the droplets measured on 3 dry samples from independent preparation batches.

Ellipsometry

Ellipsometry measurements were performed with a vertical computer-controlled Ellipsometry System EP^3 (Nanofilm Technologie GmbH, Göttingen, Germany). The angle of incidence was set to 70.0^o with a laser wavelength of 532 nm. Analyses were done on dry samples after each step of the grafting procedure in air at room temperature. Obtained data were fitted with a EP3View software. The refractive indices were assumed n=1,45 for APTES, glutaraldehyde and oligonucleotide layers. [9]

AFM

Tapping mode AFM analysis of the samples was performed using a PycoLE system from Molecular Imaging with silicon nitride cantilevers (k=42 N/m) with a scan rate of 0,5 lines/s. Different areas of the samples were imaged. All experiments were done in air at room temperature.

Surface Pretreatment

Polished silicon wafers were cut into pieces of approximately 1x1 cm^2. Chloroform rinsing of silicon wafers was chosen as a water-free non-destructive method for surface cleaning.[10] The silicon wafers were cleaned 3 times ultrasonically in chloroform for 5 minutes, rinsed with chloroform 3 times. To reveal hydroxyl groups onto the surface, samples were activated in a UV/ozone chamber (UVO-cleaner, model 42-220, Jelight Company Inc. USA) during 15 min. Clean silicon wafers were characterized by contact angle and ellipsometry measurements for determination of the thickness of the technical silicon oxide layer (data are summarized in Table 2). Topography imaging through AFM showed flat homogeneous surfaces.

2.3. Results and discussion

The first step in the optimization of the protocol was to exclude any traces of water over the course of the reaction between the silicon surfaces and APTES, which otherwise can precipitate. The reaction between the hydroxyl groups, revealed on the silicon surface through UV-ozone activation, and the ethyl-groups of APTES is the result of a condensation, which occurs in toluene, with release of ethanol. The cleaned wafers were thus transferred in a 1% v/v APTES solution prepared in water-free toluene and incubated in a sealed vessel, to prevent contamination with moisture. To limit the possibility of the formation of multilayers of APTES, the samples were then ultrasonically rinsed 3 times for 5 minutes in toluene in a waterproof ware to remove most of the non-covalently bounded APTES and 3 more times under a gentle flow of toluene. After this procedure the formation of APTES layers was assessed by contact angle, ellipsometry and FTIR measurements.

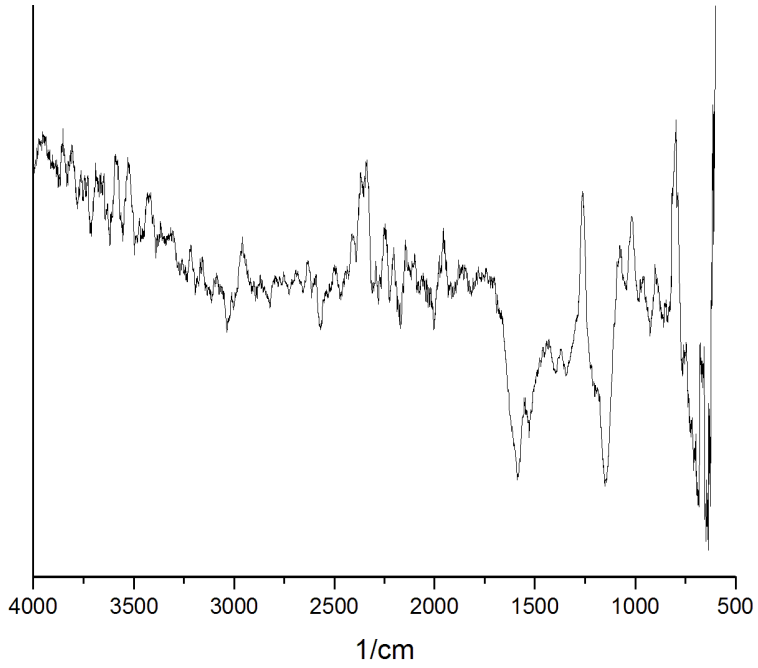

Figure 2. FTIR spectrum of the APTES-modified silicon wafer, the clean silicon wafer was set as the background.

The FTIR measurements (Figure 2) showed the characteristic peaks for APTES, which are presented in Table 1:

Frequency range (cm^{-1})	Chemical group
1110-1080	-Si-O-C-
1550-1650	$=NH_2$
2800	CH_2 stretching
2800-3000	CH_3, CH_2, CH stretching

Table 1. Characteristic band positions of the chemical groups of APTES detected by FTIR.

Ellipsometry evidenced the dependence of the thickness of the APTES layer on the incubation time. After 20 hours of incubation the thickness of the APTES layer was 11-15 nm, after 15 hours incubation – about 6-7 nm and after 2 hours incubation – 1.5 nm. Taking into account that the theoretical length of APTES is 0.8 nm, the layers cannot be considered as a monolayer. The effect of APTES polymerization on silicon surface, which can influence the APTES layer thickness, has been described in the literature[11]. However, with the shortest incubation time, as demonstrated in the following, no APTES left the surface, indicating that the molecules are covalently bound. Since it provides a homogeneous surface coverage with the lowest achievable value of the thickness, close to that of a monolayer, the incubation time of 2 hours was thus chosen to perform the following experimental steps.

As an alternative to silanization, triethoxysilylundecanal (TESUD) was investigated instead of the two-steps APTES-glutaraldehyde immobilization. Unfortunately no satisfactory response was evidenced over the course of the following immobilization steps.

The activation of the surfaces with aldehyde reactive groups was performed in a 1% aqueous solution of glutaraldehyde for 20 hours under gentle shaking and subsequent 3 times rinsing with water. The samples were stored in water until use for further oligonucleotide immobilization the same day.

The result of the reaction between APTES and glutaraldehyde is a so-called protonated Schiff base.[12] Schiff bases, which form at neutral pH, are usually unstable bonds at pH higher than 5, but could be stabilized by immersion of the samples in a solution of 50 μM sodium cyanoborohydride in water, which will reduce the Schiff base into an amine bond. Since in the course of all experimental stages described here the pH of all solutions was kept at 5, the reduction step can be done at the very last stage of the surface modification. It is also known that the glutaraldehyde can polymerize at pH around 7.4,[13] that can influence the immobilization efficiency. Therefore reaction between the amino-modified nucleotide sequences and glutaraldehyde immobilized on the surface was the most favorable at pH around 5.

The FTIR spectra of the glutaraldehyde-activated surfaces revealed the characteristic peaks of aldehyde groups at 1640 cm^{-1} and of aldehyde C-H at 2690-2840 cm^{-1} (Figure 3).

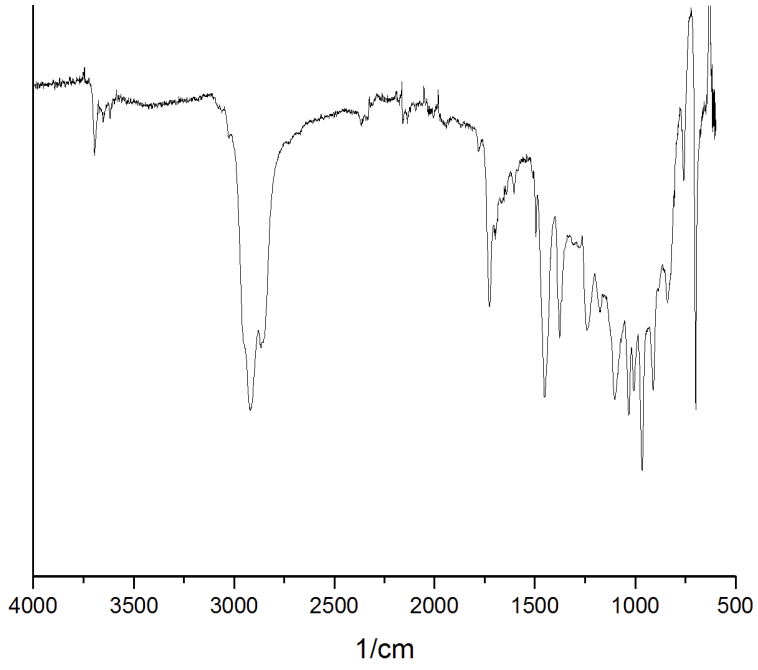

Figure 3. FTIR spectrum of the glutaraldehyde-modified silicon wafer.

The results of ellipsometry and wettability measurements are summarized in Table 2:

Sample	Static Contact Angle (°)	Thickness (nm)
Cleaned silicon wafer	27 ± 1	3.2 ± 0.8 (for SiO_2)
APTES-modification	67 ± 2	1.5 ± 0.7
Glutaraldehyde-modification	58 ± 4	1.3 ± 0.7

Table 2. Results of ellipsometry and wettability measurements after the sequential surface modifications

The thickness of the glutaraldehyde layer, obtained by ellipsometry in air, was around 1-2 nm. The results are comparable with the theoretical length of a glutaraldehyde molecule, the later being around 1.5 nm.

The wettability measurements were done after each modification steps: for the clean silicon wafer, after immobilization of APTES and after glutaraldehyde coupling. The measurements performed for the subsequent modification steps, associated with oligonucleotide immobilization and hybridization, were not conclusive due to insignificant changes of the average surface wettability.

The cleaned silicon wafer with activated hydroxyl groups has the lowest contact angle value due to the high hydrophilicity of the surface. The modification of surfaces with APTES can entail the non-coupled ethyl groups of APTES, part of them may stay dangling from the surface along with amine groups. The obtained value of contact angles is in good correlation with recently published results.[14] The following step of the surface modification procedure was the coupling of aldehyde groups, which increases the hydrophilicity of the surface.

The ellipsometry results were obtained by fitting the measured ellipsometry angles, with refractive indexes of APTES and glutaraldehyde of 1.45.[15]

For immobilization of oligonucleotide brushes the approach, described by Y. X. Jiang[8], was chosen to direct specifically the surface tethering. In this approach the amino-modified oligonucleotides were hybridized with their fully complementary sequences in solution prior to their immobilization onto the surface. Thus only the amino

groups at the 5'-terminus were available for reaction with the aldehyde groups revealed on the surface. Besides this approach prevents dense packing of the oligonucleotides in the brush, which would then lose their functional conformation for following dehybridization and rehybridization processes.

For following experiments the 24-mers oligonucleotides were chosen. The criterion of choice was the balance between two oligonucleotide chain properties: the value of the melting temperature and the conformational freedom. From one side, the shorter are oligonucleotide sequences have the lower is the melting temperature. For the 24-mers the melting temperature is 61ºC, but, for example, for 12-mers it is 38ºC, that is already too close to the experimental temperature, which will affect n the hybridization efficiency by temperature driven denaturation. From another side, there is the limited effect of surface tethering on the conformational freedom of short oligonucleotide chain. It means that the longer oligonucleotides occupy the larger area to keep the conformation of the nucleotide chain suitable for subsequent hybridization. The probe, SeqA and its fully complementary target, SeqB, were chosen as linear non self-hybridizing sequences (Table 3):

Name	Sequence	Modification
SeqA	5`-AGAGAGAGAGGGAGAGAGAGAGGG-3`	5`-Amino Modifier C6
SeqB	5`-CCCTCTCTCTCTCCCTCTCTCTCT-3`	

Table 3. Oligonucleotide sequences chosen for the experiments described herein and the whole thesis.

The solution concentration of the oligonucleotide solutions were assessed by measurement of the optical density of the solution at 260 nm. Hybridization of the complementary sequences SeqA and SeqB at concentrations of 300 nM was performed in a 50 mM NaCl solution during 1 hour at 4°C. The final concentration of double stranded oligonucleotides was 600 nM. To induce the immobilization of the oligonucleotides, the glutaraldehyde-modified surfaces were immersed in the solution of amino-modified oligonucleotides for 1 hour under gentle shaking and subsequent 3 folds rinsing steps

with 50 mM NaCl aqueous solution. Prior to AFM the samples were rinsed with water to avoid salt contamination of the surfaces.

To quench non-reacted aldehyde groups remaining active on the surface after immobilization of the oligonucleotides, the samples were immersed for 1 hour in a 20 mM aqueous solution of aminoacetic acid (glycine) under shaking and rinsed 3 times with water. Afterwards the reduction step was done.

The Schiff bases were reduced with 20 mg/ml $NaBH_3CN$ solution in 50 mM NaCl buffer. The reaction was carried out for 1 hour under shaking and followed by 3 rinsing steps with water. All depositions were done at room temperature.

To characterize the homogeneity of the layers of molecules at each stage of the immobilization protocol, assessment of the surface topography was obtained by AFM (Figure 4). The surfaces were shown as homogeneously covered at each preparation stage.

The subsequent experiments consisted in the dehybridization of the double stranded oligonucleotides immobilized onto the surface in brushes of double helices to obtain single-stranded surface tethered sequences, which can be further rehybridize. Formamide 50% and urea 4 M in 50 mM aqueous solution of NaCl were used as dehybridizing agents. In contrary to evidences obtained with urea, the experimental results showed that the single stranded oligonucleotides, obtained by dehybridization with formamide were unable to complete the following hybridization, probably, due to effects of formamide on the chemical structure of the nucleotide sequences. It is known that formamide is the agent for lowering the melting point of nucleic acids.[16] Normally, the double-stranded conformation of DNA is favorable over single-stranded, so the effect of formamide increases the stability of single-stranded conformation. This fact can bring down the efficiency of subsequent rehybridization. Therefore urea was chosen for subsequent experiments.

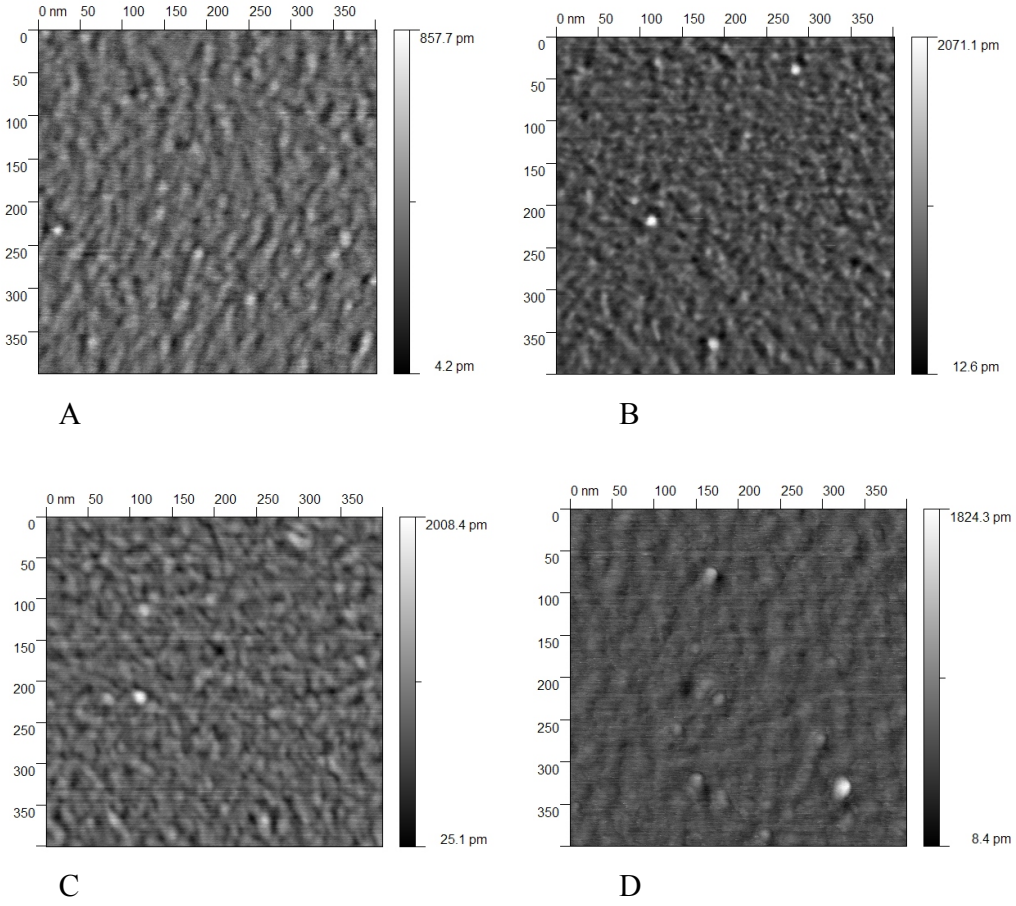

Figure 4. AFM of the topography of the surfaces:
Clean silicon wafer surface (A), APTES-modified surface (B),
Glutaraldehyde-modified surface (C), Oligonucleotide-modified surface (D)

Thus the presented experimental protocol allows obtaining brushes of oligonucleotide covalently immobilized onto silicon surfaces.

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Part 3. Optimal Hybridization Efficiency Upon Immobilization of Oligonucleotide Double Helices

3.1. Introduction

Since it has been identified as the universal carrier of the genetic information in the middle of last century, DNA became one of the most common structural materials in biotechnology. Over the last 30 years, its unique molecular recognition property, i. e. hybridization of complementary nucleotide sequences into the double helix according to Watson-Crick base pairing[1], has driven the development of DNA-based nanotechnologies.[2] The strategy of DNA-directed immobilization (DDI) of macromolecules onto surfaces is based on the hybridization process between two fully complementary nucleotide sequences, one of which, the probe, is covalently attached onto the surface, whereas the second – the target, is bound to the macromolecule of interest (peptide, protein or polymer).[3, 4] The achievement of high quality biosensors via DDI is however strongly dependant on the procedure of immobilization of the nucleotide sequences onto the surface and the subsequent hybridization efficiency.[5] The chip sensitivity, i. e. surface quality, relies on the immobilization procedure of the probe sequence as well, but in particular on the grafting density and on the resulting hybridization efficiency between target sequences with the surface-tethered probes. The amount of probes immobilized onto the surface should indeed be sufficient to induce a positive signal, but not exceed a value above which the macromolecules immobilized onto the surface are packed too densely, preventing efficient subsequent hybridization and eventually de-hybridization processes.[6-8] In these conditions solely, the oligonucleotide-conjugated biomolecules can be efficiently immobilized to possibly be removed from the surface by induced de-hybridization for regeneration of the chip and eventual subsequent cycles of use.

To achieve optimal hybridization efficiencies of surface-tethered nucleotide sequences, the immobilization of solution-hybridized nucleotide sequences prior to their

sequential de- and re-hybridization is shown here the most elegant approach to prevent steric hindrance to hybridization and non-specific interactions. Indeed, this immobilization of the double helices prior to their disassembly via denaturation is shown here, for the first time, to enable achieving nearly 100% hybridization efficiency.

However, there are no direct methods for detection of the adsorbed mass in the picogram range to quantify the number of immobilized oligonucleotides and further estimate the grafting density and hybridization efficiencies. We thus used two appropriate indirect methods: the quartz crystal microbalance with dissipation monitoring (QCM-D) and a newly developed wavelength interrogated optical sensor (WIOS, Bright Reader®). The measured physical parameters were the frequency and dissipation shifts of an oscillating quartz crystal with the QCM-D and shift of the resonance wavelength of a propagating evanescent wave with WIOS. The value of the adsorbed mass was mathematically deduced from those experimentally measured parameters. The analysis procedures were subjected to assumptions and restrictions that are described in more details in the following.

3.1.1. Theory of hybridization onto surfaces

Although the mechanism of hybridization in solution is comprehensively studied, a well-established theory of hybridization onto surfaces, experimentally assessed, is still under consideration. [9, 10] Compared to the solution process, several experimental factors influence the hybridization process onto surfaces, among which the probe grafting density that appears to play a critical role in the efficiency of the reaction.

To describe the surface behavior of hybridizing oligonucleotides, the existing theory of polymer adsorption onto surfaces was elegantly adapted by Halperin and coworkers. [9, 10]

Oligonucleotides are indeed natural polymers composed of four monomers (adenine, guanine, thymine and cytosine). Therefore the theory describing polymers as elastic body can be applied to depict the properties of oligonucleotides. Polymers can be described as chains of independent statistical segments (the freely jointed chain model, Flory, 1969). However, the Flory model fails in describing the elastic behavior of

DNA. [11, 12] Charged nucleotide sequences rather behave as relatively stiff rods of contour length L and persistence length l_p according to the worm-like chain model WLC. [13]

At physiological salt concentration the persistence length of hybridized DNA was estimated to 50 nm or 150 base pairs. [14, 15] Thus short hybridized oligonucleotides are rod-like, cylindrical macromolecules, single stranded oligonucleotides remain semi-flexible chains.

A. Halperin and coworkers modeled the immobilization of oligonucleotides onto a surface through a Langmuir adsorption isotherm. [9] The use of this model is however limited to the following assumptions: a) the probes are identical; b) each probe can hybridize with a single target and controversially; c) the targets cannot self-hybridize and; d) the probes, immobilized onto the surface, do not interact with each other.

To fulfill most of those conditions, commercially synthesized oligonucleotides of a specific composition were purchased to prevent self-hybridization in solution. To prevent inter-molecular interactions, immobilization of solution-hybridized nucleotide sequences was performed prior to subsequent disassembly of the double helix and re-hybridization. Based on these theoretical assumptions, the grafting density of the surface tethered probes was expected to lead to optimal hybridization efficiency.

The presented methodology enabled the accurate quantification of both the grafting density and the efficiency of hybridization onto a surface via measurements of the changes of adsorbed mass onto sensor surfaces in the time course of the hybridization reaction.

3.1.2. QCM-D method

In the recent years the QCM-D method was identified as a sensitive tool for the real time investigation of macromolecular adsorption at the liquid-solid interface. The sensing principle is based on the detection of changes in the electromechanical characteristics of an oscillating piezoelectric quartz crystal electrode upon mass adsorption onto the sensor surface.

The validation of this method for measurements in the liquid phase were established and discussed. The real physical values, which can be monitored with the

QCM-D, are the resonance frequency shift (Δf) and energy dissipation shift (ΔD). The resonance frequency (f) of the crystal depends on the total oscillating mass, including water coupled to the oscillation. When a film is forming on the sensor crystal, this frequency is decreasing. If the film is thin and rigid the decrease in frequency is proportional to the mass of the film. In this way, the QCM-D operates as a very sensitive balance, down to picogram/cm^2. The absorbed mass (Δm) can be deduced from a linear dependence between the absorbed mass between the measured frequency shift according to the Sauerbrey relation.

$$\Delta m = (C_{QCM}/n)\,\Delta f$$

Where $C_{QCM} = 17.7\ ng \cdot Hz^{-1} \cdot cm^{-2}$ is the mass sensitivity constant for a f=5 MHz resonance frequency quartz crystal and n = 1, 3, 5 and 7 is the overtone number.

Due to hydration of the adsorbed film with solvent molecules, the Sauerbrey relation provides the "wet" mass of the adsorbed layer, thus overestimating the real mass of adsorbed biomolecules onto the surface. In this scheme, analysis with the Sauerbrey relation does not take into account the value of ΔD, which characterizes the dumping of the energy factor due to large dissipative properties of the film. [16] In case of large values of ΔD, the Sauerbrey equation does not apply anymore. The criteria for rigidity of the ad-layer has been assessed to $\Delta D <\sim 1 \cdot 10^{-6}$. [17]

To describe a dissipative film adsorbed onto the surface, the two basic Maxwell and Voigt models were thus introduced. The Maxwell model is usually applied to a polymer solution, which can demonstrate purely liquid-like behavior whereas the Voigt model operates with macromolecules, which conserve their conformation and do not flow, therefore representing the homogeneous film. It was shown that the Sauerbrey "ideal mass thin layer" response corresponds to a purely elastic behavior using the Voigt model, i.e. zero shear viscosity. [18]

To extract data using the QCM-D method the values Δf and ΔD were thus combined to further estimate the grafting density from the evaluated adsorbed mass. For data modeling the software Qtools was used.

3.1.3. WIOS method

The WIOS (Bright Reader® technology) was very recently developed for highly sensitive measurements of small refractive index changes at the solid-liquid interface. Hence the measurement principle allows the estimation of several different biochemical events taking place at the sensor surface such as association and dissociation rates, affinity constants and specificity of biochemical reactions.

This technology is based on grating couplers in combination with an optical readout of an evanescent field sensor. The grating couplers that are used are made up of a transparent glass carrier substrate containing a diffractive grating structure that is coated with a stable high refractive index film. For this measurements tantalium pentoxide (Ta_2O_5), an optically transparent metal oxide with a high refractive index, has been used to provide an optimal wave-guiding material.

The sensor is illuminated with a laser having a specific wavelength, the so-called resonance wavelength, through which an evanescent field is created at the sensor surface. The evanescent tails of the waveguide mode penetrates a few hundreds of nanometers into the liquid, which allows the accurate detection of (bio)chemical binding events at the sensor surface. Changes, like binding of molecules to the surface, affect the resonance wavelength and are detected with high sensitivity. For continuous monitoring of the resonance wavelength, a laser diode is used for continuously tuning the wavelength, which allows fast real-time determination of the resonance wavelength during the binding events. [19]

One of the main advantages of the WIOS is the simultaneous detection of signals from 8 channels; part of them could be modified for subsequent immobilization or not, remaining passive for control studies. The measurements in the flow mode in a small volume with sensitivity up to 0.5 pg/mm^2 are appropriate for label-free and real-time monitoring of biochemical events.

The experimental data were fitted and translated into surface mass coverage according to a thin-layer sensing model. [20] This model is based on the assumption that adsorption or desorption of molecules from the sensing layer changed only the thickness of the layer while the refractive index remains constant. In our case we monitored the

growth of a thin layer of bio-molecules with a refractive index of 1.45 within a buffer having a refractive index that was close to the one of water, 1.33.

The combination of both the QCM-D and the WIOS allowed to investigate the sequential reactions for immobilization of nucleotide sequences onto the surface and to estimate the hybridization efficiency by measuring in real time two independent physical parameters in the course of the reaction processes. These two methods allowed assessing at all stages the effectiveness of the immobilization reaction *in situ*, without any contacts of the sensor with air to avoid effects of contamination or surface drying.

3.2. Experimental part

3.2.1. Material

Ultrapure water was produced via an ELGA purification system (ELGA system, Vivendi Water Systhems Ltd, Bucks, UK) up to a resistivity of 18.2 MΩ cm. All buffer solutions were filtered with 0.22 µm Millipore (Millipore Corp., Bedford, USA) membranes and degassed.

Twenty four bases long nucleotide sequences (HPLC, purity 99%) were purchased from Operon (Köln, Germany).

3.2.2. Methods

QCM-D

Frequency and dissipation shifts were monitored using the QSense D300 Quartz Crystal Microbalance (QSense, Gothenburg, Sweden). The QCM sensors consist of a disk-shaped, AT-cut piezoelectric quartz crystal coated with metallic electrodes on both sides. The QCM sensor crystal (1.4 cm diameter crystal, 1.2 cm diameter gold area, 0.2 cm^2 active area, approximately 0.3 mm thickness, roughness of the electrode < 3 nm (RMS)) operates at a resonance frequency of 4.95 MHz ± 50 kHz. We used the quartz

crystals (10 nm thick) with an active surface layer of SiO_2 (~50 nm thick). The flow cell had an 80 µL working volume.

The crystal surface was pretreated with aminopropyltrietoxysilane, APTES as described in the 'Surface Modification' section. The frequency and dissipation shifts at overtones n=3, 5 and 7 were monitored in real-time.

WIOS

For our measurements, a prototype BR-8 instrument (DYNETIX AG, Landquart, Switzerland) has been used for accurate determination of any changes of the properties of the ad-layer. The system uses a tunable laser with a wavelength of 760 nm that is tuned within 2 ms over a 2 nm range to illuminate the sensor chip. The high scanning rate allows either to monitor fast binding in real-time or averaging to increase the signal to noise ratio.

With the BR-8 instrument used for these experiments, up to 8 independent measurement channels could be used in parallel and probed several times per second. The sensor channels of the glass-based sensor chip SC-8 have a size of 1 mm^2 each. The sensing region consists of a grating with a period of 360 nm with a height of only 15 nm in order to not influence the biochemical reactions. Note that the WIOS is insensitive to any changes of the light intensity.

3.2.3. Surface modification

We applied the same surface chemistry to modify both QCM-D and WIOS sensors to enable the immobilization of oligonucleotide brushes. [21] The clean sensors were treated with a 1% (v/v) solution of APTES in water-free toluene for 2 hours at room temperature to introduce amino groups on the surface. Use of water-free toluene was necessary to avoid any precipitation of APTES and to limit the formation of multilayers. To remove most of the non-covalently bonded silane, the WIOS-sensors were sonicated 3 times over 5 min in toluene and rinsed 3 times with toluene afterwards whereas the QCM-D sensors were just thoroughly rinsed. Subsequent rinsing steps with suitable buffer after each deposition step were very important to avoid as much as possible contamination with non-covalently bonded molecules.

After the pretreatment of the sensors with APTES, all steps of the surface modification were done *in situ*. The immobilization of aldehyde groups onto the surface was performed by injecting a 1% (v/v) aqueous solution of glutaraldehyde for 1 hour, followed by 3 times water rinsing steps. The result of the reaction between APTES and glutaraldehyde is a protonated Schiff base, which forms at acidic pH and which is usually unstable at pH higher than 5. However, in our experiments all used solutions had pH not exceeding 5.

Solution concentrations of the oligonucleotides were controlled by measurement of the optical density of the solutions at 260 nm. Hybridization of the complementary sequences SeqA and SeqB (Table 1) at concentrations of 300 nM was performed in a 50 mM NaCl solution during 1 hour at 4°C.

Name	Sequence	Modification
SeqA	5`-AGAGAGAGAGGGAGAGAGAGAGGG-3`	5`-Amino Modifier C6 TFA
SeqB	5`-CCCTCTCTCTCTCCCTCTCTCTCT-3`	

Table 1. Oligonucleotide sequences

Solution hybridized oligonucleotides were injected into the sensor chamber. Kinetics of the immobilization of the oligonucleotides onto the sensor surface was recorded in real-time. After equilibrium was reached, the rinsing steps were performed.

To quench free aldehyde groups onto the surface, a 20 mM solution of aminoacetic acid (glycine) prepared in water was incubated for 1 hour, prior to rinsing steps.

With this surface chemistry we obtained surfaces with covalently bound hybridized oligonucleotide brushes. To dehybridize the immobilized double helices, incubation in a 4 M urea solution for 1 hour was performed prior to subsequent rinsing steps.

Re-hybridization between SeqB (target in solution) and the remaining oligonucleotides immobilized onto the surface, SeqA, was done by immersion of the

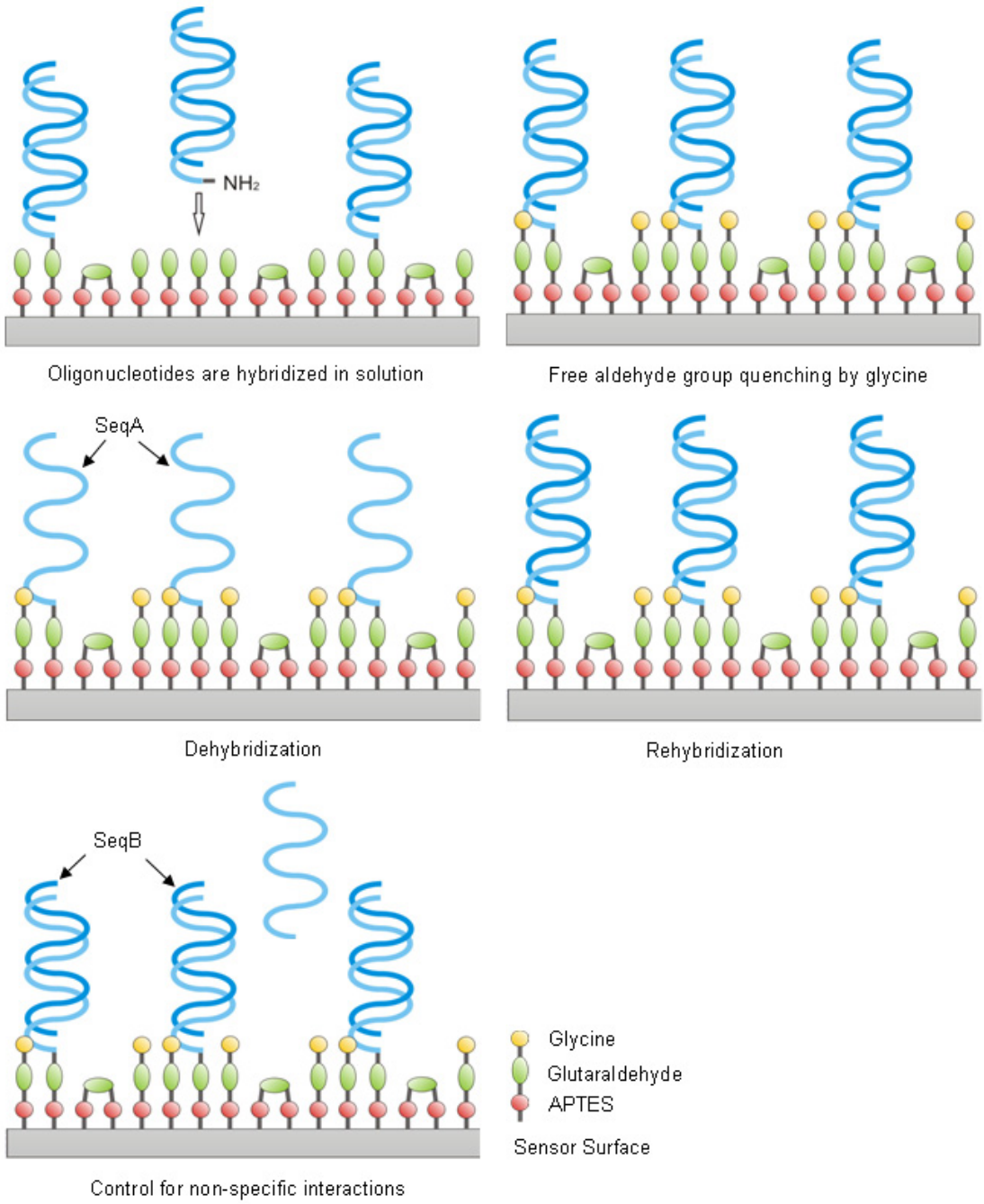
surfaces in a 300 nM solution of SeqB in 50 mM NaCl, followed by rinsing after equilibrium was reached.

As control experiments, a solution of SeqA was immobilized onto the sensors with rehybridized SeqA and SeqB to check the level of non-specific interaction between sequences.

3.3. Results and discussions

Immobilization of solution-hybridized nucleotide sequences prior to their sequential de-hybridization and re-hybridization was preferred to the tethering of the single stranded oligonucleotides to achieve the suitable density to reach optimal hybridization efficiency. This hypothesis was further supported by the choice of short nucleotide sequences, assuming that the hindrance to hybridization arises rather from macromolecular crowding of the probes tethered onto the surface than loss of conformational freedom due to their grafting. Besides, this approach limited unspecific interactions between the aldehyde groups revealed at the surface and the reactive groups along the backbone of the nucleotide sequences. Thus only the amino groups at the 5'-terminus of the probe sequence were available for reaction with the aldehyde groups present on the surface.

The QCM-D crystal-sensor surface consists of a SiO_2 coating, whereas the surfaces of the WIOS sensors are Ta_2O_5. We could therefore apply the same surface chemistry for surface modification to immobilize the oligonucleotides (Scheme 1). Modification of the surfaces with APTES, which introduces amino groups onto the surfaces, was done outside of the reaction chambers and APTES-modified sensors were prepared immediately prior to any subsequent reaction. Using the QCM-D and WIOS, we could not draw any conclusions about the formation of a monolayer or multilayer's. However, both techniques showed that during the equilibration time prior to the glutaraldehyde injection, no APTES mass left the sensor surfaces.



Scheme 1. Immobilization strategy of solution hybridized oligonucleotides followed by sequential dehybridization and rehybridization.

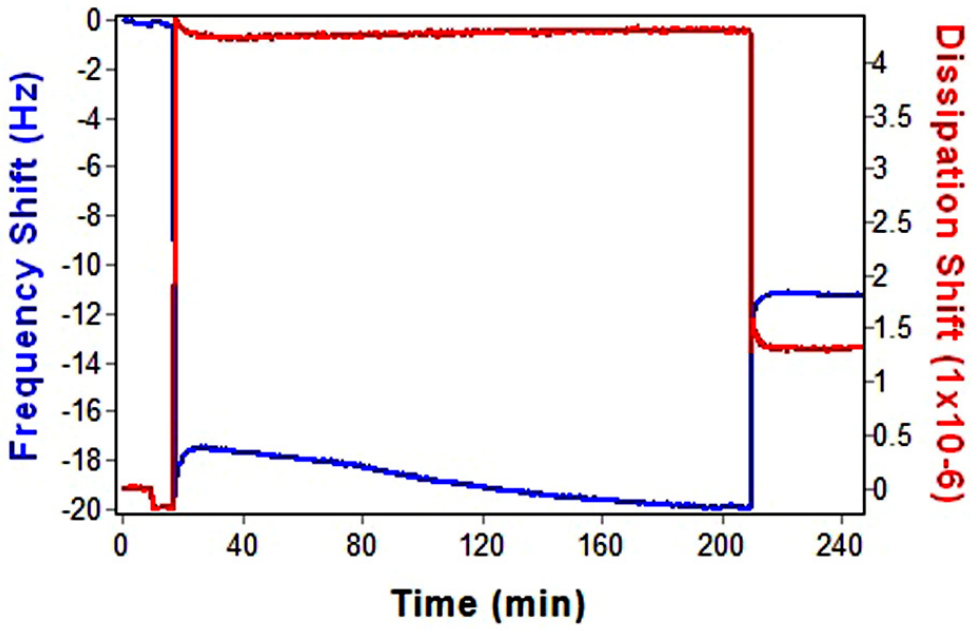


Figure 1. Time course of the frequency (blue line) and dissipation (red line) shifts recorded with the QCM-D upon binding of glutaraldehyde onto the surface

The typical kinetics of glutaraldehyde adsorption on the APTES-modified surface detected with the QCM-D is presented in Figure 1. At first, glutaraldehyde molecules adsorb on the sensor surface. Subsequent to rinsing, unbound molecules were washed away and only coupled ones remained on the surface. The evaluation of the results according to the Voigt model showed a decrease of the layer thickness from 3.6 nm to 1.8 nm upon washing. Since the glutaraldehyde density is around 1 g/cm^3, we could calculate the mass of adsorbed glutaraldehyde molecules as 179 ng/cm^2. Using the Sauerbrey equation to calculate the mass of adsorbed glutaraldehyde (after the rinsing step) we got a mass density of approximately 180 ng/cm^2. The grafting density of glutaraldehyde was around $1.2 \cdot 10^{15}$ molecules/cm^2 if we assumed the formation of a glutaraldehyde monolayer.

Since both the Sauerbrey relation and the Voigt-based model led to results in agreement we could assume that glutaraldehyde formed a thin rigid layer onto APTES-modified surfaces without any dissipative property. Besides, theoretical calculations of the molecular length predicted a monolayer thickness of 1.4 nm for glutaraldehyde.

Ellipsometry measurements further showed that the thickness of the glutaraldehyde layer is about 1.7 nm (data are not shown) in agreement with the QCM-D evaluation. We should mention here that ellipsometry measurements were done in air; i. e. molecules could collapse onto the surface, whereas measurements with the QCM-D and WIOS are performed in the liquid phase, where molecules were in hydrated layers. Correlation of the thickness values obtained via both ellipsometry and QCM-D monitoring further supported the conclusion about the formation of a thin rigid layer.

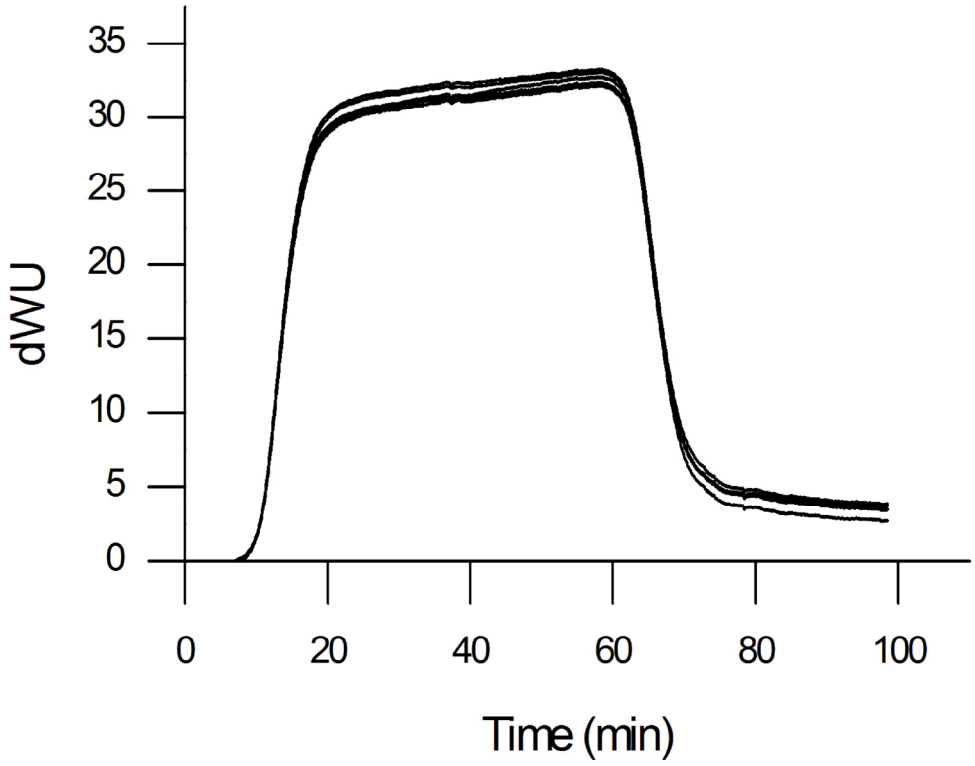


Figure 2. Time course upon binding of glutaraldehyde onto the surface monitored with the WIOS. The graph shows the result of five independent sensing channels

The kinetics of glutaraldehyde coupling to the APTES-modified WIOS-sensor is shown in Figure 2. The results are given as changes of WIOS wavelength units (dWU) versus time. The same kinetic profile was previously observed with QCM-D: adsorption onto the surface and mass loss upon washing. At saturation no more changes of the refractive index within the 200 nm sensed penetration depth occurred. The shift of the

resonance wavelength was corresponding to the change of surface refractive index, the measured unit 1 dWU corresponding to the relative change of the resonance wavelength. To translate the shift of wavelength value to the adsorbed mass the thin-layer model was applied. [20] Based on this model, the calculated value for a wavelength unit 1 WU was equal to 3.20 ng/cm^2. The results obtained with the WIOS showed that upon the adsorption of glutaraldehyde onto the surface the recorded value was in a range between 3 and 10 dWU corresponding to a surface mass coverage of 9-32 ng/cm^2. The significant difference between quantification performed either with the QCM-D or the WIOS could be explained by the effect of layer hydration on the measurements with the microbalance. Hence, adsorbed molecules as well as coupled water molecules within the glutaraldehyde layer were sensed. However, agreement between the Sauerbrey and Voight analyses indicated that the thin layer model could be applied to the characterization of the glutaraldehyde film.

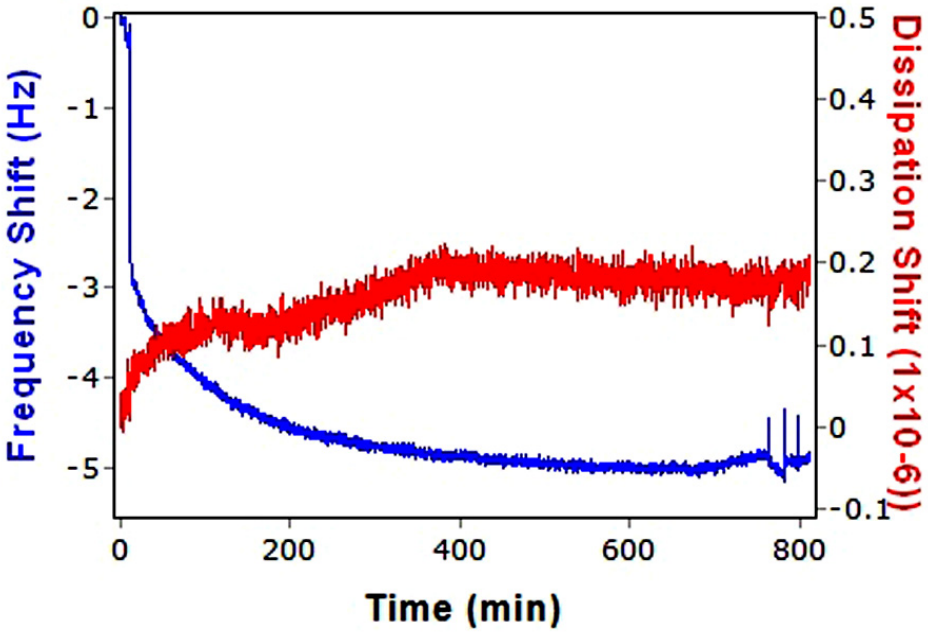


Figure 3. Time course of the frequency (blue line) and dissipation (red line) shift recorded with the QCM-D upon binding of solution-hybridized oligonucleotides onto the surface.

The QCM-D detection of the kinetics of coupling of solution-hybridized oligonucleotides to the glutaraldehyde-modified surface is shown in figure 3. This reaction was monitored during 12 hours after which the crystal surface was rinsed 3 times. Just a small amount of material was washed away, as indicated by no significant shifts of Δf and ΔD values upon rinsing. The fitting of the obtained data with the Sauerbrey equation and with the Voigt-based model gave the same values of the adsorbed mass (see below). This fact allowed considering that all material was attached onto the surface, forming a rigid nondissipative layer.

In these investigations we used 24-bases oligonucleotides. The hybridized oligonucleotides have a radius of 9.5 Å and a monomer length of 3.4 Å. The cross-section area is thus 284 $Å^2$. The corresponding parameters of non-hybridized, single stranded oligonucleotides are not well established. [22] The monomer length was estimated to 6 Å. [23] Thus in the brush regime the 24-bases hybridized oligonucleotides could be modeled as cylinders with a length of 81.6 Å and a diameter of 19 Å. If we considered these cylinders as not tilted onto the surface, the thickness of a full monolayer of hybridized oligonucleotides should be around 8 nm. Data analysis yielded a value of the thickness of the layer of around 0.8 nm at saturation. The obtained value thus indicated that only around 10% of the whole surface was covered by hybridized oligonucleotides. The analysis of the experimental data led to a mass of adsorbed hybridized oligonucleotide of approximately 85 ng/cm^2. Knowing the molecular mass of a double helix, i.e. 14887 Da, the estimation of the grafting density was approximately $3.4 \cdot 10^{12}$ molecules/cm^2. If we considered the cross-section area of one double helix as $2.8 \cdot 10^{-14}$ cm^2, the mass coverage would be around 0.095 i. e., about 10% of the whole sensor area, similar to the coverage estimated from the thickness analysis.

The time course of the immobilization of solution-hybridized oligonucleotides, measured with the WIOS, is shown in Figure 4. Analogical estimations to those performed with the data obtained via measurements with the QCM-D led to values for the adsorbed mass around 90 ng/cm^2 and a grafting density of $3.6 \cdot 10^{12}$ molecules/cm^2 matching the above model of average surface mass coverage.

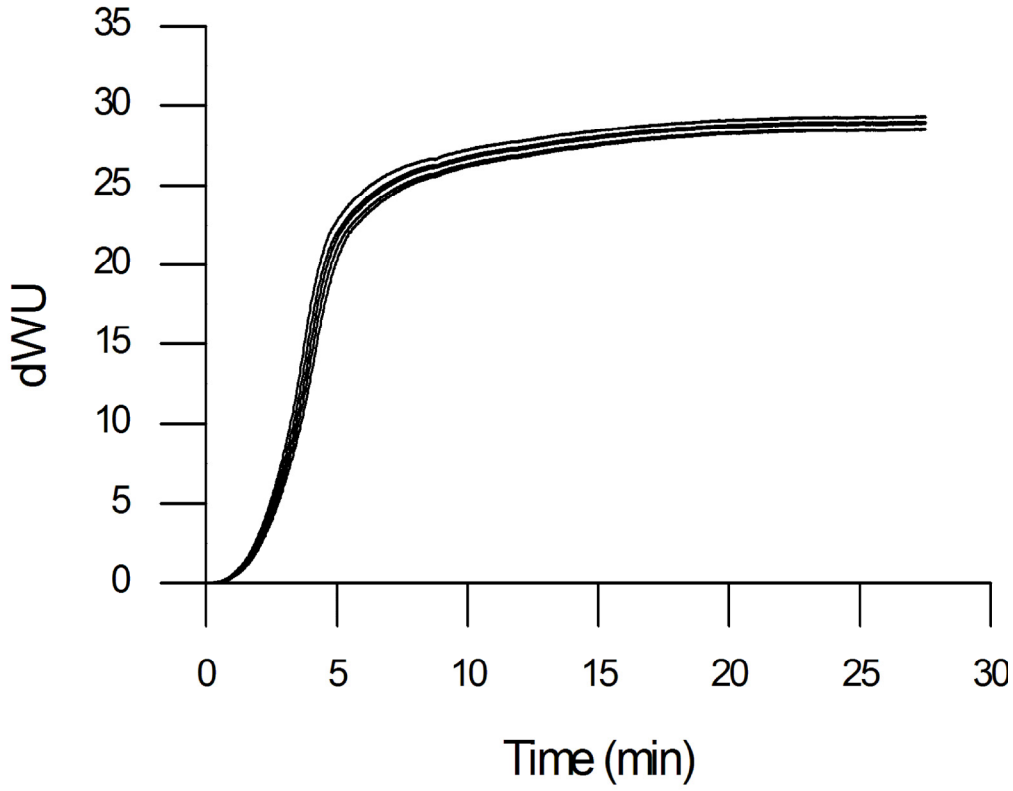


Figure 4. Time course of the binding reaction of solution hybridized oligonucleotides onto the surface monitored with the WIOS.

Quenching of free aldehyde groups onto the surface was done by injection of glycine. This short amino acid can react with aldehyde groups via their amino function, leaving the carboxylic group available. Exposure of the surface to glycine is shown in Figure 5. This small molecule was expected to diffuse within the hybridized oligonucleotide brushes and interact with the surface. Thus with the QCM-D we expected to observe a mass adsorption, i.e. a frequency decrease, but it should not influence the rigidity of the layer, i.e. dissipation shift should be negligible. Measurements with the QCM-D showed that according to the Sauerbrey relation the adsorbed mass of glycine was around 30 ng/cm^2. Knowing the molecular mass of glycine (75.07) the grafting density could be deduced, $2.4 \cdot 10^{14}$ molecules/cm^2. Monitoring of the immobilization of glycine was below the detection limit of the WIOS.

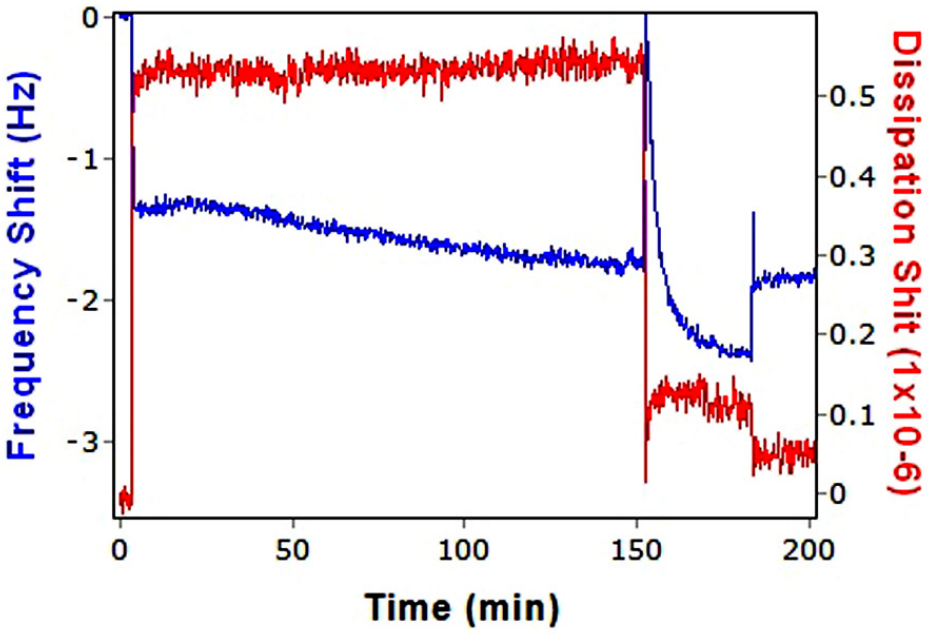


Figure 5. Time course of the frequency (blue line) and dissipation (red line) shifts recorded with the QCM-D upon binding of glycine onto the surface

Thus in our case the $1.2 \cdot 10^{15}$ molecules/cm^2 of glutaraldehyde, immobilized onto the surface, were reacted with $3.4 \cdot 10^{12}$ molecules/cm^2 of solution-hybridized oligonucleotides and further quenched by $2.4 \cdot 10^{14}$ molecules/cm^2 of glycine. Remaining molecules of glutaraldehyde were considered non-reactive because of probable cross-linking.

After this step, the de-hybridization of solution-hybridized oligonucleotides immobilized onto the sensor was performed in 4 M urea, followed by rinsing steps. Due to the high viscosity of the urea solution, the real-time kinetics was impossible to monitor.

Following sensor rinsing, the solution of complementary target oligonucleotides was injected for re-hybridization (Figure 6 and 7).

The hybridization reaction, measured with the QCM-D, should lead to an increase in adsorbed mass and a slight decrease of dissipation because of the higher rigidity of hybridized oligonucleotides (Figure 6). Due to the fact that a short nucleotide sequence can be considered as a stiff chain, no significant shift of the dissipation was detected.

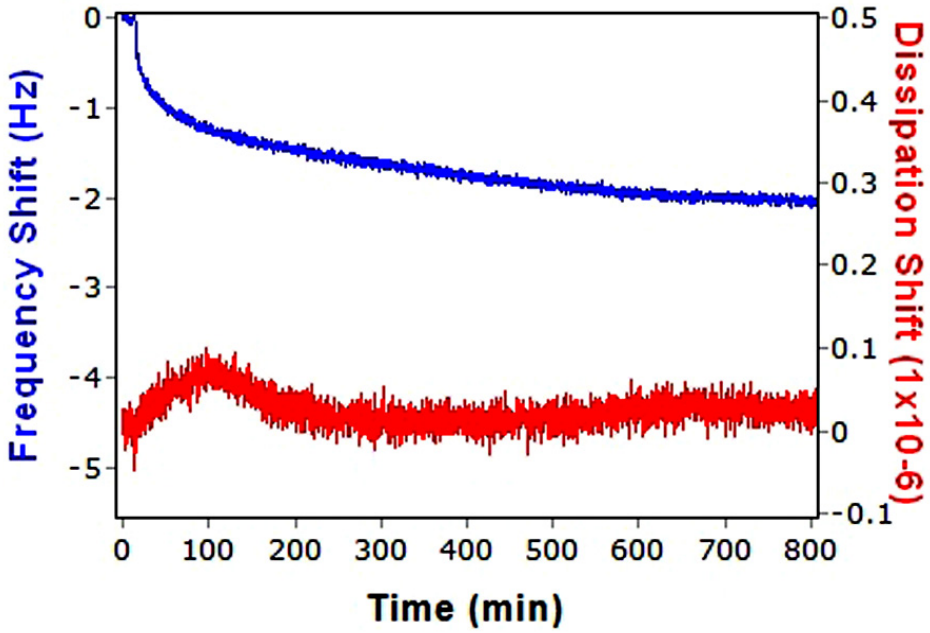


Figure 6. Time course of the frequency (blue line) and dissipation (red line) shifts recorded with the QCM-D upon the reaction of hybridization onto the surface.

The reaction of hybridization onto the surface was investigated in real-time until saturation. No material left the surface upon the following rinsing steps. As control measurements, we injected a solution of the non-complementary sequence into the sensor chamber. Both detection methods did not lead to any recording of mass adsorption onto the surface indicating that the oligonucleotides did not interact with it by selective or non-specific physisorption.

The calculation of adsorbed mass of single stranded oligonucleotides onto the sensor surface yielded 35 ng/cm^2, with the QCM-D and 45 ng/cm^2, with the WIOS. These values correspond to a hybridized surface density of oligonucleotides of $3.0 \cdot 10^{12}$ molecules/cm^2 and $3.8 \cdot 10^{12}$ molecules/cm^2, respectively. Comparing the results obtained upon grafting of solution hybridized oligonucleotides ($3.4 \cdot 10^{12}$ molecules/cm^2 with the QCM-D and $3.6 \cdot 10^{12}$ molecules/cm^2 with the WIOS) we could estimate with both methods the hybridization efficiency, which is the ratio of the numbers of adsorbed complementary sequences to the number of immobilized sequences. Via QCM-D

measurements the deduced hybridization efficiency was in average 88%, whereas with the WIOS the value was close to 100%.

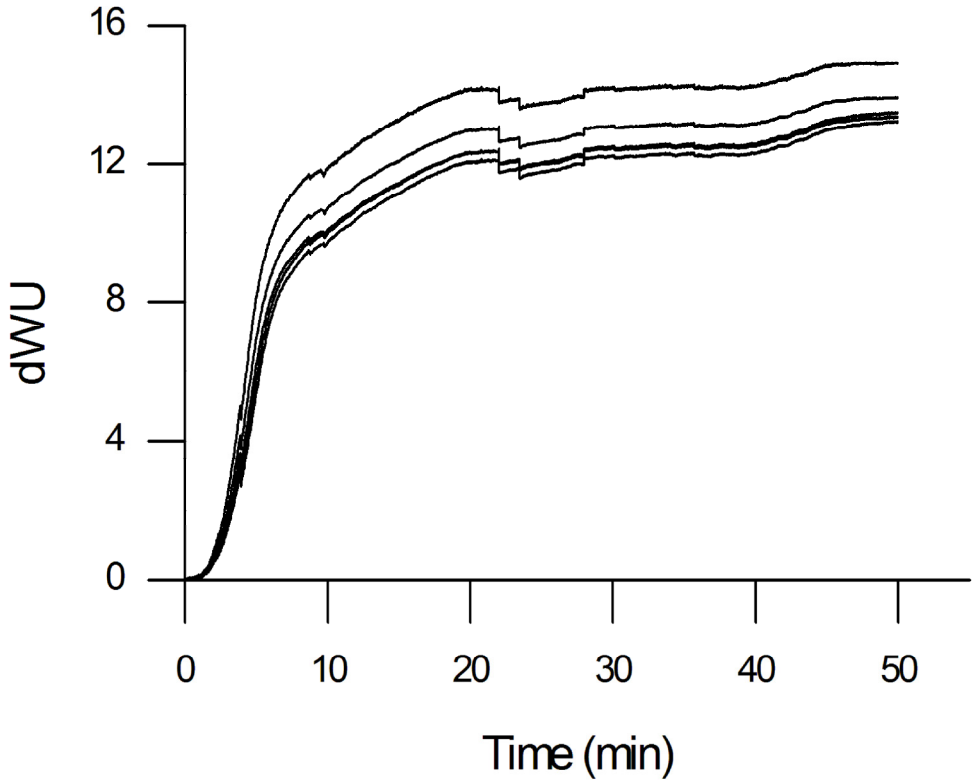


Figure 7. Time course of the reaction of hybridization onto the surface monitored with the WIOS.

Figure 8 and 9 represent the mass coverage upon immobilization of solution-hybridized oligonucleotides as well as re-hybridization subsequent to their de-hybridization. Since the mass of re-hybridizing oligonucleotides was approximately two times lower than the mass of immobilized double helices, at equilibrium the adsorbed mass ratio must reach 0.5 for the same number of immobilized double helices and re-hybridizing nucleotides.

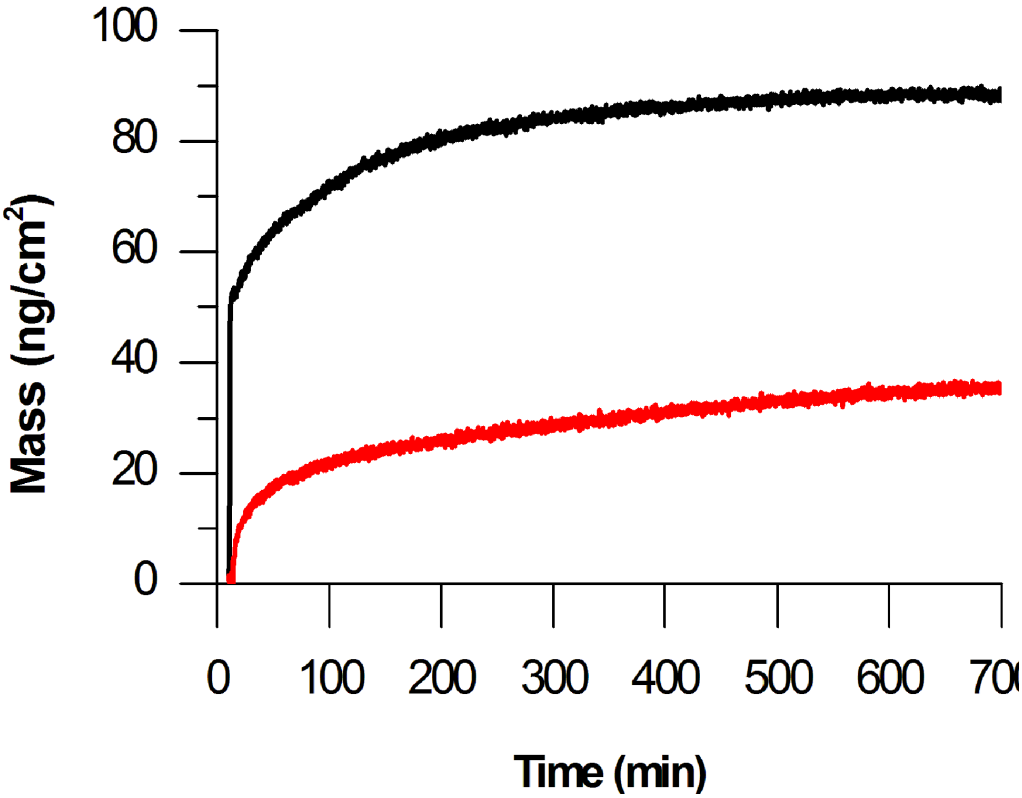


Figure 8. The adsorbed mass, extracted from measurements performed with the QCM-D, for immobilization of solution hybridized oligonucleotides (black) and for oligonucleotides upon re-hybridization onto the surface subsequent to disassembly of the double helix (red).

We should mention that with the QCM-D we worked in batch, with an initial concentration of the target oligonucleotides that decreased upon binding, whereas the measurements with WIOS were performed in the flow mode, hence the oligonucleotides concentration was kept constant during the experimental time.

However, at saturation the adsorption monitored with the QCM-D reaches equilibrium. Within the known volume of 80 µL and at a concentration of 300 nM the number of target sequences is $1.5 \cdot 10^{13}$ molecules. The estimated amount of glutaraldehyde molecules immobilized onto the 0.2 cm^2 sensing area was approximately $3.4 \cdot 10^{12}$ molecules, ensuring that we had an excess of oligonucleotides in the reaction chamber.

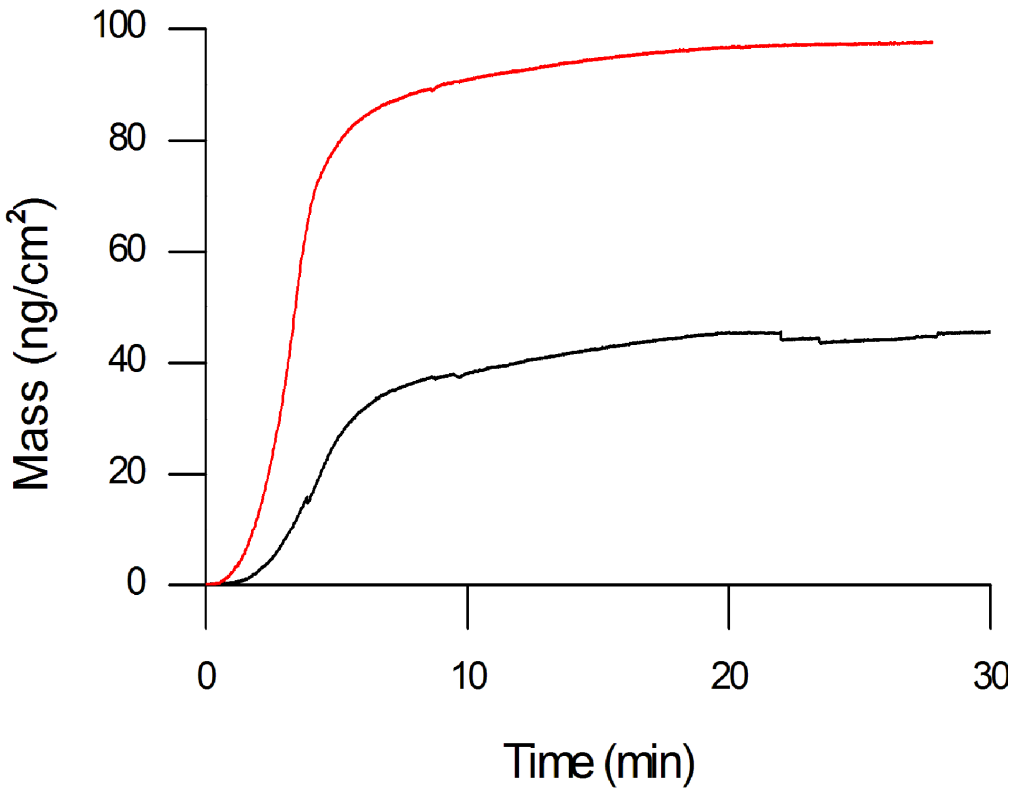


Figure 9. The adsorbed mass, extracted from measurements performed with the WIOS, upon immobilization of solution hybridized oligonucleotides (black) and upon re-hybridization onto the surface subsequent to the denaturation of the double helices (red).

With the WIOS the concentration of target sequences could be assumed as converging to infinity in the time course of the hybridization reaction. Thus the fraction of hybridized oligonucleotides was much larger than the fraction of non-hybridized probes onto the surface. The hybridization efficiency in this case was expected to tend to 1, when all probes were hybridized. At saturation in the course of an experiment conducted with the WIOS, a fully hybridized layer onto the surface was achieved. With the QCM-D we reached practically the same value of hybridization efficiency, indicating that in the conditions of Langmuir adsorption the fully hybridized state of oligonucleotides was more favorable than equilibrium between hybridized and nonhybridized states.

Why do we discuss Langmuir adsorption isotherms? In the design of oligonucleotide chips, the high hybridization efficiency is correlated to the high signal output from the sensor.

$$x_{eq} = \frac{I}{\mathrm{I}_{\mathrm{m}\,ax}},$$

with I is the signal yield, I_{max} is the maximal signal intensity of the chip.

Thus when the fraction of hybridized probes x_{eq} tends to 1, the signal from the sensor will reach the maximum value. However in modern high-density oligonucleotide chips and microarrays there are some difficulties to achieve an accurate signal due to factors influencing the hybridization reaction onto the surface such as too dense sequence packing, sequence folding or non-specific hybridization. In this work we could demonstrate the achievement of optimal surface grafting density at which the hybridization efficiency reached the maximum value via immobilization of solution-hybridized nucleotide sequences. Neglecting effects of reduced conformational freedom arising from surface tethering, which was possible through the choice of short nucleotide sequences, these studies further enabled the quantification of the grafting density to tune maximal hybridization efficiency. Sequential de- and re-hybridization of surface immobilized double helices was possible with 100% hybridization efficiency.

Eventually we calculated the probe surface density of hybridized 24-bases oligonucleotides to investigate possible models of polymer adsorption onto surfaces suitable to describe this optimal hybridization efficiency.

The probe macromolecular area assessed experimentally ($\sum_0$) was approximately 30 nm^2. In the model of hybridization of oligonucleotides onto surfaces adapted from the theory of polymer adsorption onto surfaces, the condition $\sum_0 > R_F^2$, where R_F is the Flory radius, is assumed to ensure that inter-molecular interactions are prevented to achieve optimal hybridization efficiency.

A nucleotide sequence is a semi-flexible polymer. However, the reported values of the persistence length vary over a wide range (between 0.75 and 3.5 nm). Nevertheless, the estimate, $R_F \approx N^{\frac{3}{5}} a$ with N=24 and a= 0.6 nm led a theoretical critical area $\sum_0$ of 16 nm^2, much below the experimental molecular area of a surface tethered nucleotide sequence and in agreement with an optimal hybridization efficiency owed to

the absence of inter-molecular interactions. Considering the nucleotide sequence as a rigid rod of contour length 8.16 nm or as a semi-flexible polymer of persistence length of about 2 nm led to a critical molecular area much larger than the experimentally measured, though maximal hybridization efficiencies were achieved. The 24-nucleotide long nucleotide sequences appeared to rather adopt a coil conformation than stretch even at this extremely low ionic strength (50 mM NaCl).

3.4. Conclusion

We used two indirect methods to evaluate the absorbed mass upon hybridization of surface tethered nucleotide sequences. With the QCM-D we measured the resonance frequency shift of an oscillating quartz crystal whereas with the WIOS we measured the changes of the refractive index of oligonucleotide brushes tethered onto the surface. The results obtained by both methods were in agreement. Experimental monitoring of mass adsorption with two complementary highly sensitive surface sensing methods indeed enabled the accurate calculation of molecular area of the surface-tethered oligonucleotides.

Assuming that hybridization onto surfaces is hindered by steric hindrance upon high grafting density, we overcame this issue investigating short, rigid nucleotide sequences immobilized in their double-helix secondary structure prior to de- and re-hybridization. Loss of conformational freedom upon-surface tethering did not enter into consideration since short nucleotide sequences were investigated. Besides, immobilization of solution-hybridized oligonucleotides prior to their sequential de-hybridization and re-hybridization enabled the achievement of maximal hybridization efficiencies due to suitable grafting density. In addition to the established geometric model of the double helix, the ideal grafting density obtained to reach maximal hybridization efficiencies indicated that the surface tethered nucleotide sequences adopted a coil rather than a stretched conformation.

ACKNOWLEDGMENT. NCCR and SNSF (1218229) are greatly acknowledged for financial support.

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Part 4. A Microcontact Printing Approach to the Immobilization of Oligonucleotide Brushes

4.1. Introduction

In comparison to the solution process, the study of hybridization of oligonucleotides or DNA onto surfaces has not reached yet a comprehensive understanding. However, investigations of this mechanism represent current fundamental, experimental and technological challenges. Surface hybridization is indeed the basic process ruling the DNA chip technology and has been addressed theoretically very recently.[1,2]

To enable physical chemistry studies of the mechanism of hybridization onto surfaces, facile, homogeneous and especially reusable surface immobilization of oligonucleotide brushes is a prerequisite.

As microstructures can be visualized via optical microscopy, spotting and microcontact printing have proven to be elegant approaches to assess the immobilization and subsequent hybridization of fluorescently labeled oligonucleotides.[3,4] In their early work, Vieu and co-workers demonstrated that uniformity and edge definition of spots could be obtained on glass slides functionalized with nanometer sized spherical dendrimers bearing aldehyde reactive groups at the periphery for the covalent attachment of a 5'-amino-modified sequence.[3] Recently, Reinhoudt and coworkers assessed an efficient method for transferring DNA to a substrate and facilitating the positioning of DNA with high lateral resolution by microcontact printing (μCP) based on the modification of the stamp with dendrimers.[4]

However, major hindering to hybridization arises from steric effects due to a too high density of surface tethered oligonucleotides. We thus made use of the specific property of hybridization of complementary nucleotide sequences in an iterative fashion. Complementary oligonucleotides were first hybridized in solution prior to their immobilization onto a glutaraldehyde modified silicon surface via μCP. The use of hybridized sequences ensures that grafting of the oligonucleotides occurs at a single

point, i. e. at the amino-modified 5'-terminus of the oligonucleotide to be surface-tethered. Hence, immobilization of the hybridized sequences prevents interaction between the aldehyde reactive groups on the substrate and the amino groups along the oligonucleotide backbones, which are involved in the assembly of the double stranded oligonucleotides.[5] Nevertheless, only one of the two complementary oligonucleotides is covalently immobilized onto the solid support. Sequential dehybridization and rehybridization of oligonucleotides labeled with fluorescent dyes emitting at different wavelengths could thus be followed with laser scanning microscopy to demonstrate, for the first time, the immobilization of hybridized oligonucleotides into brushes prior to dehybridization followed by efficient and selective rehybridization.

4.2. Results and discussion

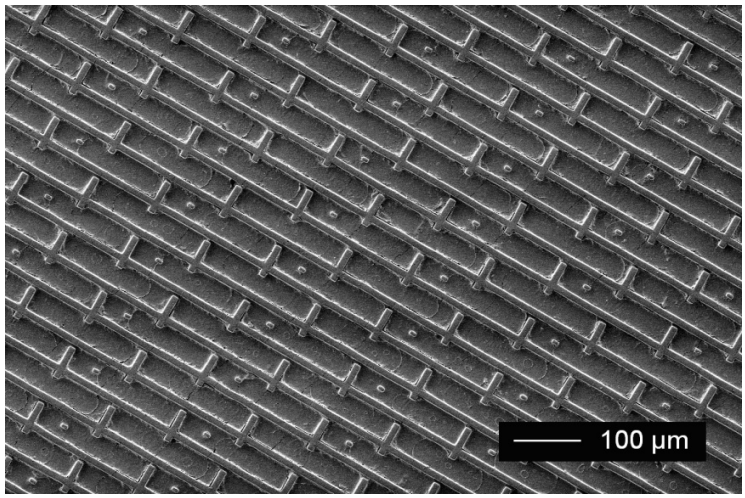
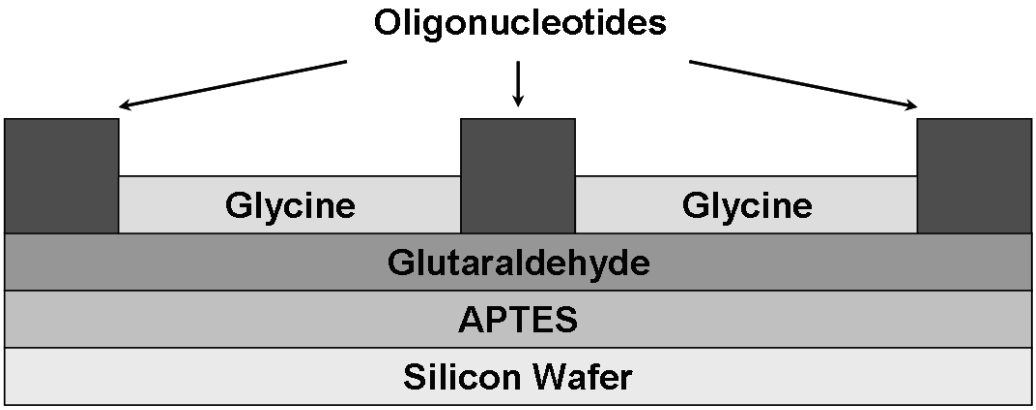


Figure 1. Scanning electron micrograph of the PDMS mask

The poly(dimethylsiloxane) (PDMS) mask, which is most commonly used for microcontact printing, is a soft polymer stamp, which allows a conformal contact between the stamp and the surface.[6,7] The scanning electron microscopy (SEM) image of the used PDMS structure is shown in Figure 1 (straps of 10 µm width distant of 30 µm). Prior to the immobilization of oligonucleotides, modification of the surface was

performed according to a widely used method of silanization of silicon oxide wafers with aminopropyltriethoxysilane (APTES) followed by coupling with amine-modified biomolecules through glutaraldehyde.[8,9]



Scheme 1. Schematic representation of the sequential chemical modification of silicon wafers.

The target surface structure is shown in Scheme 1. At each step of the preparation the samples were characterized by contact angle measurements for wettability monitoring, ellipsometry for thickness control and AFM for roughness control. The immobilization reaction was proved (data not shown) at each stage; layers of APTES and glutaraldehyde were homogeneous with thicknesses of about 1.8 nm and 1.7 nm, correspondingly. Theoretical calculations of the molecular length predicted a monolayer thickness of 0.8 nm for APTES and 1.4 nm for glutaraldehyde. Those measurements showed that APTES was deposited as a multilayer, and that the polymerization of glutaraldehyde was negligible. The samples were then further analyzed by laser scanning microscopy (LSM).

A solution of hybridized oligonucleotides, SeqA and SeqB was used for stamping (see Table 1 in the experimental section). The complementary sequence (SeqB) of the surface-tethered oligonucleotide (SeqA) is fluorescently labeled. The fluorescence images of the solution hybridized oligonucleotides, deposited by μCP, are shown in Figure 2. The detection of aggregation is associated to the resolution of this technique, which is approximately 250 nm (λ/2NA, with NA being the numerical aperture). The silicon wafer

without APTES and glutaraldehyde modification were chosen as control samples. In Figure 2A, the deposition of some oligonucleotides onto the control surface can be observed. However, the quality of μCP on the glutaraldehyde modified surfaces is conformal and demonstrates the possibility to immobilize solution hybridized oligonucleotides via μCP (Figure 2B).

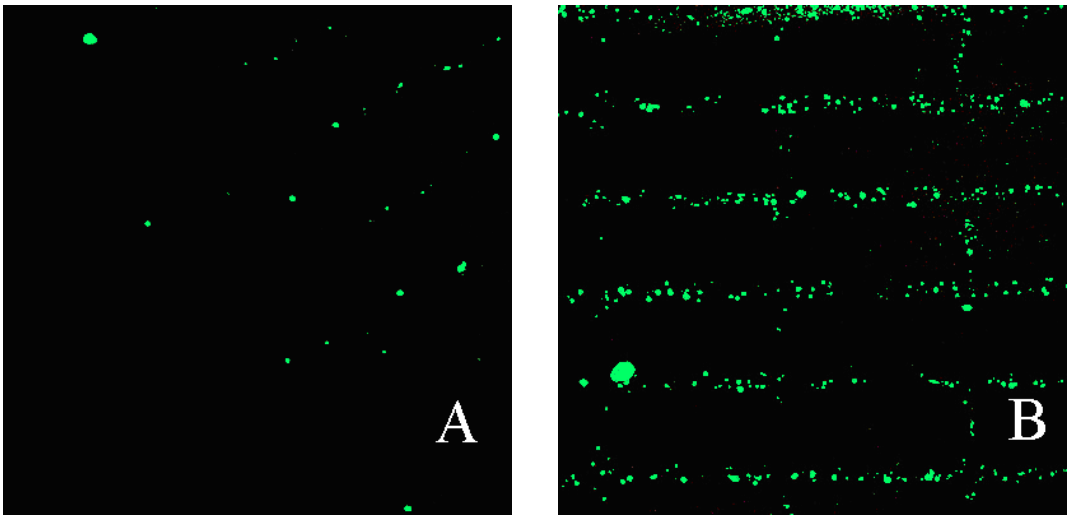


Figure 2. Fluorescence microscopy of μCP deposited hybridized oligonucleotides (SeqA and SeqB) onto control surface (A) and APTES and glutaraldehyde-modified surface (B)

Dehybridization of the stamped oligonucleotides upon incubation with a solution of urea is expected to cleave the hydrogen bonds between the immobilized complementary oligonucleotides and, following rinsing, the fluorescently-labeled complementary oligonucleotides, SeqB, should be washed away, leaving a brush of single stranded oligonucleotides covalently grafted at their 5'-terminus onto the surface (Figure 3). Because it is impossible to avoid completely non-specific interaction, the efficiency of dehybridization did not reach 100%. Fluorescent signal traces from remaining dyes on the surface can still be observed. However the intensity was significantly lower than prior to dehybridization.

The fluorescence image of immobilized oligonucleotides via μCP after re-hybridization with SeqC, which is the fluorescently labeled complementary sequence of the grafted oligonucleotides onto the surface, is shown in Figure 4. The control images of

both channels were taken at every step of the μCP deposition; the signal in the red channel was negligible except upon efficient rehybridization of the immobilized oligonucleotides with their fully complementary sequence.

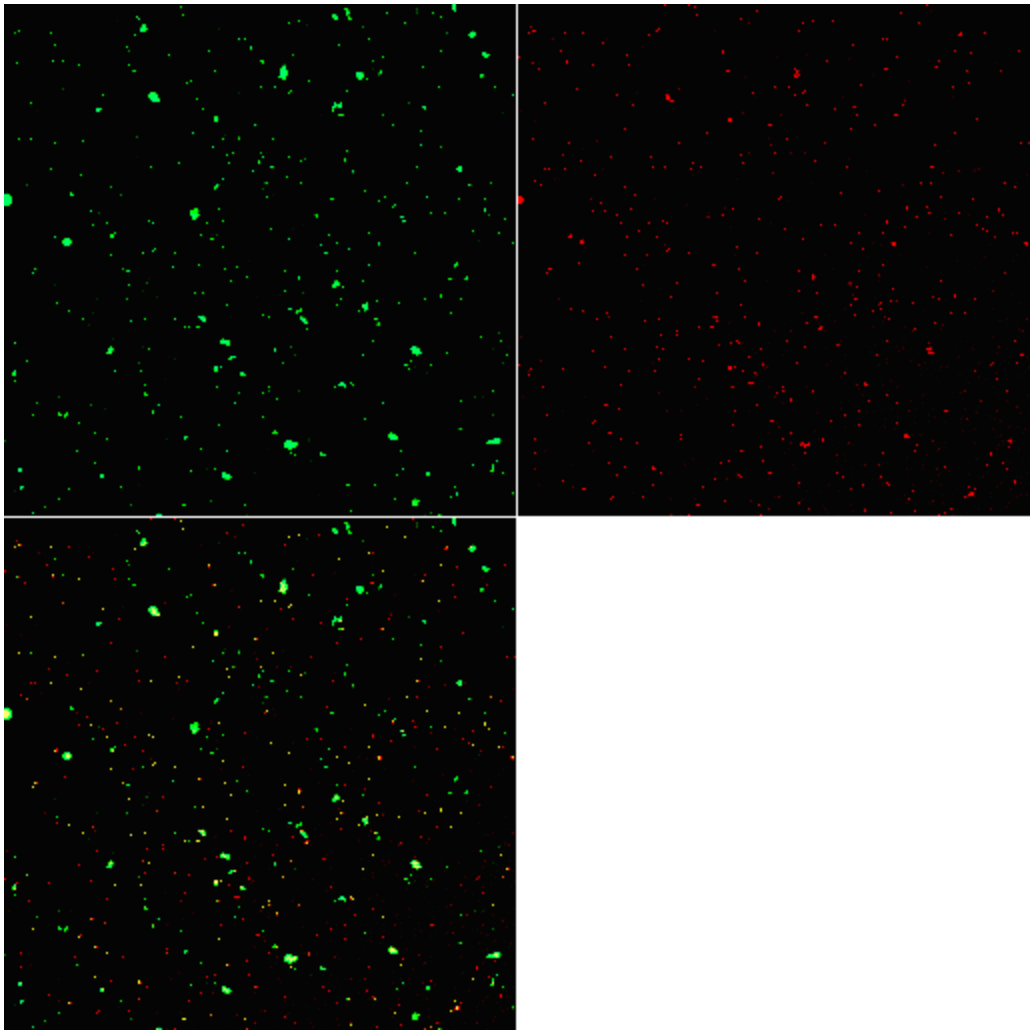


Figure 3. Fluorescent images of μCP deposited oligonucleotides after dehybridization. Top right: Green fluorescence channel for detection of immobilized oligonucleotide; top left: Red fluorescence channel, in which only the signal from rehybridized oligonucleotides is detected; Bottom: Colocalization of both green and red fluorescence signals.

The stamping of hybridized oligonucleotides into brushes would not be fully assessed at this stage without essential control experiments. First, deposition of the fluorescently labeled single stranded oligonucleotide SeqC onto the surface by μCP shows that the oligonucleotides bind to the surface. After printing we indeed observed the

structure of the mask onto the surface, which assesses the oligonucleotide interaction with the aldehyde-modified surface.

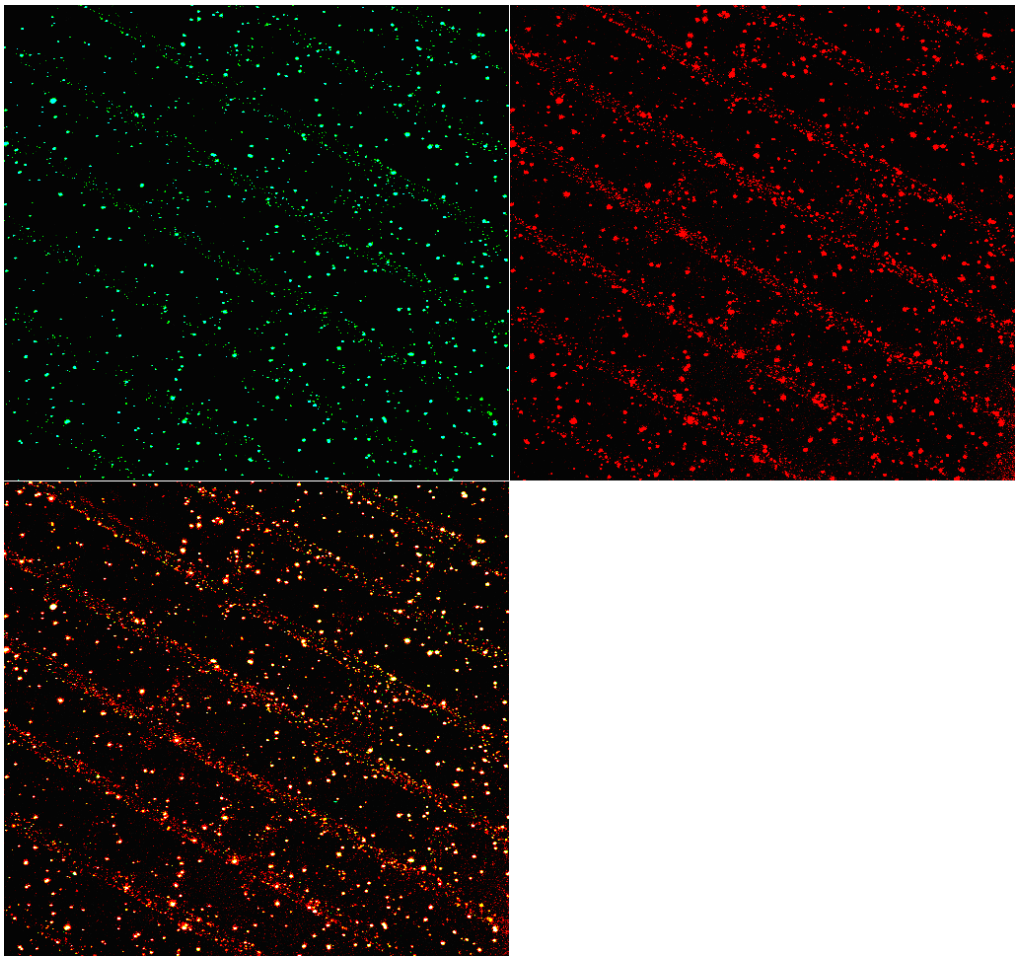


Figure 4. Fluorescent images of μCP deposited oligonucleotides after re-hybridization with SeqC. Top right: Green fluorescence channel for detection of immobilized oligonucleotides; Top left: Red fluorescence channel corresponding to the detection of rehybridized oligonucleotide. Bottom: superposition of both green and red channel signals.

However, stamping of the single stranded SeqA, followed by hybridization with its fully complementary sequence, SeqB does not enable the LSM detection of any features originating from the PDMS mask. Those observations demonstrate that for successful hybridization onto the surface the oligonucleotides should be tethered within a brush-like structured layer. If the amino groups present along the oligonucleotide backbone interact with the surface, which is the case upon immobilization of the single

stranded sequence, the chain "cross-linkage" onto the surface prevents efficient hybridization. However, upon immobilization of the hybridized sequences, the amino groups along the oligonucleotides are protected within the double strand except the function at the 5'-terminus of the oligonucleotide to be grafted onto the solid support, enabling immobilization of the oligonucleotides into a brush-like layer.

Second, the specificity of interaction between the oligonucleotides was investigated. For this purpose SeqD, which is a non-complementary sequence of the surface-grafted SeqA was used at the rehybridization step. LSM measurements did not show any mask-like structure on the surface, indicating that specific hybridization only occurs between the complementary SeqA and SeqC.

In this paper we demonstrated that an oligonucleotide brush can be obtained by covalent interactions between the surface and the terminus of the nucleotide sequence by μCP of hybridized oligonucleotides. The fact that the oligonucleotide patterns, immobilized onto the surface, can undergo sequential hybridization and dehybridization proves the stability of the immobilization of the oligonucleotides within a brush.

As several recent reports highlight cell recognition of oligonucleotides, micro-patterned surfaces are being investigated further to study cell response onto surfaces.[10,11] Being the minimal modified surface square area of interest of the oligonucleotide stamped surfaces large enough, i. e. between 10 and 30 μm, to allow the spreading of various cell types with normal functionality, oligonucleotide microcontact printing appears to have fundamental and experimental appeal and a high potential to design not only biosensors or biochips but also implants and cell-growth promoting or antifouling surfaces for tissue engineering.

4.3. Experimental Section

Surface modification

The silicon wafers were chosen for those experiments due to their low roughness and high chemical homogeneity. Silicon wafers were cut in 1x1 cm^2 peaces. The samples were treated with a 1% (v/v) solution of APTES in water-free toluene for 2 hours at room temperature to bring amino groups on the surface. Use of water-free toluene is necessary to avoid any precipitation of APTES and to reduce multilayer formation, what was shown by ellipsometrical measurements (data not shown). To remove most of the non-covalently bonded silane, the surfaces were sonicated 3 times over 5 min in toluene and rinsed 3 times with toluene afterwards. Subsequent rinsing steps with suitable buffer after every deposition step are very important to avoid as much as possible contamination with non-covalently bonded molecules. The samples were immersed in 1% (v/v) glutaraldehyde solution in water for 1 hour, rinsed 3 times with water and stored in water until use for stamping the same day. The result of the reaction between APTES and glutaraldehyde is a so-called protonated Schiff base.[12] Schiff bases, which form at neutral pH, are usually unstable bonds at pH higher than 5, but could be stabilized by immersion of the samples in a solution of 50 μM sodium cyanoborohydride in water, which will reduce the Schiff base into an amine bond. Since in the course of all experimental stages described here the pH of all solutions was kept at 5, the reduction step can be done at the very last stage of the surface preparation.

Oligonucleotide immobilization

24 bases long oligonucleotides (HPLC, purity 99%) were purchased from Operon (Köln, Germany). Probe, SeqA and its fully complementary sequences target, SeqB and SeqC, Alexa 532 and Alexa 488 modified were chosen as linear non self-hybridizing sequences, SeqD Alexa 488 modified was chosen as the non-complementary sequence of SeqA (Table 1). Concentrations of the oligonucleotide solutions were controlled by measurement of the optical density of the solution at 260 nm.

Table 1. Oligonucleotide sequences

Name	Sequence	Modification
SeqA	5`-AGAGAGAGAGGGAGAGAGAGAGGG-3`	5`-Amino Modifier C6
SeqB	5`-CCCTCTCTCTCTCCCTCTCTCTCT-3`	5`-Amino Modifier C6-Alexa Fluor 488 (ester)
SeqC	5`-CCCTCTCTCTCTCCCTCTCTCTCT-3`	5`-Amino Modifier C6-Alexa Fluor 532 (ester)
SeqD	5`-GGGAGAGAGAGA-3`	5`-Amino Modifier C6-Alexa Fluor 488 (ester)

Hybridization of the complementary sequences SeqA and SeqB at concentrations of 300 nM was performed in a 50 mM NaCl solution during 1 hour at 4°C. Thus only amino groups at the 5'-terminus were available for reaction with the aldehyde groups present on the surface. This reaction hence prevails over non-specific binding. A drop of 30 µL of hybridized oligonucleotide solution was deposited onto the PDMS stamp and slightly spread during 3 min; the excess of solution was soaked out by pipetting. The stamp was placed into uniform conformal contact with the flat substrate for 3 min and rinsed afterwards. To quench free aldehyde groups on the surface after deposition by µCP, the samples were immersed for 1 hour in a 20 mM solution of aminoacetic acid (glycine) prepared in water. Afterwards the reduction step was done. After these manipulations we obtain microstructured surfaces with covalently bound hybridized oligonucleotide brushes. As control experiments the samples without APTES and glutaraldehyde modifications were used for the stamping procedure. To de-hybridize the immobilized oligonucleotides, the samples were immersed in a 4 M urea solution for 1 hour. Re-hybridization between SeqC or SeqD and the remaining oligonucleotides immobilized onto the surface, SeqA, was done by immersion of the surfaces in 300 nM solution of SeqC or Seq D in 50 mM NaCl for 1 hour in dark condition. After rinsing, the samples were analyzed by laser scanning microscopy, LSM.

For control experiments single stranded SeqA was printed on glutaraldehyde modified silicon, prior to immersion in glycine and incubation with a solution of SeqB.

Fluorescent microscopy

A Zeiss LSM 510 laser confocal microscope was used to study the fluorescence signal from modified silicon surfaces in the epi-fluorescence detection mode. Fluorescence imaging of μCP stamping of Alexa-labeled oligonucleotides was performed relative to a background corresponding to the fluorescence of the sample modified with APTES and glutaraldehyde. The fluorescence signal from Alexa 488-labeled oligonucleotide, was registered upon emission at 488 nm wavelength with 505-550 nm emission filters (green channel) whereas the fluorescence of Alexa 532-labeled oligonucleotides, was recorded at 543 nm and 560-615 nm excitation and emission wavelengths respectively (red channel). The use of long-pass filters is not expedient in this study case since, over a wide range of wavelengths, the signal to background ratio is lowered with band-pass filters due to the background fluorescence of aldehyde and silane over the whole wavelength range of interest. All samples were investigated in the multi-track mode to avoid the cross-talk of the various dyes used for these investigations.

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Part 5. General Conclusions and Outlook

The scientific investigations, described in this thesis, were directed to improve the knowledge on the hybridization of oligonucleotides, immobilized on surfaces. A general mechanism of this process, described theoretically, is still missing experimental assessments, though potential applications, such as DNA-based biosensors are constantly in development. For this purpose the procedure of oligonucleotide immobilization was investigated stepwise and optimized. The subsequent studies of the oligonucleotide hybridization and denaturation efficiency were done *in situ* using two modern methods based on different measuring physical principles: the quartz crystal microbalance with dissipation monitoring (QCM-D) and the wavelength interrogated optical sensor (WIOS, Bright Reader®). With the QCM-D we measured the resonance frequency shift of an oscillating quartz crystal upon mass detection whereas with the WIOS we measured the changes of the refractive index of oligonucleotide brushes tethered onto the surface. The results given by both methods were in agreement. The obtained data were treated, applying the existing mathematical models, to draw conclusions on the preferred conformation of oligonucleotides attached to the surface, and the accurate calculation of the mean molecular area of the surface-tethered oligonucleotides.

The main results of this work, carried through, can be summarized in the following conclusions:

- The applied method of surface modification by silanization with APTES and following glutaraldehyde coupling was optimized for the preparation of homogeneous layers covalently bound to silicon surfaces. The oligonucleotides bind to glutaraldehyde-modified substrates in the brush-like conformation with preservation of their functional properties.

- The covalent immobilization of oligonucleotides double helices on surfaces prior to sequential denaturation and rehybridization was proven to lead to optimal hybridization efficiency. The grafting density of immobilized oligonucleotides obtained, using this approach, allows: a) to avoid the effect of overcrowding, when too dense packing of molecules

hinders their conformational freedom, necessary for subsequent denaturation and rehybridization; and in the mean time b) preserve the functionality of each immobilized oligonucleotide available.

- Applying the theory of polymer adsorption on the surfaces, it was possible to conclude that the 24-nucleotide long nucleotide sequences appeared to rather adopt a coil conformation than stretch one even at this extremely low ionic strength (50 mM NaCl).

- This access, the solution hybridization of oligonucleotides prior to their immobilization on the surface, can be also used in the microcontact printing approach to pattern surfaces. It was proved via exchange of the fluorescently labeled complementary sequence of the surface tethered nucleotide sequence. Hybridization with complementary sequences labeled with fluorescent tags emitting at different wavelengths enabled to follow the sequential dehybridization and rehybridization cycle via fluorescence microscopy.

Perspective Studies

As a result of this research work we improved the experimental approach to immobilize oligonucleotide brushes with preservation of their main functional property, which is hybridization. Moreover, this approach can be used not only to immobilize the oligonucleotides by their loading from solution, but also to use microcontact printing to structure the surface. Further research is proposed using the combination of this way of immobilization of oligonucleotides with the ability of binding other molecules of interest, through their coupling with the oligonucleotide sequences complementary of the surface-tethered one. For example, oligonucleotide-based copolymer systems were synthesized in our research group.

Using this approach, experiments to understand the biological response towards the polymers, immobilized on surfaces, can be carry out for following clinical applications in the fields of biocompatibility and biosafety. The methods, used in this

thesis, QCM-D and WIOS, can also provide data about material coupling efficiencies and kinetics of binding to surfaces. On the basis of microcontact printing, when one oligonucleotide sequence is deposited by printing and another one of different composition – by loading from solution, one can create the structured surfaces to compare two deposited materials on one substrate.

All these possibilities of surface investigation and characterization can be used in the studies of cell-material interactions. In recent publications specific oligonucleotide sequences were found to trigger cell signaling processes. The structured surfaces by microcontact printing can be applied in cell culture experiments to direct the adhesion of different types of cells according to the surface structures. The investigations of interaction of surface-immobilized sequences with cell receptors appear as very challenging.

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Curriculum Vitae

- Researcher in biophysics and physical chemistry
- Competencies in cell biology and chemistry of surfaces and interfaces
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Academic Education

2005 – 2009	University of Basel	Ph.D. thesis
1992 – 1997	Belarusian State University	Diploma in physics

Languages

English:	Fluent
German:	Basic
Russian:	Native language

Technical Skills

Biophysics	Spectroscopy, fluorescence and chemiluminescence of biological samples
	Principles of optical sensors
Physical Chemistry	Immobilization of biomolecules on surfaces
	Physical-chemical characterization of surfaces (AFM, FTIR, QCM-D, waveguiding optical sensor, ellipsometry, contact angle measurements)
Cell Biology	Blood cell isolation
	Basic cell cultures and maintenance
	Inhibitory analysis of cell metabolism
	Analysis of cell-surface interactions
	Preparation of samples (cell fixation and labeling) and for confocal laser microscopy

Professional Activities

Since 2005	**University of Basel** **Department of Chemistry** **Ph.D. thesis** Ph.D. on basic research in physical chemistry. Investigation and validation of quantitative methods for the analysis of hybridization reactions onto surfaces for DNA-based biosensor development. Assisting in laboratory course for students. Guiding student's semester work. Publication preparation.
2002 – 2005	**Belarusian State University** **Physical Faculty** **Department of Biophysics** **Junior research officer. Post-graduate study.** Investigated the influence of neopterin and 7,8-dihydroneopterin on the generation of reactive oxygen species of peripheral blood cells. Researched the influence of neopterin on aggregation, degranulation and myeloperoxidase activity of blood cells. In cooperation with clinic researchers investigated the functional activity of neutrophils of peripheral blood of patients during treatment by various medicines in post development clinical studies. Published the work in peer-reviewed journal in my field.
1997 – 1999	**Belarusian State University** **Physical Faculty** **Department of Biophysics** **Engineer.** Investigated functional activity of neutrophils of peripheral blood in healthy donors and patients during treatment by various medicines.

Award

2005 Blair-Curtius-Pfleiderer-Wachter Award for Pteridine Research: *Influence of neopterin and 7,8-dihydroneopterin on signal transduction pathways in neutrophils.*

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Abstracts:

Covalent Modification of Oligonucleotides with Synthetic, Hydrophobic Polymer
F. Teixeira, K. de França, J. Razumovitch, N. Cottenye, W. Meier, C. Vebert,
Macromolecular Colloquium. 2009, Freiburg, Germany

Oligonucleotide-based Polymers: Synthesis and Hybridization
F. Teixeira, J. Razumovitch, K. de França, F. Kehl, M.Wiki, W. Meier, C. Vebert-Nardin
Workshop on Nanoscience. 2008, Davos, Switzerland

Nucleotide-based Amphiphilic Block Copolymers: Self-assembly, Hybridization and Cell Response
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