UNIVERSITÄT FÜR BODENKULTUR

Development of quality control methods for quantitative evaluation of epoxy- and aminosilane coatings on polymeric surfaces

by

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Declaration of Authorship

I, THOMAS BUTTINGER, declare that this thesis titled, 'DEVELOPMENT OF QUALITY CONTROL METHODS FOR QUANTITATIVE EVALUATION OF EPOXY- AND AMINOSILANE COATINGS ON POLYMERIC SURFACES' and the work presented in it are my own. I confirm that:

- This work was done wholly or mainly while in candidature for a research degree at this University.
- Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated.
- Where I have consulted the published work of others, this is always clearly attributed.
- Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work.
- I have acknowledged all main sources of help.
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Signed: _____

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"All truths are easy to understand once they are discovered. The point is to discover them."

Galileo Galiei

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Abstract

Department für Nanobiotechnologie
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Grafting of reactive chemical functionalities on the surface of polymeric substrates is of great interest in diagnostic and analytical applications contained in a 'lab-on-a-chip' environment. Chemical coating and modification is performed in order to immobilize a wide range of suitable biological probes, so called capture molecules, and to suppress non-specific adsorption of other biological molecules. The performance of the resulting assays often directly correlates with the amount of reactive groups presented in a defined area. This thesis will compare different methods of determining the quality of epoxy- and aminosilane coatings on polymeric consumables produced by SONY Biosciences, to establish a reliable quality control tool for the evaluation of chemically activated surfaces.

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Contents

Abbreviations

APTES	**A**mino **P**ropyl **T**ri **E**thoxy **S**ilane
BSA	**B**ovine **S**erum **A**lbumin
CBB	**C**oomassie **B**rilliant **B**lue
COC	**C**yclic **O**lefin **C**o-polymer
COP	**C**yclic **O**lefin **P**olymer
CVD	**C**hemical **V**apor **D**eposition
DMSO	**D**i **M**ethyl **S**ulf **O**xide
ELISA	**E**nzyme **L**inked **I**mmuno **S**orbent **A**ssay
GLYMO	**GLY**cidyloxypropyltri **M**eth **O**xy silane
GMBS	**G**amma **M**aleimido **B**utryloxy **S**uccimidine ester
HCl	**H**ydro **C**hloric acid
IVD	**I**n **V**itro **D**iagnostic
KCl	Potassium chloride
LC-SPDP	**S**ulfosuccinimidyl **P**yridyl **D**ithio **P**propionamido hexanoate
NaOH	Sodium hydroxide
NaCl	Sodium chloride
$\mathbf{Na_2HPO_4}$	Di-sodium phosphate
NHS	**N**- **H**ydroxy **S**uccinimidyl
PBS	**P**hosphate **B**uffered **S**aline
PC	**P**oly **C**arbonate
PDITC	**P**henylene **D**i **I**so **T**hio **C**yanate

PDMS	**P**oly **D**i **M**ethyl **S**iloxane
PMMA	**P**oly **M**ethyl **M**eth **A**crylate
POC	**P**oint **O**f **C**are
TAMRA	Carboxy-tetramethyl rhodamine
TPE	**T**hermo **P**lastic **E**lastomer
TRIS	**TRIS**(hydroxymethyl)aminomethane
XPS	**X**-ray **P**hotoelectron **S**pectroscopy

Physical Constants

Avogadro constant $N_A = 6.022 \cdot 10^{23}\ \mathrm{Mol}^{-1}$

Symbols

A	absorbance	[AU]
AU	absorbance unit	
ϵ	molar extinction coefficient	[Mol L^{-1} cm^{-1}]

Dedicated to my Family....

Chapter 1

Introduction

1.1 ”Lab-on-a-chip”

In modern medicine and research there is high demand for flexible, robust and easy to use analytical technologies. In an approach to meet those demands, especially the ”lab-on-a-chip” is of increasing importance.

”Lab-on-a-chip” is a summarizing term related to the possibility of performing different diagnostic or analytic laboratory tasks using a miniaturized, ready to use device that offers benefits over conventional methods while still being able to generate equivalent results. [1, 2]

There are several categories of devices depending on the materials used and the application at which they are aimed. The most important ones being highly integrated structured devices for either microfluidic or microarray applications. Within these two major categories there is a wide range of devices that fit into a sub-category of their own, because of the sheer number of different possibilities: For example studying complex biochemical reactions and cellular processes or fulfilling routine diagnostic tasks in health care, often referred to as *In vitro* diagnostics (IVD) or point-of-care diagnostics (POC). [1–3]

The spectrum of functions implemented in these devices range from simple fluid manipulation in form of microfluidic pumps, valves or filters to highly integrated designs where a single chip is capable of performing complex analytical separation and detection techniques like electrophoresis, chromatography, fluorescence, and electrochemical detection as well as biological applications, including DNA sequencing and fragment sizing, PCR amplification, amino acid, peptide and protein analysis, immunoassays, cell sorting or manipulation, and *In vitro* fertilization. [1]

The main advantages associated with these "lab-on-a-chip" devices are the compact, highly integrated format which results in a high level of mobility, a low demand of laboratory space, possibilities of parallelization and ease to use which often enables even unqualified personnel to fulfill otherwise complex measurements. Furthermore there are some advantages specific to the field of microfluidics like the major benefit of low sample and reagent volumes, leading to low waste levels and overall cost reduction. [1, 2, 4–6]

Designing and manufacturing such devices is a challenging task, and demands advanced technologies and know-how. Not only is it difficult to integrate a complex assay into a compact format while still retaining the proper function, it is also very demanding to produce such disposable devices in industrial scale amounts. There is often a disparity between creating a functional prototype and mass production, especially when it comes to the choice of materials.

1.1.1 Overview of manufacturing methods

For early LOC devices dating back to the 1990s glass and silicon have been the materials of choice, using established methods deriving from the semiconductor industry. [7, 8] They were fabricated using micromachining techniques like wet etching, dry etching, photo-lithography or electron beam lithography. [1, 6] While the percentage of silicon devices diminished over time, glass devices are still widely used due to their excellent properties concerning optical transparency, flatness [9], solvent compatibility and chemical

resistance. It is also possible to realize intricate structures on a very small scale using the various aforementioned micromachining techniques. [1, 7]

Another route of production is based upon polydimethylsiloxane (PDMS). [1, 6] Fabricating the actual devices makes use of replica molding: casting the PDMS pre-polymer (a two-component mixture of base and cross-linking agent) against masters created by photolithography and curing at a moderately elevated temperature to replicate the desired features. [1, 6]

Creating those masters involves the printing of the microfluidic design on a transparency or fabricating a chrome mask which serves as the photomask for the photolithography process. Photolithography consists of spin coating a layer of photoresist (e.g. SU-8 photocurable epoxy) onto a silicon wafer followed by curing said resist via exposure to UV-light through the patterned photomask and removing the excess material, which leaves a positive or negative relief of resist on the wafer depending on the type of resist (see figure 1.1). After the replica molding procedure the ridges on the master appear as valleys on the chip. [1, 6]

To create a device with enclosed micro channels, the PDMS replica can be sealed via different methods. For example by exposing the PDMS part to an oxygen plasma as a preparative step before bonding it to glass or another piece of PDMS. [1, 2]

While the majority of published manufacturing methods revolve around glass as well as PDMS there has been increasing recognition that neither of them are suitable for mass production, driving commercial producers to seek other materials. [4, 7, 10] The high cost involved in processing glass, mainly arising due to clean room requirements, time consuming methods and material prices, is a strictly limiting factor for its usage for the production of disposable devices on an industrial scale. [1, 6, 11]

Compared to glass based methods the transition to PDMS fabrication techniques made the production faster and less expensive, mainly by eliminating

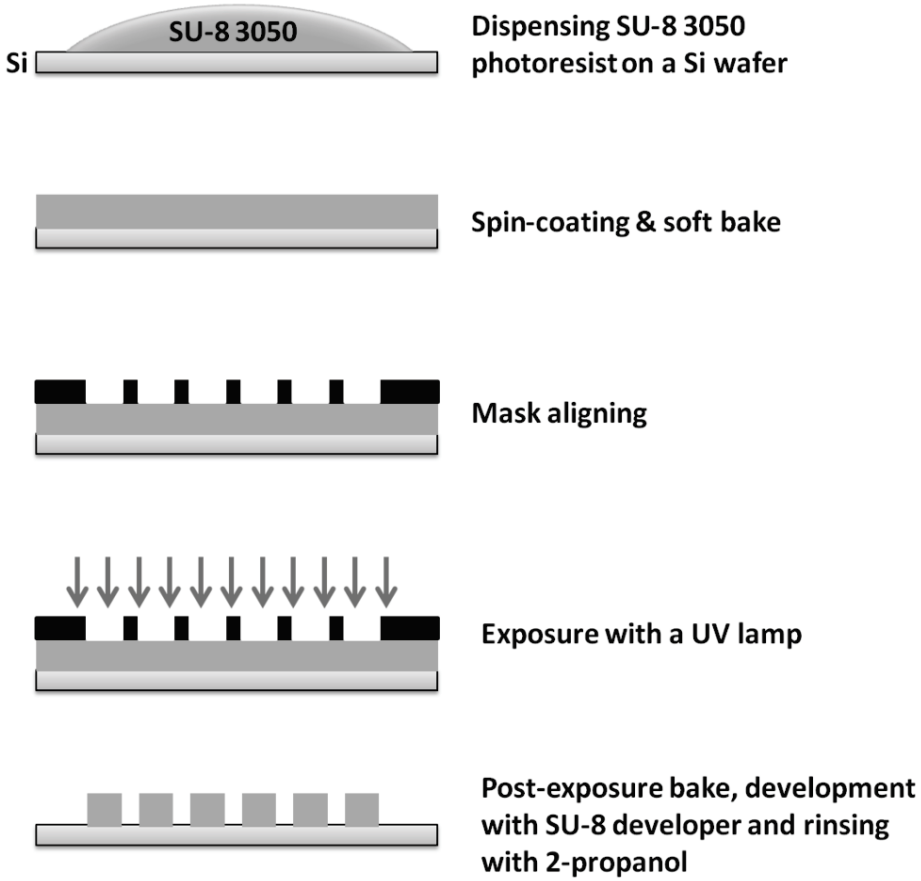

FIGURE 1.1: Schematic process flow of SU-8 photolithography

the necessity of using a cleanroom which is a main cost driver for manufacturing glass and silicon devices and by using much simpler tools and processing steps. [6]

Considering though that conventional methods for PDMS curing still take around one hour and that recent improvements still only brought down the time to 3 to 10 minutes, it is still not a scalable solution for most applications. [1, 12, 13] Therefore SONY Biosciences is working on an alternative "lab-on-a-chip" approach: injection molded polymeric consumables like chips, slides and variously shaped carrier structures as a true alternative to established glass or PDMS based devices currently available on the market. [4, 10, 14]

1.1.2 Injection molding for industrial scale production

Injection molding is a process commonly used in the plastics industry. It includes melting pre-polymerized pellets of a chosen thermoplastic (e.g. cyclic olefin polymer) and injecting it into a heated mold cavity under high pressure (see figure 1.2), where the molten material is cast against a master that is commonly produced via photolithography followed by a galvanic plating procedure. The injected parts are then cooled below the particular glass transition temperature of the material and released from the mold (see figure 1.3). [1] Casting molten plastic via injection molding under such high pressures maximizes the contact between the material and the features depicted on the master. This allows for precise replication of the intricate microfluidic structures. [1] The quality of the replication mainly depends on the quality of the master and the operational parameters, such as mold temperature, injection pressure and residence time, that need to be optimized and fine-tuned for each individual product. Once all the parameters are set it only takes a few seconds for each piece to be molded. [1]

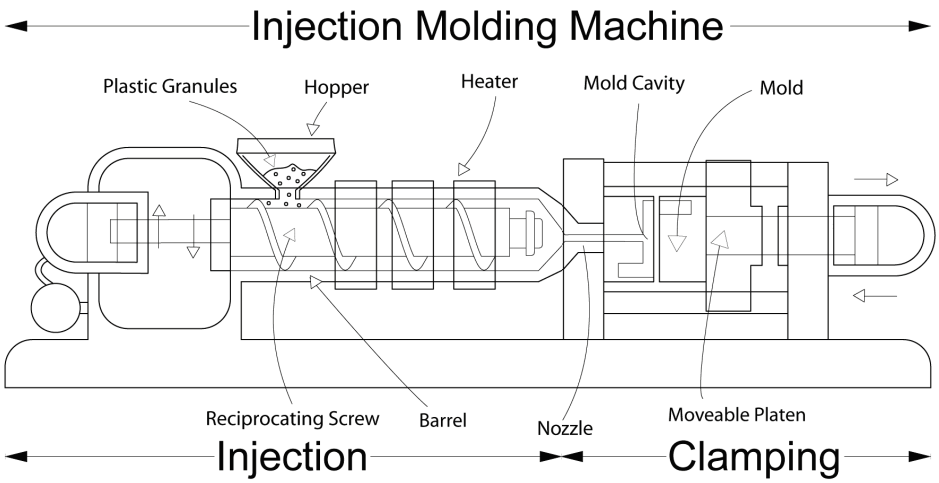

FIGURE 1.2: Schematic display of injection molding machine

The purchase of complex molding equipment and the fabrication of masters has a high start-up cost attached to it, but the high throughput and the fact

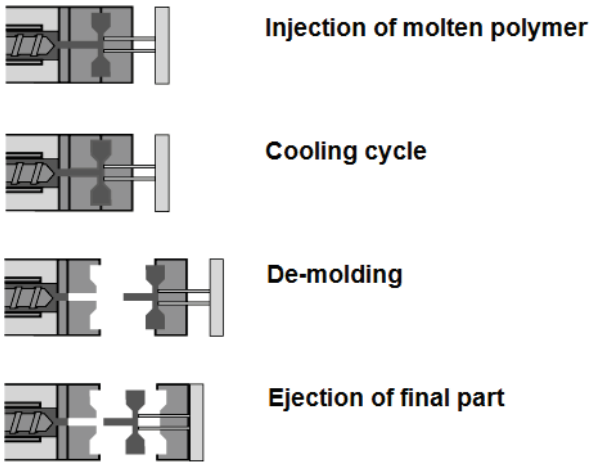

FIGURE 1.3: Schematic process flow of injection molding

that polymers are inexpensive and can be thermally sealed using relatively simple tools makes injection molding a very cost effective procedure. [1, 14]

This is the major reason for SONY Biosciences as well as other commercial manufacturers of microfluidic devices to employ plastics for achieving high-volume, low-cost production. [7, 10]

1.1.3 Functionalization

Choosing an appropriate production method solely based on its large scale manufacturing aspects is not sufficient. This is mainly related to the fact that the function of lab-on-a-chip" devices not only relies on the proper shape and microstructure of the consumable, but also on the surface chemistry. When considering plastic for a certain kind of application a few important aspects need to be considered: Some polymers might be incompatible with organic solvents or acids, while plastics in general are incompatible with high temperatures. [6] Not all polymers are transparent and if they are they often have a strong auto-fluorescence which might be problematic for assays based on fluorescent markers.

However, these issues can often be addressed by making proper use of the wide range of available plastic materials which allows the manufacturer to choose materials based on the properties that suit the specific application. [7] Though sometimes even the wide range of different materials is not enough, and there may be no perfect match for the requirements. In these cases, in order to either circumvent inherent disadvantages of these plastics, or to further improve their inherent qualities it is often necessary to modify their surface to fulfill the requirements of their complex final application. These treatments particularly involve application of different functional coatings via sputtering or chemical/physical vapor deposition.

The major reason for such modifications in the case of many assays is the need for immobilization of capture molecules like antibodies on the active surface of the device which play the role of biological probes that specifically interact with desired target molecules. [15] While it is sometimes sufficient to immobilize said capture molecules via strong non-covalent interactions between the molecule and the surface, it may as well be necessary to create a covalent chemical conjugation. There is a broad range of analytical assays for the identification or quantification of target molecules that rely on such surface chemistries. In the case of SONY Biosciences the polymeric devices on which this thesis is based are sputtered with a thin coat of silicon dioxide, mimicking the surface of well established glass microchips. A further activation step is the silanization with either aminosilane or epoxysilane via vapor phase deposition, where the silane covalently binds to the silicon dioxide. Hereby the surface is altered in a way that it shows a certain reactivity towards different functional groups, enabling it to generate covalent bonds with a wide range of capture molecules which are therefore immobilized on the surface. [15–17]

The performance of the immobilization technique depends on the ability of the capture molecules to form these covalent bonds. This ability mainly depends on the number of available reactive groups provided by the silane, within a defined surface area. A higher density means that more capture molecules can be immobilized which is favorable up to a certain threshold

where the high density of silane molecules starts to cause steric hindrance between the probes, which affects the biological activity, resulting in a decrease of affinity towards the target molecule and a limitation of the effectiveness of the assay. [16, 18, 19]

1.1.4 Aim of this thesis

Given the fact that the surface density of reactive functional groups provided by the silane layer is a major contributor to the overall performance and reproducibility of the devices, the aim of this thesis was to develop and establish a quality control routine to quantify said density of reactive silane molecules on the surface of injection molded plastic consumables.

To accomplish this task a number of different methods was compared in order to establish a reliable tool for the evaluation of the silanization process.

Chapter 2

Background Info and Theory

The following section should give a brief overview over the materials and methods relevant to this thesis. It will focus on the theoretical background and should answer basic questions such as what drove the decision to agree upon a particular procedure for accomplishing a certain task. An in depth explanation of the parameters and protocols as well as a more detailed version of all the technical informations can be found in the dedicated materials and methods section in a latter part of this thesis.

2.1 Microarray manufacturing

Microarrays are a common application for LOC-devices. A microarray is a pattern of biological capture molecules on the surface of a glass or plastic supporting structure. This pattern is then brought into contact with a diagnostic sample to allow the capture molecules to possibly react with the complementary target molecule. A following detection step evaluates the result of the experiment, namely if the target molecule was present in the sample and could be captured by the probe. In order to manufacture common microarrays a vast variety of said capture molecules needs to be attached onto the surface of different carrier structures. There are two main classes of capture molecules that function as probes in these diagnostic or

experimental assays: proteins, and nucleic acids such as DNA and RNA. For DNA based microarrays the actual assay involves the hybridization of a sample (e.g. PCR product) to allele-specific oligonucleotide probes immobilized on a carrier substrate. In comparison, common protein based microarrays make use of the attractive forces between antibodies and their respective antigens: A capture antibody is immobilized on the surface targeting a certain antigen of interest that might be present in the sample. In a subsequent step a solution containing a detection antibody against the same target antigen is applied, resulting in a signal when the antigen is indeed present. While DNA and protein microarrays are both very common, this section will be focused mainly on the immobilization of proteins, including enzymes and antibodies, in the context of IVD applications. [2, 21]

2.1.1 General aspects

Immobilization of capture molecules is a primary design and performance consideration when it comes to lab-on-a-chip devices. Successful immobilization relies on multiple factors including the surface and its properties, the sample matrix, molecular properties of the probes as well as buffer constituents. The combination of those factors determines the performance metrics of a given assay which include the analytical sensitivity, selectivity, reproducibility and reusability. It is critical to establish a strong bond between the probe and the surface and to keep the non-specific adsorption of components that are unrelated to the assay as low as possible, while retaining activity of the proteins and granting binding partners unhindered access to their active sites. [2]

2.1.2 Molecular orientation

In general surface immobilization of capture molecules can be performed either in a controlled or uncontrolled way regarding their spatial orientation. Especially for antibodies and proteins the orientation of the molecule on the surface is a critical parameter. Active sites need to be facing away from the

surface to be accessible for target molecules. Uncontrolled immobilization is a term describing a process where the attachment of probe molecules relies on their interaction with the surface via multiple generic anchoring points like amino acid residues that are natively present on the molecules outside. It can not be controlled which particular anchoring group interacts with the surface. The resulting spatial orientation of the probe molecules is therefore random and a certain fraction of them is likely not accessible for binding partners, due to their active sites facing towards the surface where target molecules can not reach because of steric hindrance. This leads to a reduction in activity causing an overall loss in signal. Besides the orientation, the conformation of proteins needs to be considered as well. The three dimensional structure needs to be intact for the proteins to retain their native function in order to guarantee a reproducible high performance assay. Random immobilization causes problems for protein conformity. Anchoring via multiple binding sites may lead to denaturation of proteins causing them to lose their activity. [2]

Controlled immobilization on the other hand is based on the introduction of particular functional groups to specific sites of the capture molecules that allow them to bind to the surface in a defined way which grants precise spatial control leading to an overall increase in protein activity and assay stability. To detect analytes in a reliable way the bio-probes need to be immobilized in a controlled way. This maximizes the surface density of capture molecules while retaining their native conformation thereby granting the activity. [2]

2.1.3 Methods for immobilization

Many different strategies have been established to achieve protein attachment. The major mechanisms of immobilization are physisorption, bioaffinity interaction, covalent bonding as well as a combination of all three mechanisms. [2]

Physisorption is the simplest approach for immobilization of capture molecules. The orientation of the immobilized molecules on the surface is random. It therefore belongs to the uncontrolled methods. Physisorption is based on intermolecular forces such as electrostatic, hydrophobic and Van der Waals interactions as well as hydrogen bonds. While it may be difficult or even impossible to isolate the influences of the different intermolecular forces, electrostatic forces in the form of ionic interactions are fundamental for molecular attraction and are a major contributor to the interaction of proteins and surfaces. Exploiting this mechanism is an efficient way to immobilize capture molecules onto the surface. This can be done via introduction of corresponding functional groups of opposite electrical charge. Examples of positively charged functional groups consist of protonated amine (NH_3) and quaternary ammonium cations ($RN_4{}^+$), while the typical negatively charged groups are carboxylic acid (COO^-) and sulfonic acid ($RSO_3{}^-$). [2]

The procedure itself is very simple and consists of applying the molecules onto the surface and incubating them for a certain period of time. During the incubation phase the molecules will attach to the surface via the interaction of the functional groups. There is no further chemistry required, but the performance heavily relies on parameters such as concentration, exposure method and time and it may not be possible to fine-tune the process to meet the requirements of industrial scale production. In comparison to more sophisticated methods the bonding force is generally weak and also strongly depends on environmental conditions such as pH, ionic strength, temperature, surface conditions during the incubation and the actual assay, which renders the procedure very sensitive to external influences. Furthermore the resulting layer of immobilized capture molecules might not be stable enough for the application. In conclusion physisorption is characterized by a very low degree of control and a lack of reproducibility. Nevertheless it is commonly used due to the simplicity of the assay procedures, the lack of toxic reagents, and the absence of complicated chemical protocols.[2]

In comparison to the simple procedure of physisoprtion, common covalent immobilization techniques require more sophisticated chemistry, often depending on bi-functional linker molecules that couple the capture molecules

to the surface via a chemical bond. Direct coupling of the target molecule is possible as well but only rarely performed. The protocols vary depending on the actual application, and can have different levels of complexity. The major advantage of covalent immobilization is the strong bond leading to very robust assays. Another way of attaching molecules to surfaces is bioaffinity immobilization which benefits from naturally occurring interactions of certain molecules that show a very strong attraction towards each other (e.g Biotin and Avidin). Bioaffinity by definition is more of a phenomenon rather than a technique and is exploited in the framework of protocols based on physisorption and covalent bonding, being the underlying principle that enforces the bonding between the target molecule and surface functional groups. Bioaffinity immobilization is not unlike regular physisorption in a sense that it requires incubation of target molecules on a surface in order to achieve anchoring to certain functional groups. While this can happen instantaneously when the k_{on} rate is high enough, more often than not bioaffinity based methods require a significant incubation time. Examples for bioaffinity partners are Avidin-biotin, protein A/G, antibody-antigen, genetically engineered protein affinity ligands, DNA hybridization and aptamers. Certain bioaffinity interactions are amongst the strongest bonds apart from true covalent bonding. [2]

Other than the strength of the bonds there are quite a few more advantages over regular physisorption, namely the high specificity and the controlled spatial orientation of the molecules which leads to a much better accessibility of capture antibodies compared to random orientation strategies, a wider range of buffer systems to choose from, compared to covalent bonding procedures based on primary amines that can not make use of popular amine based buffers (Tris, Glycine), and the possibility to reverse the immobilization using pH change or chemical treatment. [2]

What all the procedures have in common is that certain criteria have to be met regarding the surface and its properties. The surface on which the immobilization is performed plays an integral role concerning the performance of the assay. Surface modification is often required to either increase the surface energy or hydrophobicity in order to prepare the surface for

the introduction of functional groups. Common substrates for covalent immobilization chemistries are chips made of glass or silicon, whereas SONY Biosciences uses injection molded plastic consumables made of cyclic olefin polymers with a glass-like top coat in form of a SiO_2 sputter layer. This layer is relatively homogenous and mimics the well-known properties of glass surfaces. There are different methods for further modification in order to facilitate the binding of the required capture molecules, based on the underlying principles mentioned earlier inn this section. [2]

2.2 Overview of state of the art immobilization techniques

This non-exhaustive list of immobilization techniques should give an overview of several popular approaches concerning the immobilization of capture molecules on surfaces.

2.2.1 Silanization

Silanization consists of grafting a layer of silane molecules onto a surface. Silanes are categorized based on their functional end group. For amino-labeled oligonucleotides as well as for proteins there are two established attachment chemistries that are both based on silanization. The first method uses a layer of epoxy silane. The epoxide functionality of the silane has a direct reactivity towards the primary amine functionality of the amine groups of the oligonucleotides and proteins and does not need any additional steps other than properly applying the capture molecules on the surface and a few hours of incubation. [2, 15] The other method is based on an amino silane that has an amino functionality itself. In a subsequent step following the silanization procedure, the surface is treated with a solution containing 1,4-Phenylene diisothiocyanate (PDITC), a homobifunctional linker molecule with two amine reactive isothiocyanate groups on a phenyl ring. In contact with the amino silane covered surface it forms a thio-urea linkage with the

silane, while one isothiocyanate group remains free to couple with available amine groups of the capture molecules for immobilization. [15]

2.2.2 Avidin-Biotin

Avidin (66-69 kDa tetrameric glycoprotein) is a popular binding partner for Biotin (water-soluble vitamin B). While the exceptionally strong non-covalent interaction between the two molecules belongs to the bioaffinity category, it requires covalent conjugation of the target protein/ surface to the Biotin/Avidin molecules. It is widely employed to functionalize the surface with Avidin derivates such as Streptavidin or Neutravidin via physisorption or covalent bonding while using biotinylated target molecules to achieve their permanent attachment to the surface. Biotin is small enough to not affect the native function and conformation of proteins when conjugated, which makes it suitable choice when immobilizing antibodies and enzymes. The interaction is rapid and almost insensitive to pH and temperature changes as well as to proteolysis and denaturing agents. [2]

2.2.3 Affinity ligand

C or N termini of proteins can be genetically engineered to have an oligo-histidine (His) segment that specifically chelates with metal ions like Nickel ($Ni_2{}^+$). The Nickel ion is then bound by another chelating agent such as NTA (nitriloacetic acid) which is covalently bound to the surface. If desired the target molecule can be released by adding chelating agents like EDTA (ethylenediaminetetraacetic acid) which compete for the binding sites causing the removal of the target molecule from the surface. [2]

2.2.4 Protein A/G

Protein A and protein G are popular IgG antibody immobilizing reagents. Both are extracted from bacteria and specifically bind the Fc region of IgG

antibodies. The antigen binding Fab region is not affected by this conjugation and remains accessible. Protein A and G are often used in combination with spacer molecules to mitigate effects of steric hindrance. [2]

2.2.5 DNA-hybridization

Another widely adopted method for oriented immobilization of target molecules is based on DNA-hybridization. It consists of coupling single stranded DNA (ssDNA) covalently to the target molecules of choice while immobilizing the complimentary DNA (cDNA) to the surface. These DNA-oligonucleotides have various lengths and sequences. They are synthesized individually prior to immobilization. In order to perform the attachment procedure the oligonucleotides need to be chemically modified, by replacing one end of the single strand with a functional group that may form a covalent bond with a reactive group presented at the surface, like an amino group. These modified oligos are commercially available. Bringing the probe conjugates in contact with the cDNA covered surface causes hybridization of the two complimentary single stranded DNA fragments, thereby immobilizing the molecule on the surface. A major advantage of this method is the capability to create multiplexed microfluidic assays via the use of several pairs of DNA strands for different populations of capture molecules. DNA based immobilization is reversible by temperature control or alkaline denaturation which might be an advantage for certain applications. [2]

2.2.6 Hydrophobin

Hydrophobin, a cystein rich small protein (100 amino acids) extracted from filamentous fungi is capable of forming self-assembled monolayers on hydrophobic-hydrophilic interfaces. Conjugating it to capture molecules of choice enables direct functionalization of polymeric surfaces in an aqueous environment.[2]

2.3 Quantification of surface chemical groups

Surface chemical groups are of great importance for the functionalization of "lab-on-a-chip" devices, e.g. the amino groups mentioned earlier in this chapter.

There are different possibilities to make use of such primary amine moieties on surfaces: Amino groups which are mainly introduced via amino silane deposition can modify macroscopic surface properties like hydrophilicity, thereby modulating cell adhesion capabilities, or specific differentiation of stem cells on microchips. [16]

The main use of these amino groups in this context though is to chemically attach bioactive molecules such as oligonucleotides, oligosaccharides, enzymes or peptides onto the surface as mentioned in the previous section. [2]

The performance of amine based immobilization techniques mainly depends on the quality of the amine layer. One parameter that has to be set accordingly is the amino group density. There are existing methods to determine this density prior to further functionalization, thereby evaluating the chemical composition of the surface. But many established methods like X-ray photoelectron spectroscopy (XPS) where the binding energy of electrons is measured for qualitative and quantitative analysis of a surface, or fluorometry with o-phtaldehyde where a precursor reacts with primary amines resulting in a fluorescence signal are either expensive and time consuming in the case of XPS or semi-quantitative in the case of fluorometry. [16, 22, 23]

In contrast, colorimetric assays that take advantage of anionic dyes like Orange II or Coomassie brilliant blue are reported to be quantitative, inexpensive and convenient to perform on various material geometries. [16]

2.3.1 Orange II

The Orange II assay is reported to be a suitable method to evaluate the quality of an aminosilane coating, by quantifying the related amino group surface density. [16]

The mechanism of the assay is based on the reversible electrostatic interaction between the negatively charged sulfonated dye and the positive charge of the protonated amino groups in acidic solution. In a second step under alkaline conditions the neutral amino groups then induce Orange II release into the solution whose absorbance can be measured via UV-vis-spectrophotometry to evaluate the quantity of the desorbed dye. Due to its distinctive negative charge and low steric hindrance Orange II is very sensitive, and provides reliable quantification over a wide range of amino group surface densities (5 to 200 pmol/mm^2).

Excellent linear correlation of the Orange II assay to other established methods (e.g. LC-SPDP binding/ Ethylenediamine assay) has been reported, validating it as a reliable method. [16]

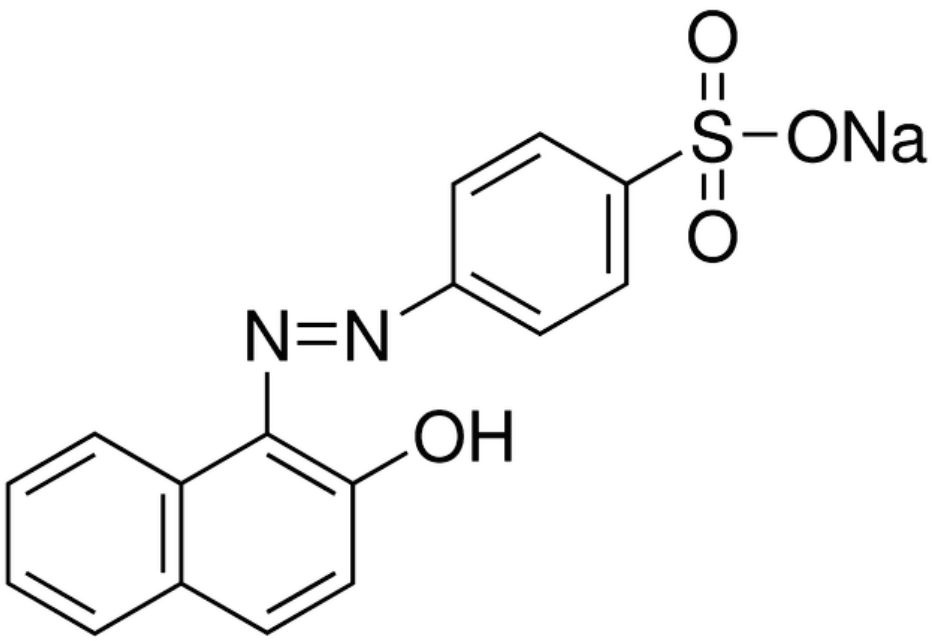

FIGURE 2.1: Chemical structure of Orange II sodium salt dye

2.3.2 Coomassie brilliant blue

Coomassie brilliant blue (CBB) is another colorimetric reagent that can be used for quantification of protonated reactive groups like the amino group. The immobilization of the dye molecule on the amino groups is a reversible process, depending on the pH. Using a comparable procedure as previously described for Orange II the concentration in the eluate can be determined to calculate the surface density of amino groups on the tested surface. [16]

Conditions like pH, incubation time, temperature and solvent are optimized for these assays. It is therefore assumed that the dye is able to detect all of the accessible amino groups. Using the same samples in the actual assay, CBB is reported to yield lower values than Orange II although it shows a higher non-specific adsorption on pristine surfaces. This suggests that steric hindrances prevent the large divalent CBB molecules from reaching all the amine moieties, whereas the small monovalent Orange II suffers less

steric hindrance and can bind more. Literature values for the resolution of Coomassie brilliant blue are reported to be about 4 amino groups groups per nanometer square. [16]

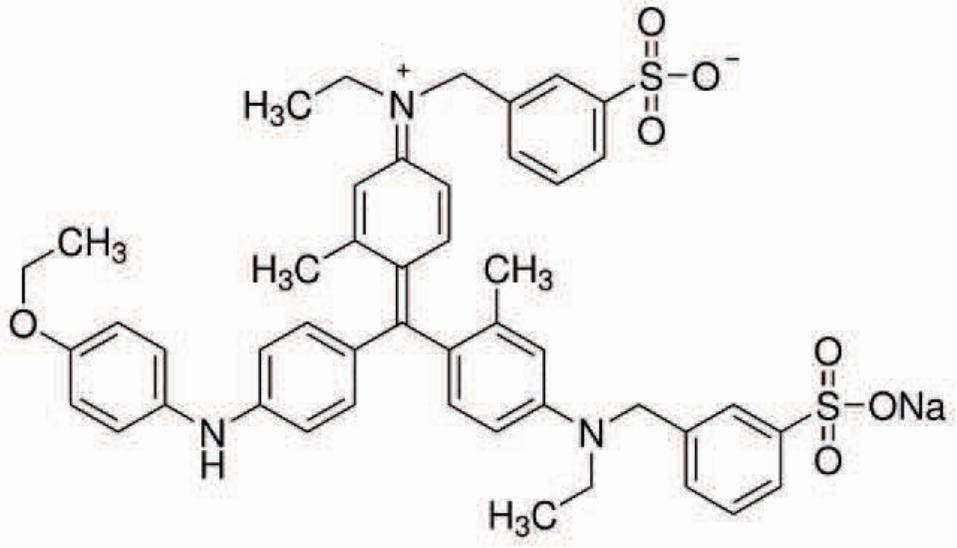

FIGURE 2.2: Chemical structure of Coomassie brilliant blue dye

2.3.3 Succinimidyl esters

An additional economic method to detect amino groups is fluorescence labeling based on succinimidyl esters (NHS-esters). NHS-esters have a high reactivity towards primary amines and are therefore excellent reagents for amine modification. When an amino group is exposed to a NHS-ester it forms a very stable formamide bond. [2]

The hydrolysis of the bond can compete with conjugation, but this side reaction is usually slow below pH 9. TAMRA^{TM} [carboxytetramethylrhodamine] is a fluorescent reporter dye that is available as an NHS-ester which makes it suitable for the detection of amino groups on surfaces.

TAMRA^{TM} Cadaverine is a TAMRA dye molecule conjugated to a Cadaverine molecule. Cadaverine is the trivial name for diaminobutane. It enables

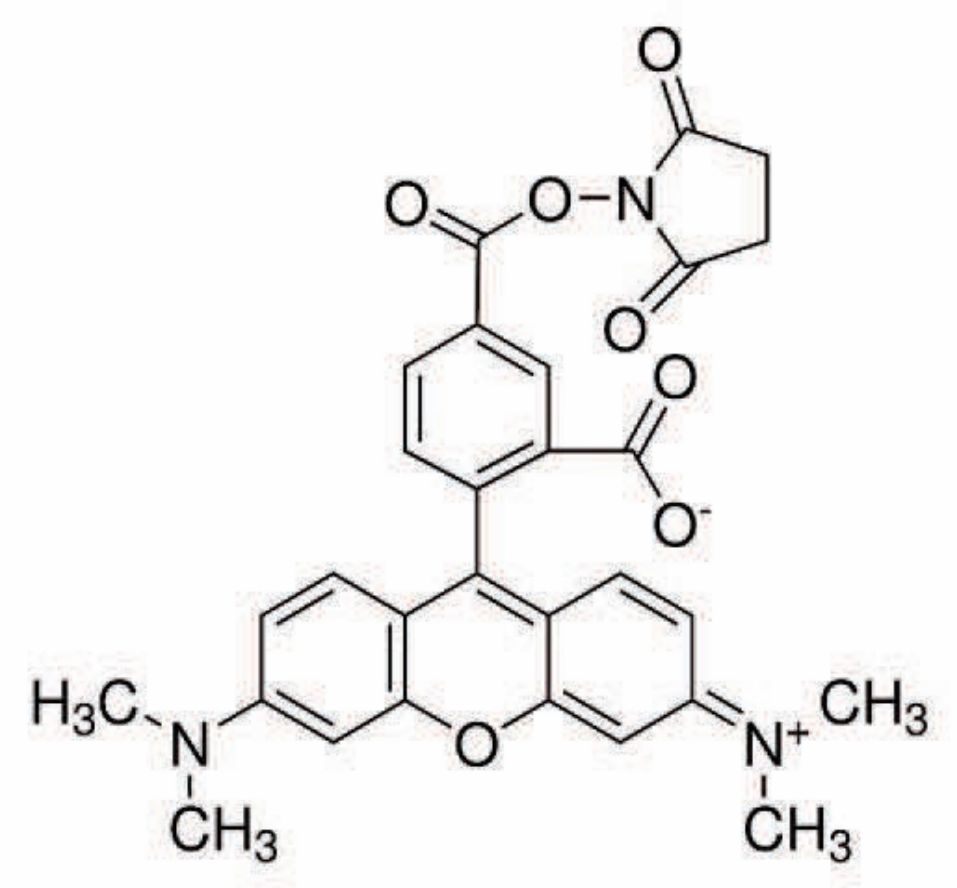

FIGURE 2.3: Chemical structure of TAMRA N-Succinimidyl-Ester

the attachment of the TAMRA dye to an epoxide via the primary amine of the Cadaverine.

In comparison to other available assays the fluorescence based methods are less preferred due to their susceptibility to surface effects like quenching as well as emission-, and absorption shifts leading to insufficient quantification and non-linearity. [24]

2.4 Cyclic olefin polymer

Glass and PDMS have been the standard materials for a long period of time when it comes to the fabrication of "lab-on-a-chip" devices for microarray or microfluidic applications. They have their disadvantages though. One of the major drawbacks of these traditional materials are the high production costs and the often complicated and time consuming fabrication processes which renders them incompatible with mass manufacturing of disposable consumables. [7, 10]

As an alternative to glass, regarding an overall reduction in production costs of portable and disposable microsystems, especially one class of materials has performed very well: Cyclic olefin (co-)polymers (COP/COC) like Zeonor 1060R (see figure 2.4). [11, 13]

These materials stand out due to their excellent properties concerning chemical resistance (acids, bases and polar organic solvents), optical clarity and transparency (300 - 1200 nm), low shrinkage, low moisture absorption and low birefringence. [25]

They fulfill the requirements for the fabrication of LOC-devices with a broad range of applications like capillary electrophoresis, chromatography, blood-, DNA and protein analysis, with the possibility to involve electrochemical or optical detection, while being able to withstand the harsh conditions during the preparation steps, the chemical modification and the final assay itself. [11]

A major disadvantage of COP is the fact that it is pure hydrocarbon and has no native surface chemical groups that could enable immobilization of capture molecules. Silanization would be a common method of introducing reactive organofunctional groups on chemically inert COP surfaces, preparing them for biological functionalization, but it depends on available hydroxyl groups. To be able to perform silanization on COP it is necessary to include a pre-treatment step. In general plasma oxidation would be a simple method that introduces oxygen based reactive groups like carbonyl (C=O), ether (C-O-C), and hydroxyl (-OH) groups. Despite the presence of

oxygen atoms it has been reported though, that there is no evidence for the presence of the demanded hydroxyl groups on plasma oxidized COP samples. Therefore it is reasonable to introduce a SiO_2 sputter layer on top of the plain COP that functions as a base layer in order to enable silanization. [15]

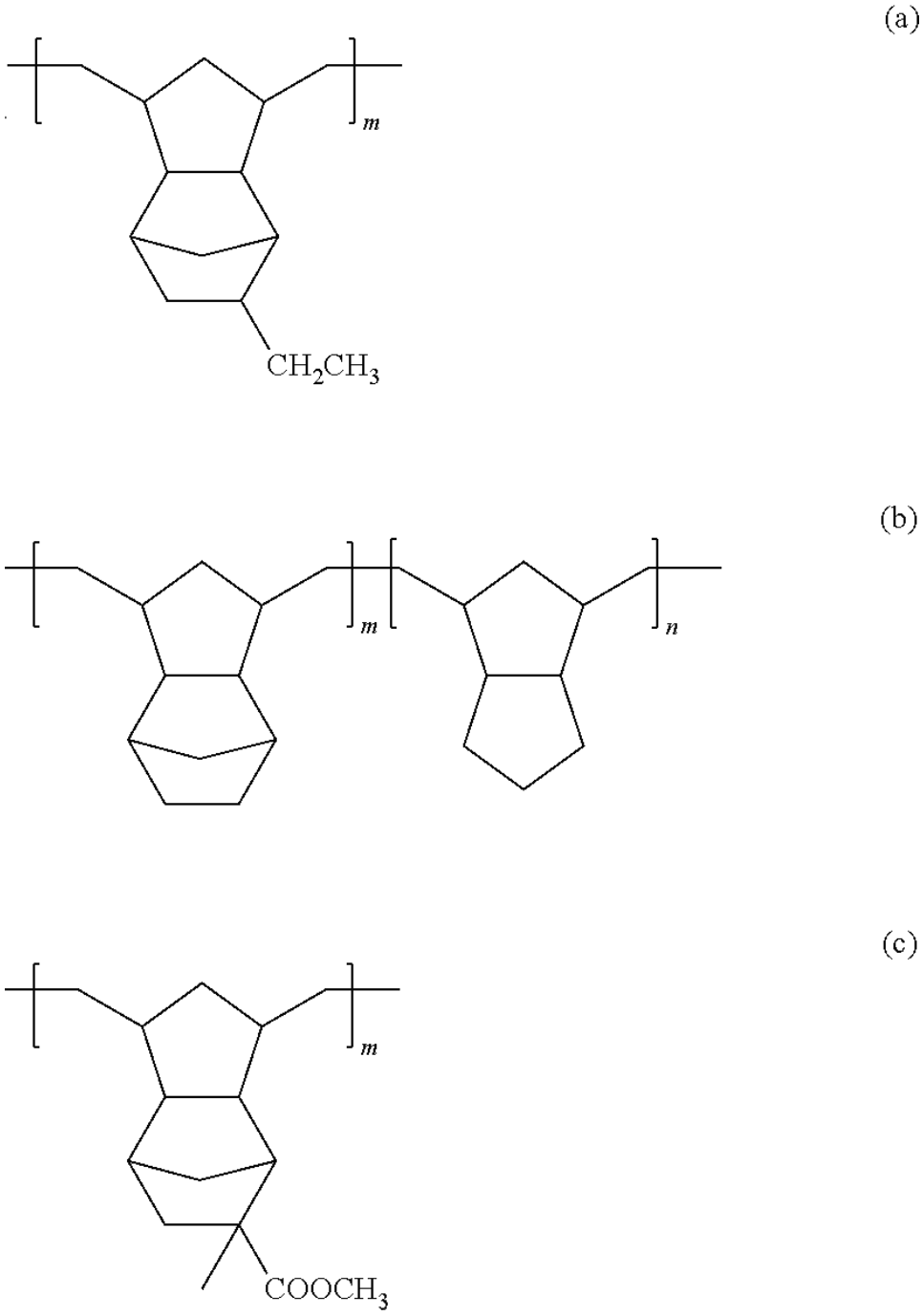

FIGURE 2.4: Chemical structure of Zeonor 1060R cyclic olefin polymer

2.5 Sputtering

The deposition of thin films can be commonly performed using one of three general approaches: Physical or chemical vapor deposition, or the deposition from liquids.

For applying thin films of silicon based oxides especially the physical vapor deposition is of great importance, namely sputtering (see figure 2.5).

Sputtering is a technology based on ionic interaction. Energetic ions are generated via a glow discharge process performed by applying a voltage in order to ionize a low pressured working gas. The sputtering gas can vary, but mostly inert gases like Argon are used. Reactive gases like Oxygen can be used as well depending on the application.

The energetic ions are accelerated within the working chamber, resulting in an ionic bombardment of a target which is attached to a negative potential and consists of the material that should be deposited. [26]

Upon bombardment, atoms of the target material are ejected and thereby undergo a phase transition from solid to gaseous. The molecules from the vapor can then move freely in the chamber and condense on the substrate that is placed opposite to the target. The energy required for the formation of the film on the substrate is provided by the deposited atoms themselves, which have been energetically excited by the collisions resulting from the ion bombardment. [26]

This intrinsic energy of the particles is sufficient for film growth and renders additional energy sources obsolete. Therefore sputtering can be used to deposit films of high quality even at room temperature, which is favorable when using plastics as a substrate. [26]

Using sputtering for Silicon oxide deposition has two main advantages. There are no hazardous process gases involved and the temperature is significantly lower than for other PVD processes. This is benefitial for cost reduction and general safety. [26]

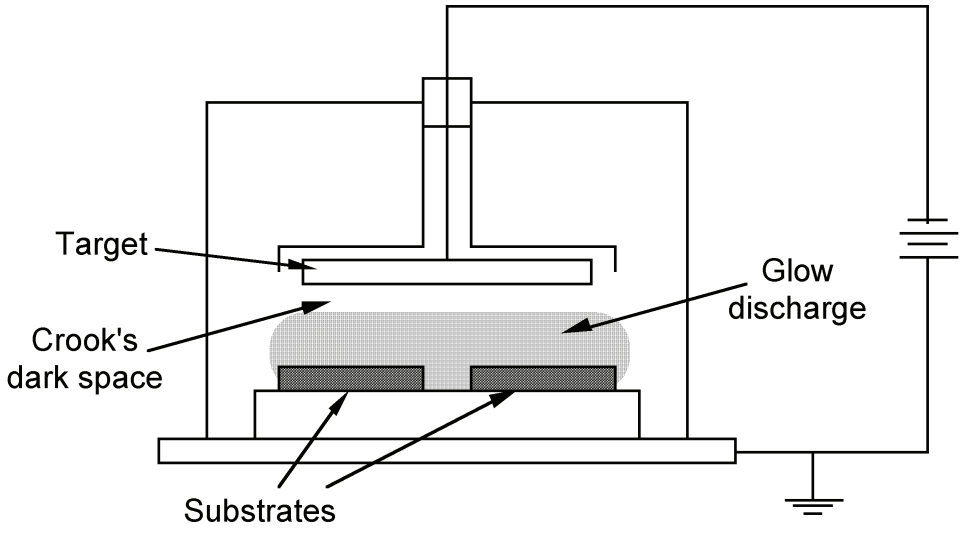

FIGURE 2.5: Sputtering of Silicon dioxide on polymeric substrates

2.6 Silanization

Silanization has many applications in microarray-, and microfluidic systems such as biosensors because of the valuable capability of silanes to provide reactive groups for further immobilization of target molecules onto a surface. It is a main method for the surface attachment of biological probes like proteins or DNA fragments, required for diagnostic or analytical "lab-on-a-chip" assays. [18]

Silanization is a surface modification technique based on the formation of self assembled monolayers (SAMs) composed of silane molecules on hydroxylated substrates, like glass, silicon wafers, or SiO_2 coatings. Silane molecules with a strong and specific affinity for the substrate react with the available chemically active groups, thereby forming the SAM. These monolayers are applied in order to alter or enhance the physical and/or chemical properties of the surface. [18]

There are different methods of applying silanes, like deposition from anhydrous solution, spray application or vapor phase deposition. The method

has an influence on the final properties of the resulting surface layer such as the number and distribution of chemically active groups available for further processing. "A suitable method needs to efficiently and covalenty attach a layer of uniform probe density across the surface", while being reliable, reproducible and easily transferable to mass production scale. [27]

As important as the right choice of silanization method is the right choice of silane. The silanes that are mainly used typically have a linear morphology. At one end they provide a surface reactive group while having an organo functional group at the other end. The most important requirement is the high affinity for the surface which they are assembled on, while the functional group is chosen according to the final application. [18]

An important class of silanes is alkoxysilanes such as aminosilanes and epoxysilanes. They feature at least one alkoxy group: an alkyl residue consisting of a carbon and hydrogen chain attached to an oxygen atom. Alkoxysilanes with an organo functional group are often referred to as organosilanes. [27]

2.6.1 Solvent deposition

Solvent deposition of silanes usually consists of submerging the sample in a solution of an organic solvent containing the silane. While being a simple method with low setup costs, the deposition of silanes via solution has some major drawbacks. Although highly purified solvents are used, strict anhydrous conditions are difficult and costly to guarantee. The presence of water causes the silane molecules to cross-polymerize in the solution. The deposition of these previously polymerized silanes from the surrounding solution onto the surface leads to inhomogeneities in the structure of the layer, often resulting in the formation of heterogenous multilayers. The resulting films are unstable. Decomposition might easily occur. [17, 18, 28]

These factors lead to a largely irreproducible product where layer thickness and surface density cannot be precisly controlled. Other phenomena like

non-covalent hydrogen bonding of silane precursors with silanols on the surface in addition to the previously mentioned problems lead to a point where the process becomes wholly unpredictable. [19]

2.6.2 Vapor deposition

Another commonly used method for applying silane monolayers is the chemical vapor deposition (CVD). CVD is a silanization technique where the silane precursor is deposited onto the substrate from the gas phase, where it forms a covalent bond by reacting with surface chemical groups. It relies on increasing the partial pressure of the silane within a closed system to increase its concentration in the gas phase. This is achieved by either heating the closed container and/or by lowering the base pressure using a vacuum pump.

Generally speaking the temperature should be maintained above 50 °C and below 120 °C to promote reaction, while the pressure is kept below 100 mbar. The time needed for the deposition and reaction of the silanes ranges from 30 minutes for amino-silanes like APTES to 4-24 hours for epoxy silanes like GLYMO. Elevated temperatures over 120 °C promote evaporation and purging of physisorbed molecules from the surface and should therefore be avoided. [19]

The silane precursor is provided via an open reservoir containing the purified silane solution. The mechanism of the deposition of the molecules from the gas phase onto the substrate can be described as chemisorption. It is a controlled way to deposit surface saturated layers, without the use of organic solvents like toluene while also drastically reducing the volume of reagents needed. [18, 19]

In addition the concentration of silane in the gas phase is usually much lower than in the liquid phase. Therefore the amount of silane molecules in close proximity to the surface is lower. This reduced availability of silane molecules has the positive effect of reducing non-covalent adsorption of silane. Using the CVD process also excludes already polymerized silane

molecules from the attachment reaction, because they can rarely perform the transition into the gas phase in the first place. In comparison to the liquid phase deposition the CVD therefore favors the formation of monolayers instead of multilayers. [19, 27]

Depending on how the procedure is carried out though, it is entirely possible that multilayers can be formed even while using CVD, but these layers are usually of higher order and quality in comparison to solvent based methods. [18] The formation of monolayers can be achieved on planar as well as on porous surfaces, though the density might be lowered on porous surfaces due to small pores that can't be penetrated by silane molecules. [29, 30]

Concerning mass production, the fact that the CVD procedure does not involve additional solvents is a serious advantage for upscaling the process as a whole. Preparation and handling of the samples is much simpler, while tedious operations like solvent removal and recovery can be completely avoided. [19]

During the CVD procedure, the first step of the deposition is the adsorption of the silane from the gas phase onto the surface. Upon exposure to surface bound trace amounts of water the reaction between the silane and the reactive groups of the sample is catalyzed, thereby forming the SAM. [18]

The underlying chemical mechanism of the permanent attachment is the immediate hydrolysis of silane alkoxy groups whereby reactive intermediate silanol groups are formed under the release of alcohol. These activated silanol groups can now interact with available hydroxyl groups on the target surface (mostly other silanol groups from the silicon dioxide) covalently attaching to it via a condensation reaction that forms a siloxan bond. The number of alkoxy groups available for hydrolysis determines the number of possible interactions with the surface. [27]

Commonly used trialkoxysilanes usually form either one or two siloxane bonds with the surface, while the remaining silanol groups form crosslinks

to other silane molecules in close proximity. This is called horizontal polymerization. It is enforced via thermal curing of the samples and increases the stability against hydrolysis. [28]

In comparison, the cross polymerization of surface bound silane molecules with unbound silane molecules from the solution, as well as the polymerization of unbound silane molecules prior to the actual deposition should be avoided by protecting the stock solutions from humidity. This enhances the quality of the deposited monolayer and prolongs the shelf life of the chemical. [18] Furthermore it is advised to distill the silane solutions on a regular basis when a batch is in use over a long period of time. [28]

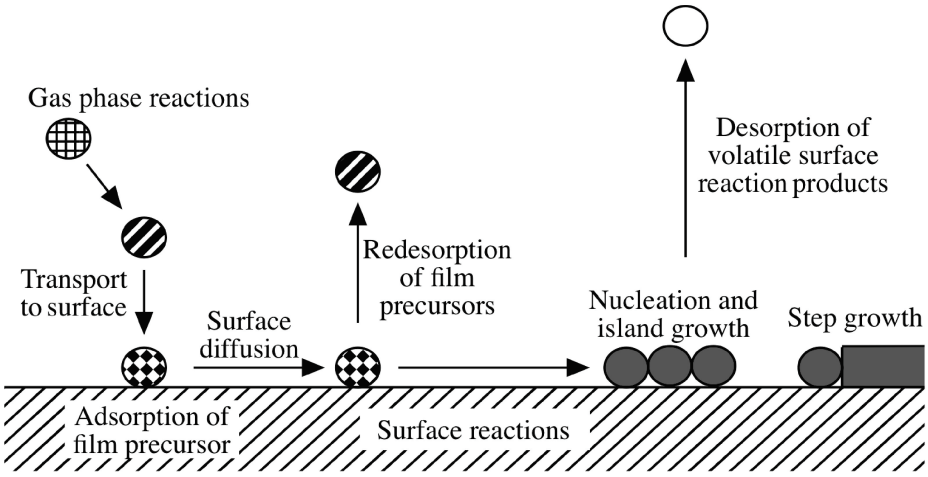

FIGURE 2.6: Schematic mechanism of chemical vapor phase deposition

2.7 Silanes

Aforementioned trialkoxysilanes are the most often used silanes for commercial silanization processes. [19] They interact with silica or glass-like surfaces through a site adsorption mechanism. After initially adsorbing to the surface the silane with its three alkoxy groups has different possible bonding modes: Mono-, bi- and tridentally. [19]

When deposited from the gas phase the preferred number of attachment sites for the most common monomeric trialkoxysilanes has been observed to be either two or sometimes only one while the third silanol always remains free. [29–31]

Under suboptimal conditions this free silanol group is capable of reacting with unbound silanes that are not attached to the the surface which may cause polymerization that leads to heterogeneity in the layer, as mentioned before. Although this represents a disadvantage when preparing homogenous surfaces for LOC chips and biosensor applications, they are still preferred over monoalkoxysilanes which only have one possibility to react with the surface and are therefore very likely to be cleaved off due to hydrolysis.

2.7.1 Aminosilane

The aminosilane APTES [(3-aminopropyl)triethoxysilane] is an organofunctional trialkoxysilane deriving from the group of the ethoxysilanes that introduces surface amino groups. [18]

The main use of the primary amines is to propagate the immobilization of proteins onto the surface under exposure of a cross-linking reagent like GMBS (Gamma-maleimidobutryloxy succimidine ester). APTES like other alkoxysilanes reacts with available hydroxyl groups of oxidized metals (e.g. silanol group) by SN_2 exchange of silane ethoxy groups for the silanol oxygen resulting in a siloxane bond. [28]

The hydrolisation of the alkoxy group, which generates the active silanol group of the silane prior to forming the siloxane bond with the connective silicon dioxide layer, releases ethanol as a side product. [18]

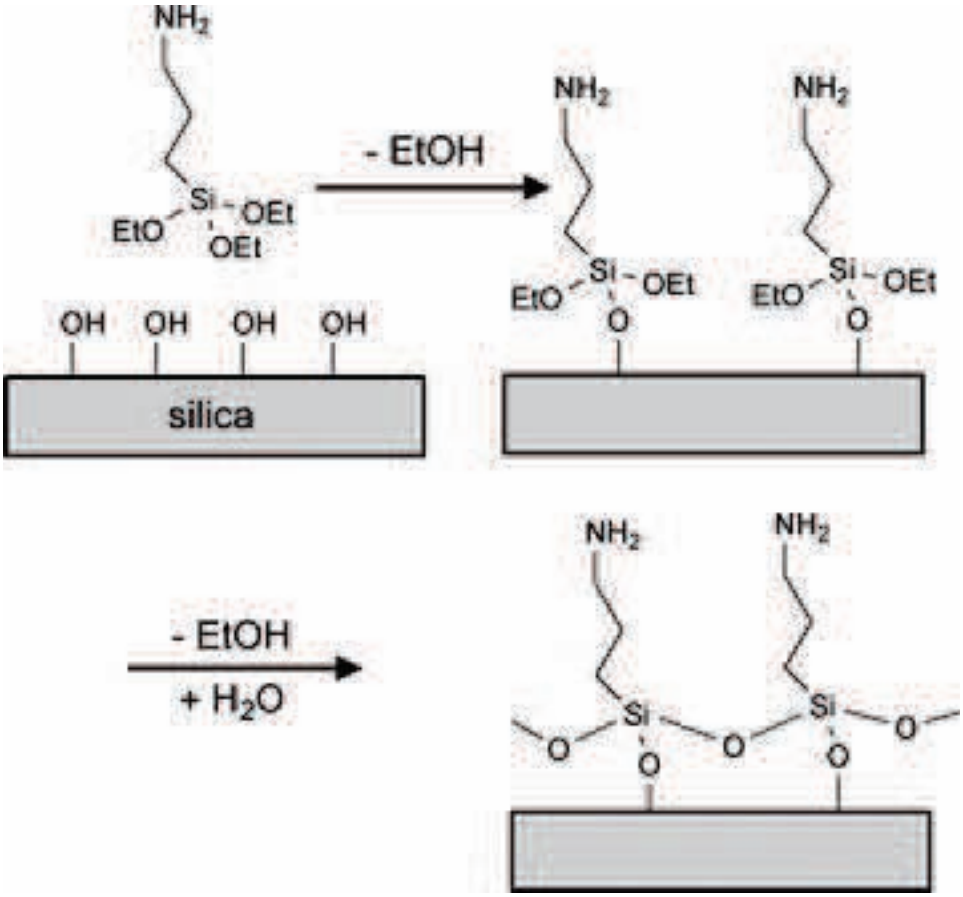

FIGURE 2.7: Mechanism of silanization, APTES

This reaction of the APTES molecules during the chemical vapor deposition is self catalyzed by the amino group in the organic chain. The electron rich amine functionality can form hydrogen bonds or ionic interactions with surface silanols and catalyzes the condensation reaction that forms the covalent siloxane bond. External catalysis is therefore unnecessary. [19]

For a high performance APTES layer the reported densities range from 2 to 3 aminosilane molecules per nm^2 for gas phase deposition. [29, 30]

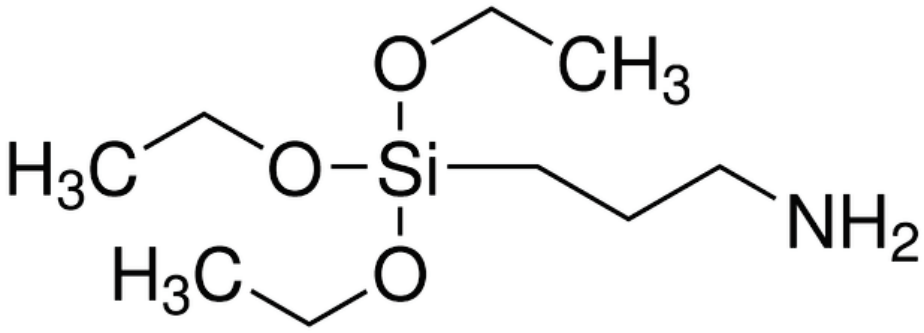

FIGURE 2.8: Chemical structure of (3-aminopropyl)triethoxysilane - APTES

2.7.2 Epoxysilane

The epoxysilane GLYMO [(3-Glycidyloxypropyl)trimethoxysilane] is an organofunctional trialkoxysilane that introduces surface epoxide rings. [32]

GLYMO is covalently attached to hydroxyl groups under the release of methanol, the bonding mechanism being the same as previously described for APTES. [18, 28]

Epoxysilane coating is a main method of immobilizing DNA and proteins as capture molecules onto the solid surface of a "lab-on-a-chip" device. The coupling mechanism relies on the epoxide rings high reactivity towards amino groups. [33]

Amino groups on functional targets can be found in form of the N-terminus of proteins, and as side chains of certain amino acid residues (e.g. Lysine). Under physiologic conditions the amino groups carry a positive charge and are therefore usually facing outwards of the globular protein structure, making them accessible for the coupling reaction. [2] For the immobilization of nucleic acids, one can use NH_2 modified oligonucleotides as well as single stranded nucleic acids that have available amino groups as part of the bases Adenine, Cytosine and Guanine. During the incubation most proteins and modified oligonucleotides will form a covalent bond with the ring structure of the epoxysilane. [2, 32]

The method is very robust but since epoxysilane has such a high reactivity towards primary amines, one should avoid buffer solutions that contain amines, like TRIS. Those would compete with the primary amines of the target molecules, thus causing a lower surface density of the spotted molecules which results in a decreasing signal.

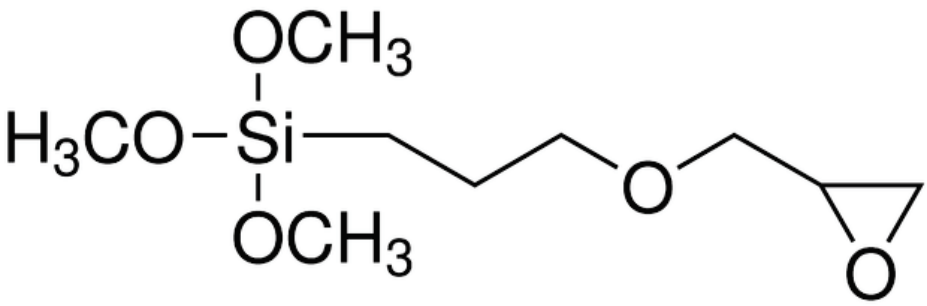

FIGURE 2.9: Chemical structure of (3-Glycidyloxypropyl)trimethoxysilane - GLYMO

Since the GLYMO epoxysilane has no amino groups it is by definition incompatible with the Orange II assay as well as with the Coomassie brilliant blue assay. To circumvent this problem and to potentially enable the two colorimetric assays to be used for epoxysilanes as well, the GLYMO can be chemically modified using diamines. The high reactivity of the epoxy functionality results in a reaction with the diamine molecule, leaving the silane with a functional amino group deriving from the other end of the diamine molecule instead of the epoxy group.

2.8 Micro-dispensing

The manufacturing of common microarrays consists of printing or spotting a liquid containing the probe onto the surface of the substrate by creating small droplets. While there are different means of creating these droplets, commonly used instruments make use of a micro-nozzle. [32, 34]

This micro-nozzle is attached to a robotic arm that can move in X, Y and Z direction. A pump attached to the nozzle enables it to take up liquids from wells containing the medium that needs to be printed. The droplets are deposited at designated locations determined by a CAD file. The pattern of spotted probes that is generated by this procedure is known as a "grid". After activating the surface by spotting the grid, the resulting microarray is then ready to receive experimental or clinical samples. This relatively simple approach can be easily customized. The grid can be changed for the requirements of every run, and the probes can be varied to the certain needs of a particular experiment.

The fluids that need to be spotted in order to create a microarray mostly consist of biological polymers like DNA (oligonucleotides, cDNA or small fragments of PCR products) and proteins in appropriate buffer solutions. These probes are designed to match the target molecules that are either other DNA fragments, other proteins or small molecules like various types of antigens. [34]

The probes are synthesized prior to the deposition. For industrial applications in particular this is mostly performed by commercial manufacturers.

The printing of the respective microarrays can be performed in-house, but requires the appropriate equipment. Miniaturization of assays in form of LOC-devices also calls for a miniaturization of liquid handling to a comparable degree. [35]

Microdispensing is the term related to the creation of small dosages of liquid media fulfilling this need of miniaturization. It is divided into contact- and non-contact dispensing. For industrial applications especially the non-contact dispensing is of great importance. Its characterized by the method of droplet formation. In contrast to the contact-dispensing where the drop is created directly on the surface, the droplet coming from the end of the nozzle during non-contact dispensing is created at sufficient distance from the substrate so it can be fully ejected before it hits. [35]

Non-contact micro-dispensing systems are able to accurately deposit small and uniform spots of biological fluid with a high throughput, while creating only minimal waste which makes it economical and environmentally friendly. It is a convenient method for manufacturing of microarrays with a high degree of complexity and miniaturization. Non-contact micro-dispensing can be divided into two different methods: Jet-forming dispensing and dynamic drop dispensing. What to choose mainly depends on the properties of the medium that needs to be spotted like viscosity, density, surface tension and vapor pressure. Jet-forming dispensing is based on the application of a static pressure to generate a jet of liquid that gets ejected from the orifice of the nozzle. It is a well-established method, but it is only partially compatible with very small volumes of liquids with higher viscosity and therefore suboptimal for the spotting of microarrays. [35]

This problem can be circumvented using another type of non-contact dispensing: Dynamic drop dispensing is based upon the separation of the drop from the nozzle via a dynamic process. A common method is to use a piezoelectric dispenser, consisting of a glass or quartz capillary tube with a very fine tip to which a piezoelectric collar is attached. The capillary is attached to a fluidics system, usually a syringe pump or a reservoir. [36]

Upon activation of the piezoelectric mechanism via application of a shaped electrical pulse, the ring shaped piezo crystal contracts. The resulting compression wave results in the ejection of a droplet from the tip of the nozzle which is thereby deposited on the substrate underneath. Via adjusting of the voltage it is possible to tune the dispensed volume and accuracy of dispensing. [37]

Piezoelectric dispensing technology is adaptable to a wide range of biological reagents, biomaterials, and biocompatible polymers. The range goes from nanoliters down to about 100 picoliters. The technology is highly reliable and accurate, concerning dosage as well as placement. Clinical acceptance is a problem though, because drop uniformity does not necessarily equal uniform sample deposition. [36]

Other than non-uniform deposition the most severe problem that might occur during the spotting of biological probes is film creation. In the time between the fluid uptake and the actual dispensing the solvent can evaporate. The drying liquid leaves a film at the orifice of the nozzle which leads to dispensing failure or drop instability over time. A way to circumvent this problem is to increase the humidity of the surrounding, or to decrease the concentration of the probe in the spotting medium.

2.9 Spectrophotometry

Spectrophotometry is a method to quantitatively analyze the concentration of target compounds in a sample solution. This is done by emitting light upon the sample, measuring the fraction of light that passes through the solution over the whole spectrum. Most compounds do have a characteristic spectrum of wavelengths. Depending on the application, one significant peak can be chosen for target identification and quanitification. The extinction value for corresponding wavelength is directly correlated to the concentration of the analyte in the solution. [38]

The measurement is performed within a spectrophotometer. The basic components of such a device are a light source, a monochromator, a sample cuvette and a detector. The light source consists of a lamp that emits light of a certain bandwidth of wavelengths depending on the application (e.g. ultraviolet, visible, infrared). Light from the source lamp is passed through the monochromator consisting of an entrance slit, a dispersion device (a grating or a prism) and an exit slit. The monochromator diffracts the light into a "rainbow" of wavelengths. Depending on the position of the movable diffraction grating, only a narrow bandwith of this diffracted spectrum is able to leave the exit slit and pass through the sample cuvette at a given time. By turning the diffraction grating stepwise, the whole spectrum can be measured by sequentially sending light with discrete wavelengths through

the cuvette containing the test sample. In a last step the photon flux density (watts per metre squared) of the transmitted light is measured with a photodiode, a charge coupled device or another type of light sensor. [38]

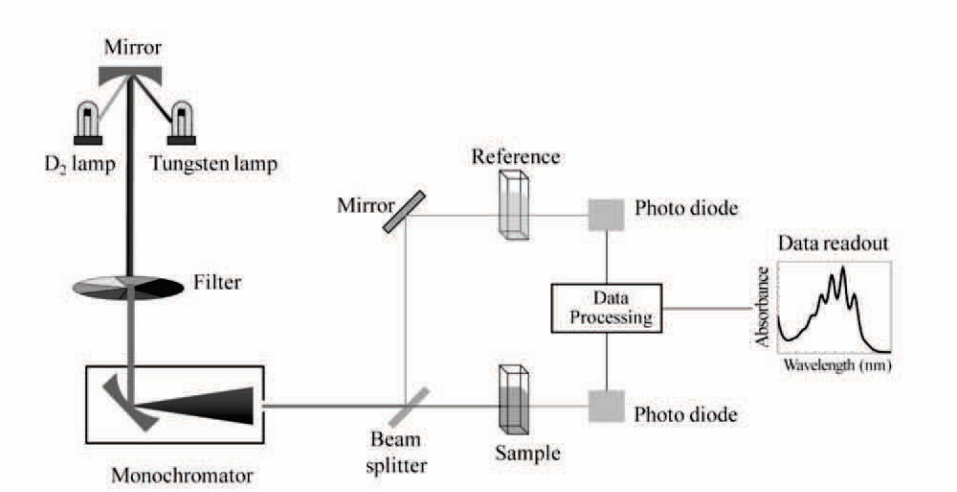

FIGURE 2.10: Schematic display of a UV/vis - Spectrophotometer

There are two major classes of spectrophotometers depending on how the actual concentration of a sample is determined: single beam and double beam spectrophotometers. A double beam spectrophotometer splits the light emitted from the light source into two paths: One passing through a reference sample, and one passing through the test sample. It then compares the difference in signal intensity between the two. A single beam spectrophotometer in comparison measures the reference sample and the test sample sequentially and substracts the determined base value from the first and every consecutive sample. Although the measurements from double beam instruments are easier and more stable, single beam instruments can have a larger dynamic range and are optically simpler and more compact. [38]

The most common spectrophotometers cover the UV and visible regions of the spectrum. Visible region spectrophotometry with a range of 400 to 700 nm is very important for colorimetric assays. Common dyes such as Coomassie Brilliant blue and Orange II both have their absorption maxima

within this range of wavelengths. Regular visible region spectrophotometers are not capable of detecting fluorescent signals. Important features of spectrophotometers are the spectral bandwidth and the linear range of absorption or reflectance measurements. [38]

What actually is measured by the spectrophotometer is the transmission. The transmission is the ratio of the signal intensity of the light that leaves the cuvette to the light that originally entered the cuvette and ranges between 0 and 1. For convenience most devices do not plot the transmission but the extinction of a solution as a function of the wavelength. [38]

The extinction is a physical property of a solution and is defined as the negative decadic logarithm of the transmission. It is a combined effect deriving from absorption, scattering, reflection and refraction (which can be neglected in this setup). It is indicative of the concentration of the target chemicals in the solution. Molecules hinder light of a certain wavelength to pass through. If the compound is more concentrated, more light will be absorbed by the sample, leading to higher values for the extinction. For analyte concentrations lower than 10 mM it linearly correlates with the concentration, following the Lambert-Beer law. [38]

$$A = c \cdot d \cdot \varepsilon = \log(\frac{Io}{I}) \tag{2.1}$$

2.10 Contact angle

The contact angle is a metric for the evaluation of the hydrophilicity of a given surface. It is a quantitative measurement of the wettability (degree of wetting) of a solid by a particular liquid.

When placing a drop of liquid on a surface, the contact angle θ is the angle formed between the tangent lines of the liquid/solid interface and the liquid/vapor interface.

For a given ideal system consisting of a particular liquid on a defined solid surface, at a set temperature and pressure, a correlating equilibrium contact angle θ^Y can be defined via the Young's equation:

$$\gamma^{LV} \cdot \cos(\theta^Y) = \gamma^{SV} - \gamma^{SL} \tag{2.2}$$

The three thermodynamic parameters γ^{LV}, γ^{SV}, and γ^{SL} represent the liquid-vapor, solid-vapor, and solid-liquid interfacial tensions, respectively. The contact angle θ^Y of a liquid drop on an ideal surface is defined by the mechanical equilibrium of the drop under the action of these three interfacial tensions.

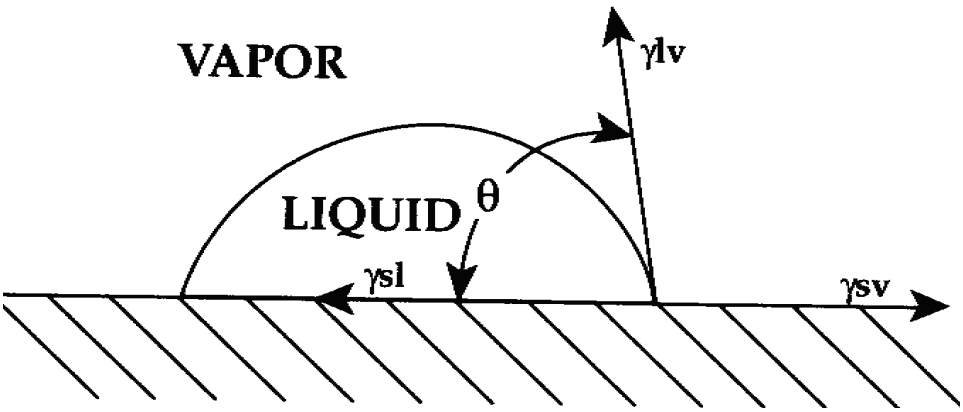

FIGURE 2.11: Relevant parameters of the Young equation

However, due to the complexity of contact angle phenomena, the experimentally observed contact angles are usually not equal to Young's contact angle θ^Y. [39]

Chapter 3

Material and Methods

For evaluating the silanization processes at SONY Biosciences two different types of injection molded devices have been used: On the one hand the SONY MS-slide with an attached SONY multi-well upper structure, on the other hand the Anagnostics Hybcell core, a custom designed cylindrical device. In preparation for the actual silanization the parts have been sputtered with an intermediate layer of silicon dioxide using reactive oxygen sputtering.

Different methods of pre-treatment have been compared regarding an optimization of the silanization procedure. The metric for evaluating the amino and epoxy group density on the surface as a measure of the overall quality of the process was a photospectrometric measurement deriving from colorimetric assays, as well as fluorescence based assays. Additional data for the characterization of the silane coating was collected by measuring the contact angle and by performing the dedicated application of the LOC-devices.

Materials and reagents used within this thesis included the following:

Reagent	Purity	Supplier
Zeonor 1060R	n/a	Zeon corporation
Zeonor 1420R	n/a	Zeon corporation
Kraton 1645	n/a	Kraton corporation
Orange II	≥85%	Sigma-Aldrich
Coomassie brilliant blue	pure	Sigma-Aldrich
TAMRA-NHS	pure	Sigma-Aldrich
Diaminopropane	≥99%	Sigma-Aldrich
Ethylenediamine	≥99%	Sigma-Aldrich
Ammonium	25% in H_2O	Sigma-Aldrich
Aminopropyl-triethoxy-silane	≥98%	Sigma-Aldrich
Glycidyloxypropyl-trimethoxy-silane	≥98%	Sigma-Aldrich
Sulfuric acid	≥98%	Sigma-Aldrich
Hydrogen peroxide	30% in H_2O	Sigma-Aldrich
Acetic acid	≥99%	Sigma-Aldrich
Dimethylsulfoxide	anhydrous, ≥99.9%	Sigma-Aldrich
Potassium chloride	≥99%	Sigma-Aldrich
Sodium chloride	≥98%	Sigma-Aldrich
Hydrochloric acid	(36.5 - 38% v/v	Sigma-Aldrich
Sodium hydroxide	≥98%	Sigma-Aldrich
Acetone	≥99.9%	Sigma-Aldrich
Methanol	≥99.9%	Sigma-Aldrich
TAMRA-cadaverine	pure	Invitrogen
TAMRA	pure	Invitrogen
Ethanol	≥96%	Merck
Isopropanol	≥99.5%	Merck
Hellmanex III	pure	Hellma

Table 3.1: Results - molar extinction coefficients

All glassware was cleaned by submerging the parts in 1% (v/v) Hellmanex cleaning solution for 15 minutes, followed by a rinse with ultrapure water (diH_2O). The parts were then left to dry or immediately dried under a stream of compressed nitrogen.

Instruments used for this thesis were the following: Magnetron sputter Oerlikon ARQ - 21 RF, Single layer sputter Shibaura Stella S-1000, Diener Electronic Nano LFG 40 plasma chamber, Binder VD 23 vacuum drying

oven, Espec LHU-113 temperature humidity chamber, Scienion sciFlexarrayer S3, Tecan LS Reloaded microarray scanner, Krüss DSA30 drop shape analyzer.

3.1 SONY slides and multi-well upper structure

The SONY MS-slide is an injection molded polymeric consumable made of Zeonor 1060R. It is a flat rectangular slide with the dimensions of 75 x 25 mm. The injection molding process of these slides was performed isothermally. The mold temperature was kept constant at around 80 °C while the melt flow temperature was set around 250 °C. The injection pressure was set at 1600 bar. The global flatness requirements were 50 μm or below.

The SONY multi-well upper structure is an injection molded polymeric consumable made of Zeonor 1060R mixed with 3% Plasblak PS4256 black carbon mastermix to mitigate the light transmittance, with an adhesive foil on the bottom. It can be applied on top of a microscope slide of choice in order to introduce a 16-well structure that can be used for any kind of biological assay.

The colorimetric assays performed as part of this thesis required the application of a distinctive volume of liquid onto a defined surface area. Using the SONY upper structure on top of a generic injection molded plastic microscope slide seemed to be the easiest setup to achieve this requirement. With each well being 7 x 7 mm the cumulated area of the 16 wells is 784 mm^2.

Attaching the upper structure permanently to a slide is possible due to a foil layer on the bottom made of ARseal 90880 polypropylene with a silicone based pressure sensitive adhesive. After removal of the PET release liner, the upper structure can be attached to any kind of plain or coated microscope slide via applying a firm pressure. The assembled consumable can be used immediately for intended experiments without any further preparative steps. Relevant features of the adhesive foil are a low moisture transfer rate which prevents changes in the concentration due to absorption of the sample solution during the assay, as well as its chemical inertness and wide operating temperature range of -40 °C to 120 °C.

Additionally it is chemically resistant to diluted solutions containing DMSO, acids, alcohols or organic solvents. As with all pressure sensitive adhesives

the surface to which it is to be applied needs to be clean, dry and free of oil, moisture and dust. To ensure these requirements the slides were stored in appropriate boxes to avoid contamination.

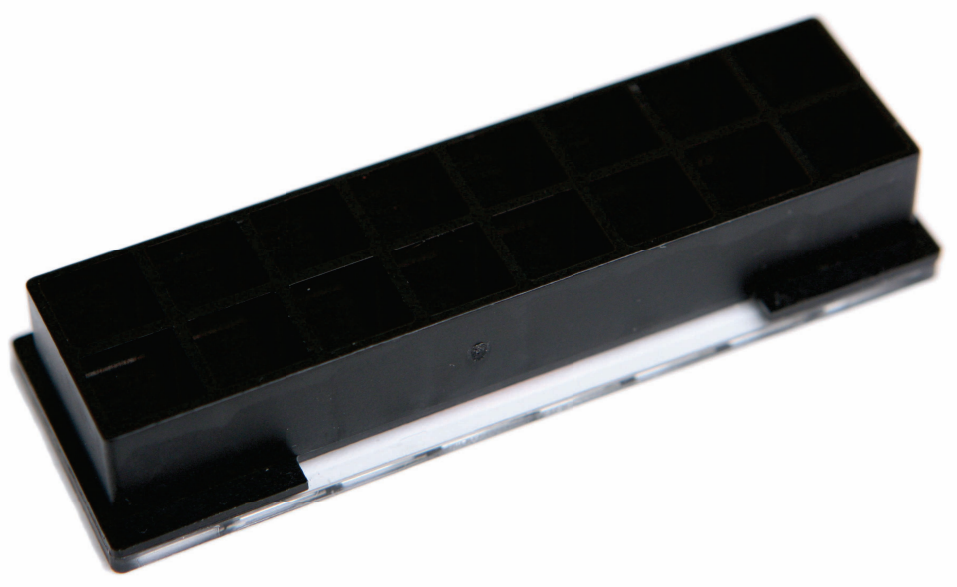

FIGURE 3.1: SONY multi-well upper structure - Polymeric consumable for introduction of microwells on microscope slides

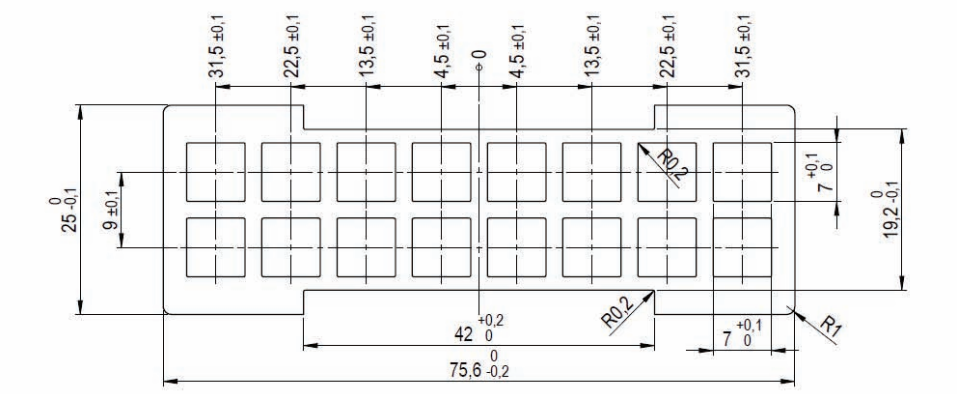

FIGURE 3.2: SONY multi-well upper structure - Technical drawing

3.2 Hybcell cores and tubes

The Anagnostics Hybcell core is an injection molded cylindrical polymeric consumable that undergoes reactive sputtering with a silicon target and chemical vapor deposition of GLYMO epoxysilane to be used as a substrate for printing oligonucleotide- and protein microarrays for IVD-applications such as the Hyborg drugs of abuse screening. The Hybcell core is made from the cyclic olefin polymer Zeonor 1420R mixed with 2% Plasblak PS4256 mastermix to reduce the light transmittance. Zeonor 1420R differs from Zeonor 1060R mainly concerning the chain length of the polymer, while the optical transmittance and the water absorbency are comparable.

FIGURE 3.3: Hybcell core - Polymeric consumable for microarray applications

The Anagnostics Hybcell tube is a transparent injection molded polymeric consumable that is used as a containment for the Hybcell core during the actual Hyborg assay. It is a clear plastic cylinder made from Zeonor 1420R without any additives.

The Hyborg assay is a multiplex, competitive immuno-assay for quantitative detection of drugs of abuse. Target substances can be detected directly from urine, saliva or serum samples with only minor sample preparation requirements. A set of antibodies targetting 21 classes of drugs which represents

over 100 single substances are immobilized on the surface of the Hybcell core via micro-dispensing.

The Hybcell core is assembled into the Hybcell tube containing 100 μL of the sample as well as 120 μL of the reagent kit that mainly consists of the competitor antigens: BSA protein conjugates of the target substances with a fluorescent label.

As soon as the Hybcell core is placed in the sample solution, drug molecules that are present in the sample compete with the labeled protein conjugates for the binding sites of the immobilized antibodies.

The more drug molecules are present in the sample the fewer protein conjugate molecules can bind to the antibodies and will be removed during the washing steps of the procedure. During the read-out the presence of drug molecules is detected in form of a decrease in signal for the respective substance.

Among the drugs that can be detected are amphetamines, cocaine, methadone, opiates, THC (Cannabis), barbiturates and antidepressants.

3.3 Cyclic olefin polymer

The COP grades chosen by SONY Biosciences for the manufacturing of the polymeric devices used in this thesis are ZEONOR 1060R and Zeonor 1420R. The standard COP microscope slides as well as the Hybcell cores and tubes used in these experiments were fabricated via injection molding.

The high optical transmittance of Zeonor 1060R in the range of 300 to 800 nm is a basic requirement for the Hyborg assay. The low water absorbency compared to polycarbonate (PC) and poly(methyl methacrylate) (PMMA) is an important criteria for the injection molding process. Since the water uptake during the storage is low the material does not need to be dried as long, prior to the molding process. It also reduces the susceptibility to geometric changes due to swelling.

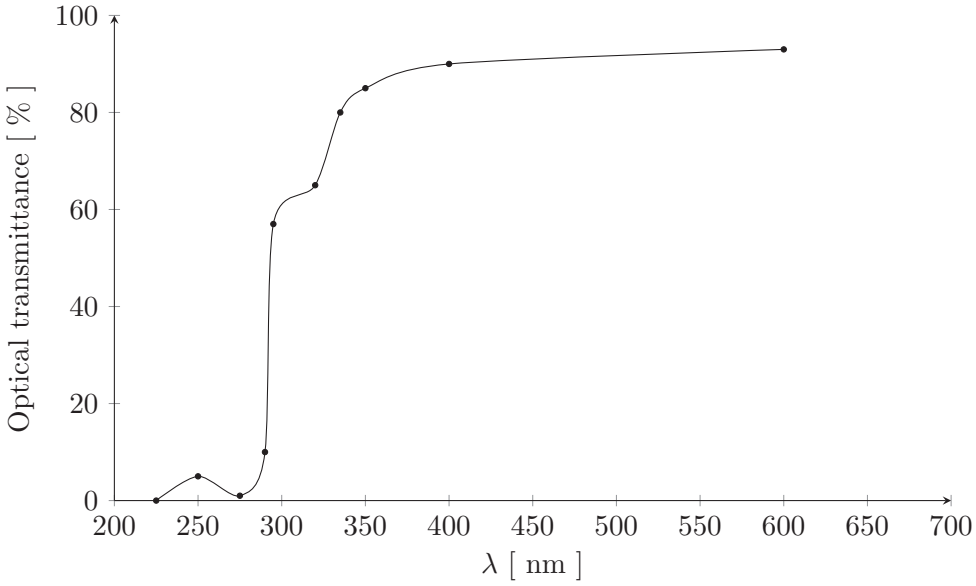

Table 3.2: Data Zeonor 1060R - Optical transmittance

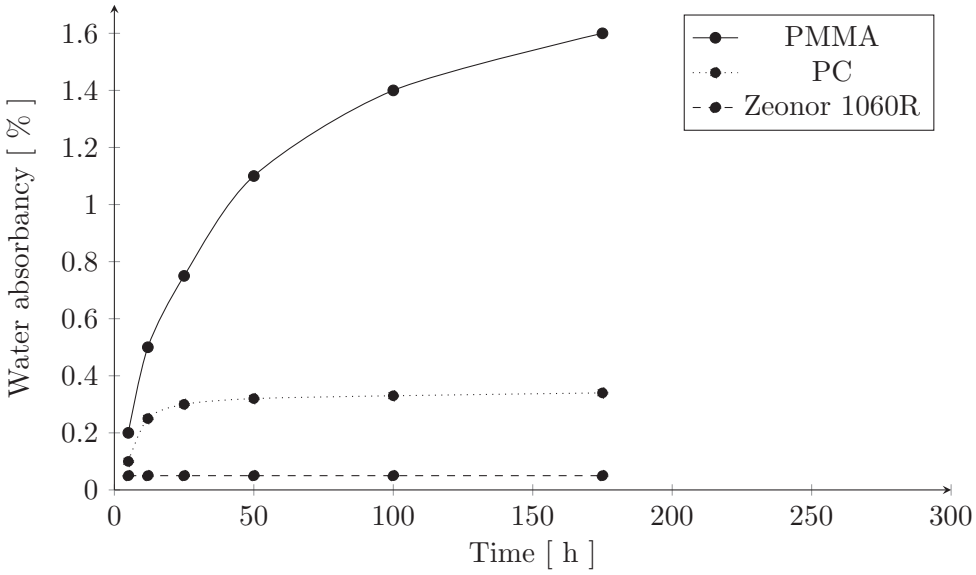

Table 3.3: Data Zeonor 1060R - Water absorbancy comparison

3.4 Sputtering

The SONY MS-slides were sputtered using a silicon target. The power was set to 3 kW. The gas flow was set to 9 sccm (standard cubic centimer per minute) for the argon influx and to 25 sccm for oxygen.

The sputtering process was carried out in 3 subsequent pulses of 5 seconds each, with 3 seconds in between to achieve a layer thickness of 30 nm SiO_2.

The Hybcell cores were sputtered using a silicon target. The power was set to 1.1 kW. The gas flow was set to 15 sccm for the argon influx and to 8 sccm for oxygen. The sputtering process was carried out in one 75 second pulse to achieve a layer thickness of 15-40 nm SiO_2. The variance in thickness derives from the three dimensional geometry of the plastic part.

Layer thicknesses were determined via ellipsometry. For the transparent slides the ellipsometric measurement was carried out on a small silicon die treated in the same sputter chamber.

The sputtered devices and the thickness measurements were provided by Gottfried Aichinger.

3.5 Cleaning and oxidation

Silanization is based upon covalent bonds between hydroxyl groups like the silicon based silanol groups. Upon exposure to different environmental conditions these groups may chemically change over time and lose the ability to bind the silane.

Therefore in order to clean the surface and regenerate the silanol groups of the sputtered silicon dioxide layer an oxidizing pre-treatment is required.

Commonly used methods are strongly oxidizing acids, oxygen-plasma or UV-radiation. Treating the surface with oxidizing acid and a plasma treatment were compared in this thesis.

3.5.1 Plasma activation

The devices were placed in the working chamber of the plasma activation unit between the two electrodes.

Using the vacuum pump the chamber pressure was lowered to 0.1 mbar in order to remove the remaining air. In the next step the chamber was flushed with O_2 until a pressure of 4 mbar was reached to provide sufficient amounts of oxygen. The pressure was then lowered to a working pressure of 0.3 mbar. After the power was set to 100 W the plasma was ignited and the slides were treated for 30 seconds. To equilibrate the pressure in the chamber it was then flushed with nitrogen.

Plasma treated devices were immediately used for further testing. Since plasma treated surfaces are highly reactive it is suggested to not store them for elongated periods of time, to provide reproducible conditions.

3.5.2 Piranha acid

The sputtered devices were initially cleaned by rinsing them with isopropanol (70%) and deionized water (diH_2O) to remove any organic residues that would cause violent reactions with the acid. In order to regenerate the silanol groups on the surface the slides were then immersed in Piranha solution [(1:3 mixture of hydrogen peroxide (45%) and sulfuric acid (96%)] for 10 minutes. After the immersion time the slides were subsequently rinsed in diH_2O 5 times.

In a second experiment the slides were additionally ultrasonicated in diH_2O for 15 minutes to fully remove all of the residual reagent.

Finally all the devices were blown dry under a stream of nitrogen and put inside the vacuum oven for further drying. For this procedure the vacuum oven was set to 30 °C and the pressure was lowered to 50 mbar. [11]

3.6 Silanization - Chemical vapor deposition

For the silanization procedure SONY Biosciences makes use of a closed chamber process performed in a vacuum oven, where the substrates are placed in close proximity of an open silane reservoir. [18]

In order to protect silane stock solutions from exposure to humidity, only bottles with an airtight seal were used. Whenever silane was transferred from the bottle to a reservoir, a small syringe was used to pierce the seal and collect the required volume to avoid unnecessary contact with air.

Mild curing conditions were applied after the silanization to enhance horizontal polymerization.

See Appendix A for a flowchart of the general silanization process.

3.6.1 Aminosilane

To prepare the vacuum oven for the silanization the working chamber was heated to a temperature of 60 °C. The devices were already placed inside the reaction chamber during this step. The temperature equilibrium of the parts and the oven is important for the reproducibility. [17]

After the oven reached the required temperature an open reservoir containing 100 μL of APTES was placed inside the reaction chamber. [18]

The oven was then flushed with nitrogen for around one minute. Vacuum was applied while the nitrogen influx was held constant, to purge remaining air out of the chamber, up to the point where the nitrogen influx and the efflux caused by the vacuum pump reached an equilibrium. The nitrogen valve was then closed and the chamber was fully evacuated to a pressure of 50 mbar. [27]

To enable the APTES to covalently bind to the surface the devices were incubated in the oven for 30 minutes. [18]

To equilibrate the pressure inside the oven after the silanization was complete, it was flushed with nitrogen. During the first minute of flushing, the vacuum pump was turned on to remove harmful silane fumes from the reaction chamber. The pressure was then fully equilibrated by turning the vacuum pump off. The devices were taken out and the silane remaining in the reservoir was appropriately disposed. [18]

Thermal curing for 1 h around 100 °C causes condensation of unreacted silanol groups to siloxane bonds which creates a cross linked monolayer with reduced susceptibility to hydrolysis. [27]

3.6.2 Epoxysilane

The silanization procedure for using GLYMO epoxysilane is similar to the one using APTES aminosilane. The main differences are the chamber temperature of 80 °C and the incubation time of 60 minutes.

Post processing conditions are the same as for the APTES silane, namely 1 h of further incubation at 100 °C with the reservoir removed.

3.6.3 Amination of epoxysilane

To enable the use of the amino group dependent Orange II assay on epoxy silanized slides and cores the devices were treated with diamine solutions of different types and concentrations. Ethylenediamine and diaminopropane were diluted with ethanol (70%) in a ratio of 1:8 (v/v) and 1:80 (v/v). Slides and cores were incubated for 24 hours in the diluted diamine solutions to complete the amination reaction. After the incubation the slides and cores were washed using ethanol (70%) and blown dry under a stream of compressed nitrogen before being used for the colorimetric assays.

3.7 Colorimetric assay

The colorimetric assays were performed in order to determine the amount of silane molecules within a defined surface area. This was done by saturating the surface with negatively charged dye molecules. These dye molecules then attached to the functional groups of the silane molecules in a stoichometric manner. After washing off all unbound dye molecules the bound molecules were eluted.

The eluate then ideally contained an amount of dye molecules similar to the amount of silane molecules on the tested surface. Via spectrophotometry it was possible to determine the concentration of the dye in the solution, and thereby the absolute number of dye molecules for a known volume of eluate.

Putting the absolute amount of dye molecules in relation to the particular area of the tested surface yields the corresponding amount of silane molecules within this area: the surface density.

The sensitivity of the assay mainly depends on the surface density of the functional groups and the size of the dye molecules, assuming stability of the binding and completeness of elution. For dyes with a higher molecular weight and high surface densities of functional groups, steric hindrance may occur, yielding a determined number of silane molecules, lower than the actual amount.

The functional surface on the slides with the attached upper structure was calculated to be 784 mm^2. The functional surface of the Hybcell cores was 565 mm^2.

3.7.1 Molar extinction coefficient and linearity

To measure the molar extinction coefficient ϵ of the dyes used for the colorimetric assays, and to evaluate whether the correlation between concentration and absorbance is linear, a series of dilutions was prepared from the stock

solutions of Orange II (14 mg/mL) at pH 3 and Coomassie brilliant blue (0.5 mg/mL).

These stock solutions were diluted in linear increments and their absorbance was measured with the spectrophotometer. For a given concentration the absorbance value allows for the calculation of the molar extinction coefficient using the Lambert-Beer equation.

$$A = c \cdot d \cdot \varepsilon = \log(\frac{Io}{I}) \tag{3.1}$$

Plotting the concentration of the dyes against the absorbance determines the linearity of the correlation between the two parameters.

3.7.2 pH-dependency of spectrophotometric measurement of the Orange II assay

During the Orange II assay the pH of the solution has to be adjusted from pH 12 to pH 3. It was tested if the change in pH has an influence on the resulting absorbance value.

3.7.3 Orange II assay on slides

The acidic wash solution consisted of diH_2O adjusted to a pH of 3 using 3 M hydrochloric acid (HCl).

The alkaline elution solution consisted of diH_2O adjusted to a pH of 12 using 3 M sodium hydroxide (NaOH).

The saturated Orange II dye solution had a concentration of 14 mg/mL. It has been prepared dissolving 1.4 g of Orange II sodium salt powder in 100 mL of acidic wash solution at room temperature. This stock solution was stored in a light protected environment to avoid decay of the dye. Storage time should not exceed one week. The solution is susceptible to infection by *Aspergillus Niger*.

In a first step dust and particles were removed from the slides and upper structures under a stream of compressed nitrogen.

After removing the protective foil from the multi-well upper structures they were attached onto the slides. This had to be done carefully to make sure that the two components were properly aligned. After attaching the upper structure onto the slide, a firm pressure had to be applied for the pressure sensitive adhesive to seal off the gaps to prevent leakage during the actual assay. The assembled parts were then cleaned again with a stream of compressed nitrogen to remove any particles that may have ended on top of the active surface during the handling. Using a multi-channel pipette, 100 μL of the saturated Orange II solution were transferred into each well of the slides. A control slide was filled with 100 μL acidic wash solution per well containing no Orange II. After filling the wells of the slides they were incubated for half an hour in a climate chamber at a relative humidity of 80% at 40 °C to enhance the binding of Orange II to the amino groups.

When incubation was complete the dye solution was removed from the wells using a multi-channel pipette. Special care was taken to avoid droplets of the solution getting on the wall of the wells, which facilitates the following cleaning steps.

After removing all the liquid from the wells, 400 μL of acidic cleaning solution were transferred into the wells. The upper structures were sucked dry using the pipette. The rinsing and drying had to be performed 3 times with 400 μL of the acidic cleaning solution. After the third time of removing the cleaning solution the slides were blown dry completely under a stream of nitrogen.

Rinsing the slides removes unbound dye molecules that are not corresponding to a silane on the surface. In the next step each well was filled with 65 μL of the alkaline elution solution using a single-channel pipette. Incubation was performed for 10 minutes at room temperature.

After the elution was completed the eluate, that contained the dye molecules corresponding to a silane, could be transferred from the wells to an Eppendorf-tube. The eluate contained in the wells of one slide was collected in a single Eppendorf-tube.

The used slides could be disposed. The pH of the eluate was adjusted to pH 3 via adding 1% (v/v) of 12,3 M HCl prior to measuring the extinction in the UV/vis - spectrophotometer at a wavelength of 485 nm. [16]

3.7.4 Orange II assay on cores

For the colorimetric assay on cores the acidic wash solution, the alkaline elution solution and the saturated Orange II dye solution were prepared in the same manner as for the experiments using the slides.

In a first step dust and particles were removed from the cores and tubes under a stream of compressed nitrogen. For every core one tube was prepared.

Using a regular single-channel pipette, 450 μL of the saturated Orange II solution were transferred into each tube. For the control core a tube was filled with 450 μL acidic wash solution containing no Orange II.

After the tubes were filled the cores were placed within them and were submerged in the solution. The cores were then incubated for half an hour in a climate chamber at a relative humidity of 80% at 40 °C to enhance the binding of Orange II to the amino groups.

When the incubation was complete the dye solution was removed from the surface of the cores by removing them from the tube and submerging them in 2 mL of fresh acidic solution. This step was performed three times for each core, using fresh acidic wash solution for every step.

After the third washing step the cores were blown dry completely under a stream of nitrogen. A fresh Hybcell tube was prepared for every core.

In the next step each tube was filled with 450 μL of the alkaline elution solution using a single-channel pipette. Incubation was performed in a climate chamber at a relative humidity of 80% at 40 °C.

After the elution was completed the eluate could be transferred from the Hybcell tubes to an Eppendorf-tube. The used cores and tubes could be disposed.

The pH of the eluate was adjusted to pH 3 via adding 1% (v/v) of 12,3 M HCl prior to measuring the extinction in the UV/vis - spectrophotometer. [16]

3.7.5 Coomassie brilliant blue assay on slides

The acidic wash solution consisted of diH_2O/ methanol/ acetic acid (85:10:10 v/v) adjusted to a pH of 2.2 using 3 M hydrochloric acid (HCl).

The alkaline elution solution consisted of 0.125 M potassium carbonate in diH_2O/ methanol (50:50 v/v) adjusted to a pH of 11.25 using 3 M sodium hydroxide (NaOH).

The saturated Coomassie brilliant blue dye solution had a concentration of 0.5 mg/mL. It has been prepared dissolving 50 mg of Coomassie brilliant blue powder in 100 mL of acidic wash solution at room temperature. This stock solution was stored in a light protected environment to avoid decay of the dye. Storage time should not exceed one week.

In a first step dust and particles were removed from the slides and upper structures under a stream of compressed nitrogen. Assembly and preparation of the slides and mutli-well upper structures was carried out as mentioned in section 3.7.3.

The assembled parts were then cleaned again with a stream of compressed nitrogen to remove any particles that may have ended on top of the active surface during the handling.

Using a multi-channel pipette, 100 μL of the saturated Coomassie brilliant blue solution were transferred into each well of the slides. A control slide was filled with 100 μL acidic wash solution per well. After filling the slides they were incubated for 5 minutes in a climate chamber at a relative humidity of 80% at room temperature to enhance the binding of Coomassie brilliant blue to the amino groups.

When the incubation was complete the dye solution was removed from the wells using a multi-channel pipette. Special care was taken to avoid droplets of the solution getting on the wall of the wells, which eases the following cleaning steps.

After removing all the liquid from the wells, 400 μL of acidic cleaning solution were transferred into the wells. The upper structures were sucked dry using the pipette.

The rinsing and drying had to be performed 3 times with 400 μL of the acidic cleaning solution. After the third time of removing the cleaning solution the slides were blown completely dry under a stream of nitrogen.

Rinsing the slides removes unbound dye molecules that are not corresponding to a silane on the surface. In the next step each well was filled with 65 μL of the alkaline elution solution using a single-channel pipette. Incubation was performed for 10 minutes at room temperature.

After the elution was completed the eluate, that contained the dye molecules corresponding to a silane, could be transferred from the wells to an Eppendorf-tube. The eluate contained in the wells of one slide was collected in a single Eppendorf-tube.

The used slides could be disposed.

The pH of the eluate was adjusted to pH 3 via adding 7.5% (v/v) of 3 M HCl prior to measuring the absorbance in the UV/vis - spectrophotometer at a wavelength of 620 nm. [16]

3.8 Fluorescence based assay

As an alternative to the colorimetric assays that are based on the spectrophotometric measurement of dye molecules there are also fluorescence based methods to verify the modification, functional density, and homogeneity of silane coated slides by spotting a dye with a reactive group that will covalently couple to the functional groups. For this kind of assay detection was performed by using a microarray scanner that uses light of a certain wavelength for the excitation of the dye molecules while determining the intensity of the corresponding emission signal.

3.8.1 TAMRA-NHS spotting, TAMRA Cadaverine spotting

The TAMRA-dyes for the detection of amino-, and epoxysilane coated surfaces were delivered in a lyophilized state. For aminosilane coated slides TAMRA-NHS was used, for the epoxysilane coated slides it was TAMRA Cadaverine.

The lyophilized dyes were reconstituted in DMSO setting the concentration of the stock solutions to a value of 100 mM. To ensure the reproducibility of the measurements the reconstituted dye stock solutions were stored in a freezer at $-20\,^{\circ}\mathrm{C}$.

To confirm the exact concentration of the stock solutions, they have been measured via spectrophotometry. Concentration values were calculated from the absorbance value at 559 nm for the TAMRA-NHS and at 545 nm for TAMRA Cadaverine via the Lambert-Beer law.

The PBS spotting buffer was prepared using 137 mM of NaCl, 10 mM of phosphate and 2.7 mM of KCl.

The amine washing buffer consisted of PBS + 0.01% Tween-20 detergent set to a pH of 7.5 while the epoxy washing buffer consisted of 50% (v/v) 0.05 M Na_2HPO_4, 11% (v/v) of 0.1 M NaOH, 39% (v/v) diH_2O and 20 μL (v/v) Tween-20 detergent, set to a pH of 11.5.

Using the dye stock solutions a series of dilutions was prepared, namely 50 μM, 40 μM, 25 μM and 10 μM in spotting buffer. For every test run these dilutions were freshly prepared. 1 μL of each dilution was spotted across the slides in triplicates using a single-channel pipet.

The slides were then incubated in a light protected climate chamber at 80% relative humidity at 40 °C for 15 minutes. After the incubation the slides were thoroughly rinsed with washing buffer. Subsequently the slides were washed 3 times each in a jar containing 40 mL of washing buffer.

The wet slides were then washed 3 times in 40 mL of diH_2O and blown dry under a stream of compressed nitrogen.

After drying, the slides were immediately examined in the microarray scanner to keep the decay of the fluorescent dye to a minimum.

The Cy5 laser was used for the measurement. The excitation wavelength was set to 532 nm. The emission wavelength was 580 nm according to a SONY Biosciences protocol. Evaluation was performed via using an imaging software. The metric was the integrated net intensity of the spots.

3.9 Hyborg assay

To back up the data generated via the colorimetric and fluorescence based assays a Hyborg measurement was performed.

For this thesis a simplified and slightly altered version of the Hyborg assay was performed. A surface control (SC) reagent that consisted of a protein conjugate with a fluorescent label was printed onto the surface of plasma treated GLYMO coated Hybcell cores to test for available epoxy functionalities.

The reagent was diluted with spotting buffer 25 fold, 100 fold and 1400 fold respectively before being printed onto the surface of the Hybcell cores via microdispensing. Each spot consisted of around 200 pL.

The metric for the assay is the emitted fluorescence signal. In contrast to the actual diagnostic Hyborg assay were the read-out is the loss in signal due to target molecules competing with the fluorescent protein conjugates, a higher surface density of available amino groups equals a higher signal during this simplified measurement, due to the increased number of sites to which the fluorescent probes can directly bind.

3.10 Contact angle

To further characterize the silane coatings, the contact angle of diH_2O on differently treated and untreated surfaces was measured using the sessile drop technique. This optical method consists of a direct determination of the tangent angle at the three-phase contact point of a static liquid drop on a flat surface, based on the shape of the drop. It is widely used because of its simplicity and the fact that only small amounts of liquid (a few microliters) and small surface substrates (a few square millimeters) are required. [39]

The measurements were performed using a Krüss DSA 30 goniometer. The setup consisted of a horizontal stage for placing the sample, a motor-driven syringe for the droplet formation, an illumination source and a high resolution camera connected to a computer with the appropriate software for analysis of the drop geometry.

After generating a drop with the syringe it is placed upon the sample slide that is placed on the horizontal stage.

For each type of surface 3 drops of 1 μL were placed across the length of a SONY MS-slide.

The angle formed between the liquid/solid interface and the liquid/vapor interface is the contact angle. It was determined using the Drop shape analysis (DSA) software from Krüss.

The camera derived image of the drop was directly transferred to the software tool where in a first step a contour recognition was carried out, based

on a grey-scale analysis of the image. Subsequently a geometrical model describing the drop shape was fitted onto the contour.

The circular segment method that was used in this case assumes that the shape of a sessile drop describes a circular arc on the surface. The software matches the parameters of a circular arc equation to the optically determined drop shape. The contact angle is calculated as the angle between the circular arc and the baseline intersecting it. This method is more suitable for smaller contact angles as the drop shape deviates from the circular arc as the contact angle increases, but can be used for large contact angles as well when high accuracy is not required. [40]

Chapter 4

Results

4.1 Molecular extinction coefficient and linearity

The absorbance values of a linear series of dilutions of Orange II and Coomassie brilliant blue in acidic solution (pH 3) were determined at a wavelength of 485 nm and 620 nm, respectively (table B.1 and B.2). Linearity was determined by the square of the Pearson correlation coefficient (R^2) as can be seen in the following plots (table 4.1 and 4.2).

From the absorbance values, the molar extinction coefficients (ϵ) for the dyes used in the colorimetric assays were determined using the Lambert-Beer law.

Within a concentration range of 0.5 to 10.0 nMol/L the absorbance values for the spectrophotometric measurements of Orange II correlated to the corresponding concentration values in a linear manner (table 4.1).

CBB did not show linear correlation of concentration and absorbance in the most important concentration range of 0.5 to 5 nMol/L and may therefore not be suitable for determining amino group density in the experimental setup of this thesis.

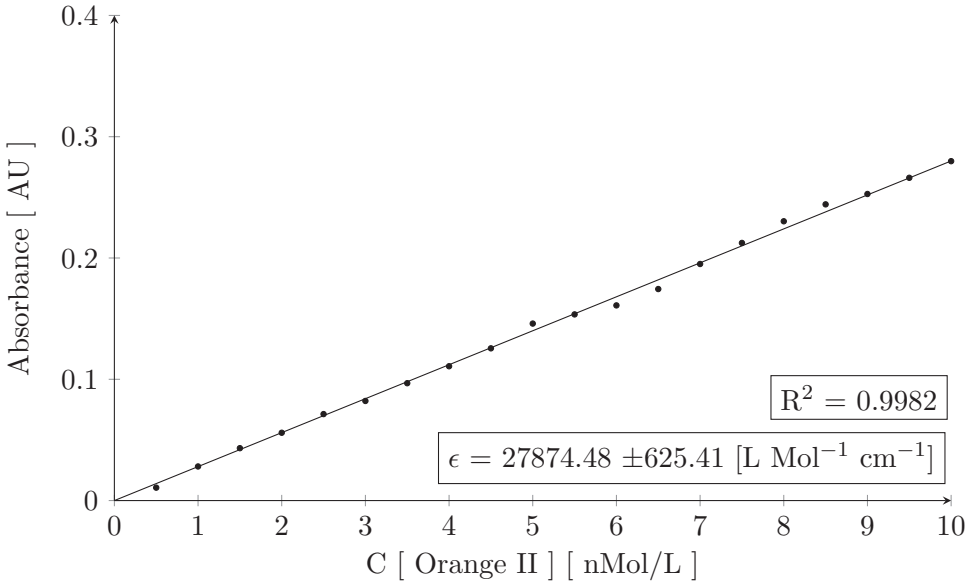

TABLE 4.1: Results Orange II - Linearity of concentration and absorbance

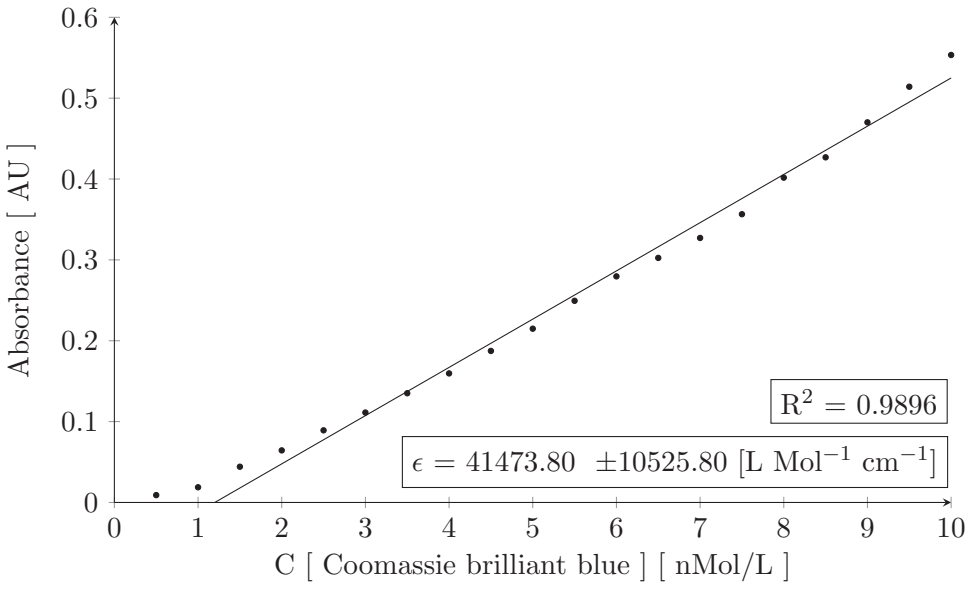

TABLE 4.2: Results Coomassie brilliant blue - Linearity of concentration and absorbance

4.2 Absorbance - pH dependency

In the last step of the Orange II colorimetric assay the pH needs to be adjusted from 12 to 3 before the spectrophotometric measurement. To determine whether this has an undesirable influence on the absorbance values derived from this measurement a comparative experiment was set up. A series of 5 different concentrations were prepared in acidic solution (pH 3) and checked for their absorbance. In a subsequent test a series of the same 5 concentrations were prepared in alkaline solution (pH 12) and then adjusted to a pH of 3 as described in the Orange II assay section. It was then evaluated if the samples with the same concentrations yielded comparable absorbance values despite the differences in sample preparation.

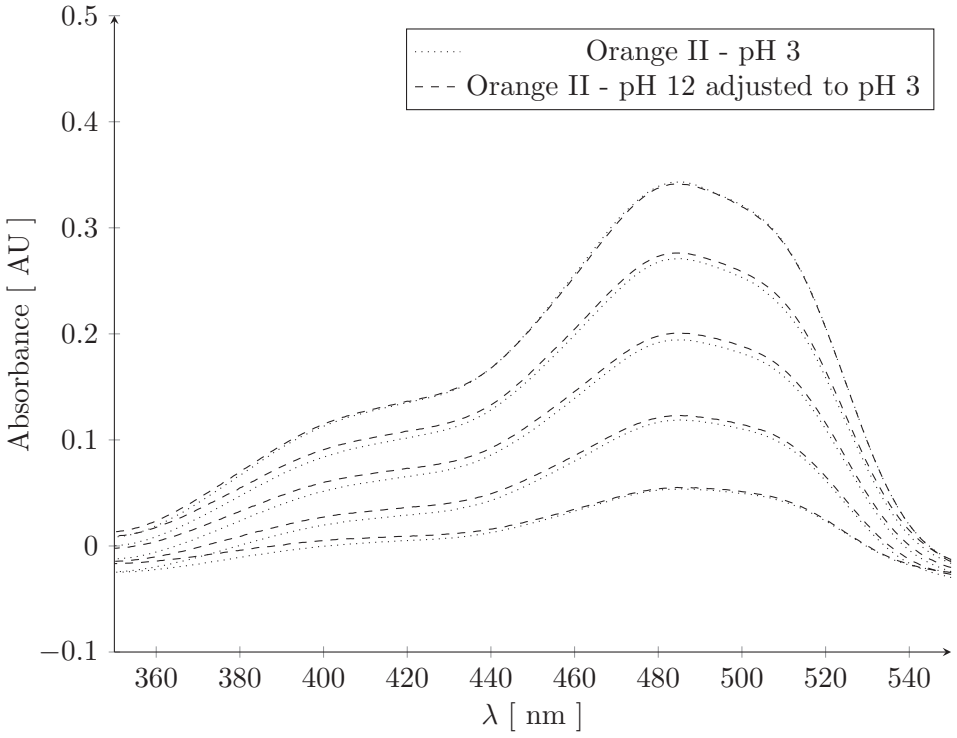

Table 4.3: Results Orange II - pH dependency spectra

The values for the molar extinction coefficient (table 4.4) that were calculated from the collected absorbance values did not show a clear difference.

Concentration [nMol/L]	ϵ at pH 3	ϵ at pH 3 (adjusted)
2.0	26850.09	27505.01
4.5	26380.12	27326.67
7.0	27748.57	28660.11
9.5	28505.26	29069.47
12.0	27770.83	27614.17
X	**27450.93**	**28035.06**
σ	$\pm$838.23	$\pm$777.89

TABLE 4.4: Results Orange II - pH dependency

For Orange II it could be shown that the adjustment of the pH during the assay does not significantly affect the outcome of the spectrophotometric measurement for a given dye concentration.

4.3 Orange II colorimetric assay on slides

All the experiments followed the procedure of the Orange II colorimetric assay on slides as described in section 3.7.3 if not stated differently. The baseline value was always the absorbance of the eluate of the control slide. Raw data of all measurements can be found in Appendix C.

The metric to describe the surface density of functional amino groups in this thesis is the amount of molecules per nm^2. It is determined by the ratio of the substance amount fraction of the dye in the eluate which is directly calculated from the absorbance measurements and the area of the plastic consumable.

Literature values for the surface density of amino groups on functionalized surfaces determined by colorimetric assays are reported to be between 3.6 molecules/nm^2 and 95.7 molecules/nm^2 [16]. Amino group surface desities for vapor deposited silanes are usually around 3 molecules/nm^2 while surface density values of around 0.3 molecules/nm^2 and below are classified as deriving from non-specific interactions. [16, 29, 30]

4.3.1 COP - unsilanized

The slides in this experiment have been Si-sputtered but not coated with APTES silane. The assay was carried out to determine the degree of non-specific binding of the dye to either the walls of the upper structure, the adhesive layer in between the plastic components that may be exposed to the solution, or the slides themselves. Assuming a low degree of non-specific binding the signal during the assay should ideally be indistinguishable from the noise during the measurement. Any detected Orange II has to derive from non-specific binding such as physisorption. The experiment was carried out three times with four slides each.

N=12	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
X	**0.02024**	**7.26441 E-07**	**0.6**
σ	±0.00162	±5.82023 E-08	±0.05

Table 4.5: Results COP - unsilanized - on slides

While the values are a bit higher than what the literature states for unsilanized surfaces the results for COP slides that have not been coated with silane (table 4.5) suggest that non-specific adsorption of Orange II molecules is still low.

4.3.2 COP - APTES

Si-sputtered COP slides that have not undergone any preparative treatment were coated with APTES using the CVD process previously described. They should give a significantly higher signal than unsilanized slides, proving the principle of the Orange II assay. Three sets of four slides have been prepared and tested.

N=12	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
X	**0.05160**	**2.53328 E-06**	**2.0**
σ	±0.00338	±1.68274 E-07	±0.13

Table 4.6: Results COP - APTES - on slides

Comparing the results of silanized and unsilanized slides, one can clearly see that there is a significant increase in the absorbance signal (table 4.6). It was shown that available amino groups deriving from the silanization are a basic requirement for the Orange II assay to work. Being able to clearly distinguish between silanized and unsilanized slides based on the signal renders the Orange II assay a valid tool for detection and quantification of amino functionalities.

4.3.3 COP - Plasma treatment/ APTES

The Si-sputtered COP slides were treated in a plasma chamber under a saturated oxygen atmosphere to regenerate silanol functionalities of the sputter layer as previously described in section 3.5.1. The oxidizing pre-treatment is thought to improve the homogeneity of the surface, to remove remaining organic impurities and to restore chemical reactivity thereby enhancing the quality of the silane layer.

N=12	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
X	**0.09535**	**4.73612 E-06**	**3.7**
σ	±0.01393	±6.92276 E-07	±0.55

Table 4.7: Results COP - Plasma treatment/ APTES - on slides

Comparing the results of the plasma treated slides to the silanized slides that have not undergone preparative treatment one can see that the results for the plasma treated slides are significantly higher (table 4.7). This demonstrates that the oxidative pre-treatment is beneficial when higher surface densities of silane molecules are required.

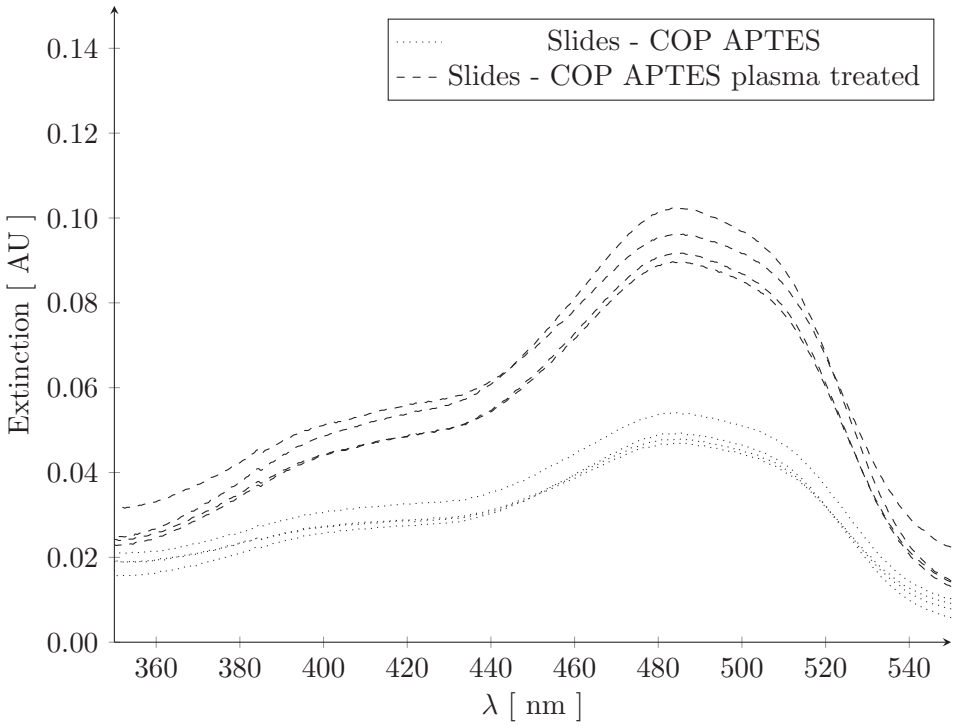

TABLE 4.8: Results - Orange II assay on slides

4.3.4 COP - Piranha treatment/ APTES

As an alternative to the plasma treatment, strongly oxidizing Piranha acid was tested as a preparative agent in order to regenerate the silanol groups on the surface of Si-sputtered slides. The procedure of the Piranha acid preparative treatment was described in section 3.5.2 of this thesis. It is thought to improve the signal in comparison to untreated slides.

After the first trial of the Piranha preparative treatment a visible residue could be observed on the surface of all four slides. Performing the Orange II assay on these slides resulted in an intense orange colored staining in those areas where the residual smear was located (table 4.9). Apparently there were significant amounts of Orange II dye bound. Measuring the eluate of these slides in the UV-vis-spectrophotometer led to extraordinarily high values for the concentration of Orange II. Seemingly the amount of Orange

II dye molecules did not correlate to the actual number of aminosilane functionalities present on the surface. Instead it can be assumed that physical adsorption to the crystalline residue is an explanation for the high values deriving from the assay.

N=4	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
X	**0.3629**	**1.80251 E-05**	**14.4**
σ	±0.05575	±2.76954 E-06	±2.21

TABLE 4.9: Results COP - Piranha treatment/ APTES - on slides

During a second trial of the Piranha preparative treatment on a set of 5 slides the goal was to avoid formation of a smear by performing an additional cleaning step. The cleaning procedure was improved via ultrasonication of the slides subsequent to the regular rinsing process. No more residue was observable after this additional post-treatment. The absorbance values of the Orange II assay were in the expected range (table 4.10). In direct comparison to the plasma treatment the Piranha treatment delivered similar results.

To evaluate the influence of Piranha acid on unsilanized surfaces a set of five slides was treated with Piranha acid and subjected to the Orange II assay to test for non-specific binding of dye molecules induced by the treatment (table 4.11).

N=5	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
X	**0.0976**	**4.84975 E-06**	**3.9**
σ	±0.02459	±1.22170 E-06	±0.97

TABLE 4.10: Results COP - Piranha treatment + ultrasonication/ APTES - on slides

With the exception of one outlier the values for these unsilanized slides were lower than for the APTES silanized ones, but still higher than for unsilanized plain Si-sputtered slides. It is therefore assumed that the Piranha treatment enforces non-specific interaction of Orange II molecules with the underlying surface that do not directly correlate with the presence of amino functionalities.

In addition to the insufficiently significant outcome of the experiment the handling and disposal requirements of Piranha acid are not suitable for industrial scale production. The hazards and the effort are too high to render a pre-treatment with Piranha acid useful.

N=5	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
X	**0.0794**	**3.94477 E-06**	**3.2**
σ	±0.04217	±2.09478 E-06	±1.67

TABLE 4.11: Results COP - Piranha treatment + ultrasonication/ No silane - on slides

4.3.5 COP - GLYMO

Si-sputtered COP slides that have not undergone any preparative treatment were coated with GLYMO epoxysilane using the CVD process previously described. No further chemical modification was performed. Considering the fact that the Orange II assay specifically relies on available amino moieties this test shall give a low signal deriving from non-specific physisorption.

N=5	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
X	**0.01744**	**8.66538 E-07**	**0.7**
σ	±0.00134	±6.70470 E-08	±0.07

TABLE 4.12: Results COP - GLYMO - on slides

The absorbance values for the Orange II assay on GLYMO coated slides (table 4.12) were lower than for APTES coated slides proving the point that the Orange II dye molecules specifically rely on available amino groups for binding. The values were comparable to unsilanized slides.

4.3.6 COP - GLYMO + Ethylenediamine 1:8

Si-sputtered COP slides that have not undergone any preparative treatment were coated with GLYMO epoxysilane using the CVD process previously described. The slides were then treated with an ethylenediamine solution

to introduce an amino group for every available epoxy functionality. This potentially allows the use of the Orange II assay to be used for the evaluation of epoxysilane coated surfaces.

N=4	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
X	**0.0383**	**1.90235 E-06**	**1.5**
σ	±0.0028	±1.41769 E-07	±0.1

Table 4.13: Results COP - GLYMO + Ethylenediamine 1:8 - on slides

The calculated surface density values are higher than for bare GLYMO surfaces (table 4.13). Derivatization with the ethylenediamine solution appears to have introduced available amino groups. Compared to APTES coated slides the values for the surface density of functional groups are lower.

4.3.7 COP - GLYMO + Diaminopropane 1:8

The epoxysilane coated COP slides were treated with a diaminopropane solution prior to the Orange II assay as previously described. Diaminopropane introduces amino functionalities for every available epoxy group in a comparable way as ethylenediamine. The treated slides should therefore yield a significantly higher signal than bare GLYMO coated slides due to the introduced amino groups whose amount should correlate with the number of silane molecules on the surface.

N=4	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
X	**0.11476**	**1.7372 E-06**	**1.4**
σ	±0.0036	±1.81566 E-07	±0.12

Table 4.14: Results COP - GLYMO + Diaminopropane 1:8 - on slides

The amino derivatized epoxysilane coating again yielded higher values than the native GLYMO surface (table 4.14). Comparing the results for diaminopropane with those for ethylenediamine in a 1:8 dilution one can see that they are comparable. In comparison to APTES silanized slides the surface density of GLYMO is again lower, indicating that the number of available epoxy groups in a defined area is lower than for aminosilane.

4.3.8 TPE - APTES

Investigating the suitability of chemical modification of thermoplastic elastomer materials the Kraton 1645 TPE foil was coated with APTES aminosilane and tested for available amino functionalities using the regular Orange II assay. An oxidizing plasma treatment step was carried out in preparation for the chemical vapor deposition of the silane. Considering the TPEs lack of hydroxyl groups the experiments were expected to only give low signals during the tests.

After coating of the thermoplastic elastomer foil with APTES aminosilane the 16-well upper structure was assembled on top of it to enable the Orange II assay.

N=3	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
X	**0.02131**	**1.05846 E-06**	**0.8**
σ	±0.00292	±1.45175 E-07	±0.11

Table 4.15: Results TPE - APTES - on slides

N=1	Extinction	Concentration [Mol/L]	Surface density [molecules/nm^2]
X	**0.02003**	**9.94884E-07**	**0.8**

Table 4.16: Results TPE - untreated - on slides

Silanized TPE foil (table 4.15) yielded signals in the same range as unsilanized TPE foil (table 4.16) during the colorimetric assay. TPE has no native hydroxyl groups on the surface. Considering the fact that silanes rely on available hydroxyl groups to form a covalent bond during the chemical vapor deposition a low signal was expected.

The values for TPE were higher than for unsilanized COP which leads to the conclusion that there is a certain degree of non-specific binding of Orange II to the TPE surface. This level of physical adsorption is still low.

4.4 Orange II colorimetric assay on cores

The Hybcell cores were tested for compatibility with the Orange II assay just like the slides with the attached SONY 16-well upper structures. Comparable experiments were carried out to confirm the feasibility. All the experiments followed the procedure of the Orange II colorimetric assay on cores as described in the section 3.7.4, if not stated differently. The baseline value was always the absorbance of the eluate of the control core.

4.4.1 COP - unsilanized

The Si-sputtered cores in this experiment have not been coated with APTES silane. They were used to determine the degree of non-specific binding of the dye to the outside and inside walls of the core. Assuming a low degree of non-specific binding the signal deriving from the assay should ideally be indistinguishable from the noise of the measurement. Any detected Orange II has to derive from non-specific binding like physisorption.

N=18	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
X	**0.02301**	**1.14323 E-06**	**0.5**
σ	±0.00318	±1.57960 E-07	±0.07

Table 4.17: Results COP - unsilanized - on cores

The values for unsilanized cores (table 4.17) are comparable to the results for unsilanized slides. The amount of Orange II molecules bound to the Si-sputtered surface is negiable.

4.4.2 COP - APTES

Si-sputtered COP cores that have not undergone any preparative treatment were coated with APTES using the CVD process previously described. They should give a significantly higher signal than the unsilanized cores.

N=18	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
X	**0.11425**	**5.67518 E-06**	**2.7**
σ	±0.01425	±7.07892 E-07	±0.33

TABLE 4.18: Results COP - APTES - on cores

The increase in signal between the untreated cores and the ones treated with APTES aminosilane (table 4.18) clearly indicates that the cores are suitable to be used in an Orange II assay. The values are comparable to the respective measurements that were performed using the SONY MS-slides.

4.4.3 COP - Plasma treatment/ APTES

The COP cores were treated in a plasma chamber under a saturated oxygen atmosphere to regenerate silanol functionalities of the sputter layer as previously described in section 3.5.1. The oxidizing pre-treatment was used to remove impurities from the surface of the polymer and to regenerate the silanol groups of the sputter layer.

N=18	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
X	**0.18602**	**9.23978 E-06**	**4.4**
σ	±0.00927	±4.60790 E-07	±0.22

TABLE 4.19: Results COP - Plasma treatment/ APTES - on cores

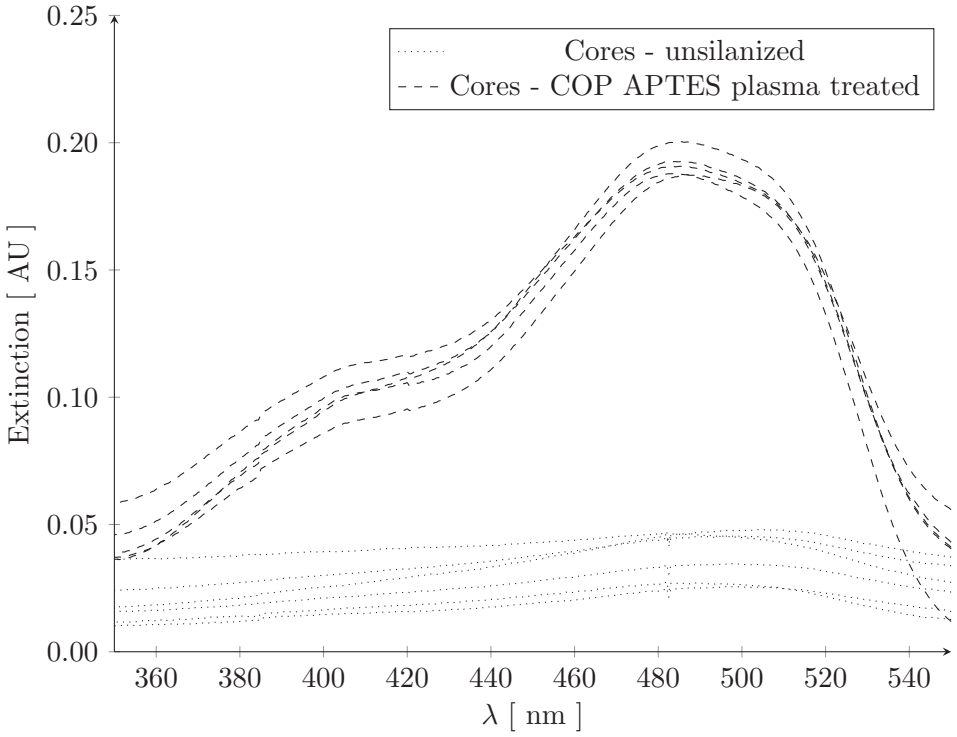

TABLE 4.20: Results Orange II - Assay on cores

One can clearly see that the values for plasma treated cores (table 4.19) are significantly higher than for the cores that have not undergone an oxidizing pre-treatment. To achieve silane layers with a higher surface density plasma treatment is recommended.

4.4.4 COP - GLYMO

Si-sputtered COP cores that have undergone a plasma treatment were coated with GLYMO epoxysilane using the CVD process previously described. No further chemical modification was performed. Considering the fact that the Orange II assay specifically relies on available amino moieties that are not present when using GLYMO this test shall only give a low signal deriving from non-specific physisorption.

N=4	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
X	**0.02630**	**1.30669 E-06**	**0.6**
σ	±0.00492	±2.44449 E-07	±0.11

TABLE 4.21: Results COP - Plasma treatment/ GLYMO - on cores

The surface density of silane molecules on pristine GLYMO (table 4.21) is lower than for an APTES layer, again confirming that the Orange II assay does only work for primary amines. The values are comparable to unsilanized cores determining that non-specific adsorption is low.

4.4.5 COP - GLYMO + Ethylenediamine 1:8

The Si-sputtered COP cores were plasma treated and coated with GLYMO epoxy silane before derivatization with an ethylenediamine solution as previously described in section 3.6.3 to enable an evaluation of the silane surface density via the Orange II assay.

N=18	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
X	**0.07839**	**4.01785 E-06**	**1.9**
σ	±0.00507	±2.52100 E-07	±0.12

TABLE 4.22: Results COP - GLYMO + Ethylenediamine 1:8 - on cores

The results for the surface density of functional groups (table 4.22) is significantly higher than for GLYMO surfaces without the amination of the epoxy groups suggesting that the procedure can be used to enable the Orange II assay for the evaluation of GLYMO coated surfaces. The assay delivers comparable results to the corresponding experiment on derivatized MS-slides.

4.4.6 COP - GLYMO + Diaminopropane 1:8

The Si-sputtered COP cores that have undergone a plasma treatment and the deposition of GLYMO were derivatized using a diaminopropane solution as previously described prior to Orange II assay.

N=18	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
X	**0.07473**	**3.71193 E-06**	**1.8**
σ	±0.01010	±5.01883 E-07	±0.24

TABLE 4.23: Results COP - GLYMO + Diaminopropane 1:8 - on cores

The values for the surface density of functional groups deriving from the tests (table 4.23) are comparable to the results using an ethylenediamine solution of the same concentration.

4.4.7 COP - GLYMO + Ethylenediamine 1:80

There is the possibility that the treatment with a diamine solution introduces amino groups not corresponding to an available epoxy group via sticking of diamine molecules to the surface in a non-specific manner like physisorption, thereby yielding a false positive signal.

To evaluate this risk the Si-sputtered COP cores that have undergone plasma treatment and GLYMO silanization were treated with an ethylenediamine solution as previously described prior to the Orange II assay. The concentration was lowered to avoid potential false positive results due to physisorption.

N=18	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
X	**0.07661**	**3.80547 E-06**	**1.8**
σ	±0.00694	±3.44775 E-07	±0.16

TABLE 4.24: Results COP - GLYMO + Ethylenediamine 1:80 - on cores

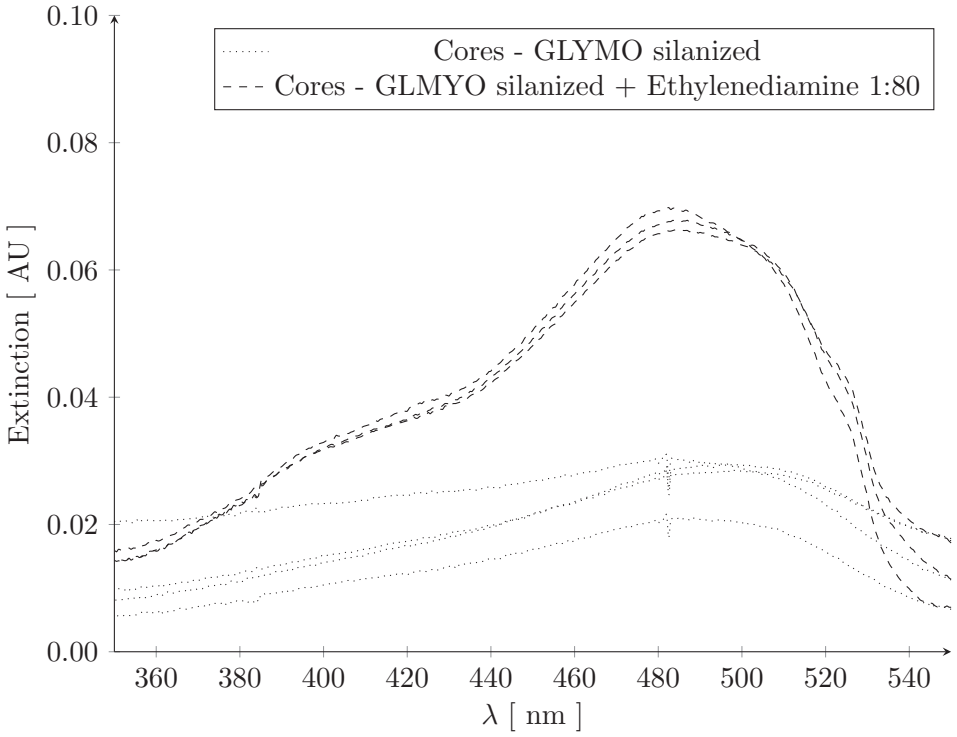

TABLE 4.25: Results Orange II - Assay on ethylenediamine 1:80 derivatized cores

The results (table 4.24 - 4.25) were similar to the outcome of the previous tests with higher concentrations of the ethylenediamine solution. This supports our conclusion that the same amount of available epoxy groups were aminated regrdless of the concentration. Higher concentrated ethylenediamine solution does not introduce additonal non-related amino groups available during the Orange II assay.

4.4.8 COP - GLYMO + Diaminopropane 1:80

The COP cores were treated with a diaminopropane solution as previously described prior to Orange II assay.

Comparable to the experiments with ethylenediamine the diaminopropane treatment with the lower concetrated solution (table 4.26) yielded similar

N=18	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
X	**0.07766**	**3.85768 E-06**	**1.8**
σ	±0.00869	±4.31906 E-07	±0.20

TABLE 4.26: Results COP - GLYMO + Diaminopropane 1:80 - on cores

results compared to the treatment with the higher concentrated solution, again suggesting that a 1:80 diluted diamine solution is sufficient for the amination of available epoxy groups.

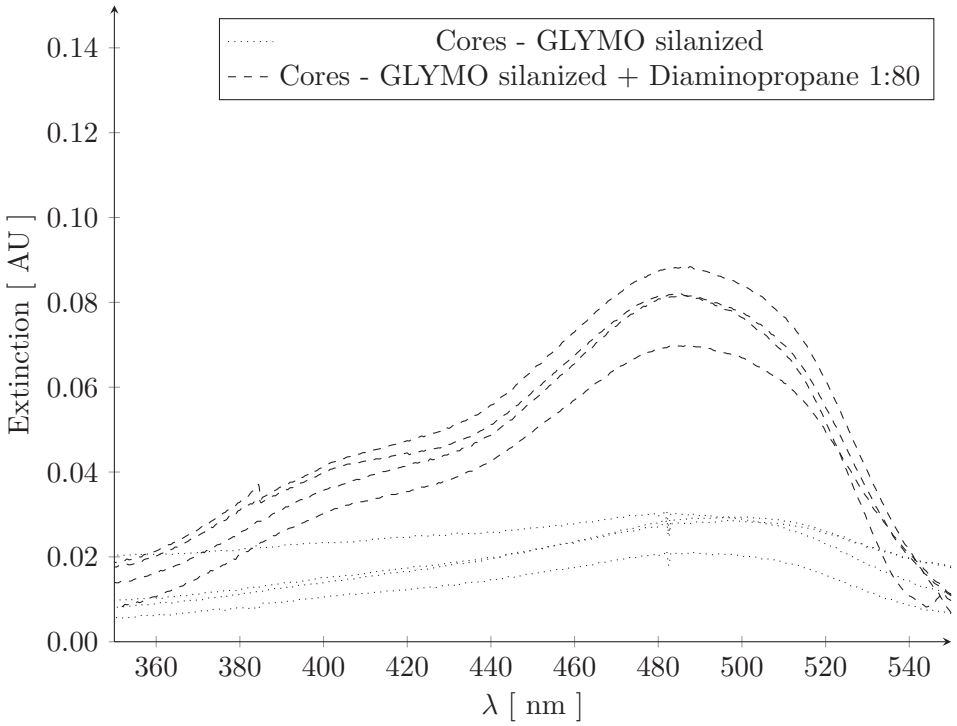

TABLE 4.27: Results Orange II - Assay on diaminopropane 1:80 derivatized cores

4.4.9 COP - unsilanized + Diaminopropane 1:80

To investigate the effect of a diamine solution on pristine Si-sputtered COP surfaces, cores that have not been silanized were treated with a diluted diaminopropane solution as previously described. The Orange II assay

was performed to see if diamine molecules attach to the surface in a non-specific way generating a false positive signal without the presence of silane molecules. The decision was made to use diaminopropane for these tests because of its lower reactivity.

N=18	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
X	**0.01438**	**7.14637 E-06**	**0.3**
σ	±0.00401	±1.99320 E-07	±0.09

TABLE 4.28: Results COP - unsilanized + Diaminopropane 1:80 - on cores

According to the measurements (table 4.28) the adsorption of diamine molecules to surfaces without epoxy functionalities is negliable. The derivatization of epoxysilane coated surfaces with a 1:80 diluted diaminopropane solution seems to be a suitable tool to enable the Orange II assay without generating false positive results.

4.4.10 COP - unsilanized + Diaminopropane 1:8

The effect of a diaminopropane treatment on a bare Si-sputtered COP surface was repeated with a diaminopropane solution of higher concentration. Again the Orange II assay was performed to determine the amount of available amino groups present on the surface due to the treatment.

N=18	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
X	**0.04687**	**2.32829 E-06**	**1.1**
σ	±0.00373	±1.85626 E-07	±0.09

TABLE 4.29: Results COP - unsilanized + Diaminopropane 1:8 - on cores

According to the measurements (table 4.29) adsorption of diamine molecules to surfaces without epoxy functionalities does occur for diamine solutions of higher concentration. Using a diaminopropane solution that has been diluted 1:8 yielded values of around 1.1 functional groups per nm^2 falsely

suggesting the presence of derivatized epoxy groups on the unsilanized substrates. It can be concluded that diamine solutions above a certain concentration threshold generate false positive results due to molecules attaching non-specifically to the surface.

4.5 Coomassie brilliant blue colorimetric assay on slides

All the experiments followed the procedure of the Coomassie brilliant blue colorimetric assay on slides as described in the section 3.7.5.

Similiar to the Orange II assay the metric of the measurements is the surface density of amino groups. The absolute amount of molecules per nm^2, which is represented by the ratio of the substance amount fraction of the CBB dye in the eluate (directly calculated from the extinction measurement) and the functional area of the plastic consumable.

4.5.1 COP - unsilanized

The Si-sputtered slides in this experiment have not been coated with APTES aminosilane. They were used to determine the degree of non-specific binding of CBB to the surface of the slides or the 16-well upper structure. The values deriving from the assay were assumed to be low.

The absorbance values were compared to the results of the linearity measurement of CBB. The molar extinction coefficient of 18171 was chosen accordingly.

N=12	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
X	**0.00665**	**3.66105 E-07**	**0.3**
σ	±0.00117	±6.47391 E-08	±0.06

Table 4.30: Results COP - unsilanized - on slides

The CBB assay on unsilanized Si-sputtered slides yielded no significant signal (table 4.30). Non-specific adsorption of CBB to the surface is very low.

4.5.2 COP - APTES

Si-sputtered COP slides that have not undergone any preparative treatment were coated with APTES using the CVD process previously described. They were expected to give a significantly higher signal than the uncoated slides deriving from the CBB assay.

N=12	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
X	**0.06339**	**N/A**	**N/A**
σ	$\pm$0.03789	$\pm$N/A	$\pm$N/A

Table 4.31: Results COP - APTES - on slides

The highly heterogeneous results for the CBB assay on slides (table 4.31) made it impossible to assign a definite molar extinction coefficient from the linearity measurements. It was therefore not possible to calculate the concentration in the eluate. Values for the surface density of amino groups could not be determined. The high variability of the values deriving from different slides expose fundamental issues of the assay. It was therefore decided not to proceed with further experiments for the CBB assay.

4.6 Fluorescence based assay

For the fluorescence based assay TAMRA-NHS and TAMRA Cadaverine were spotted in 4 different concentrations onto APTES and GLYMO coated slides respectively according to section 3.8. Spotting buffer (PBS) without any fluorescent dye was used as a control. The raw data deriving from the measurements can be found in Appendix A.

In accordance to the QC guidelines of SONY Biosciences the threshold for acceptable quality of vapor deposited APTES coatings is an integrated density of optical units of around 30.000 RFU.

The coefficient of variation (CV) needs to be under 10% between multiple slides.

$$CV = \frac{\sigma}{X} \tag{4.1}$$

4.6.1 TAMRA-NHS

The net intensity of the fluorescence signal of TAMRA-NHS was determined after washing according to the protocol described in section 3.8. The average net intensity within each slide has been determined as well as the average over all slides for each concentration of fluorescent dye.

It was observed that an increase in concentration of the fluorescent dye resulted in higher values for the fluorescence signal (table 4.32). The values reach a plateau when using 50 μM of TAMRA-NHS dye. It is therefore concluded that for a concentration of 50 μM all available amino groups are occupied by a dye molecule.

For all the different concentrations one can observe a loss in signal from slide to slide which equals a loss in signal over time. The time for the scanning of 6 slides with the required resolution takes around 1 hour. The critical threshold of 30.000 RFU can be reached for the first slides, confirming that the overall quality of the deposited layer is sufficient, but drops below this

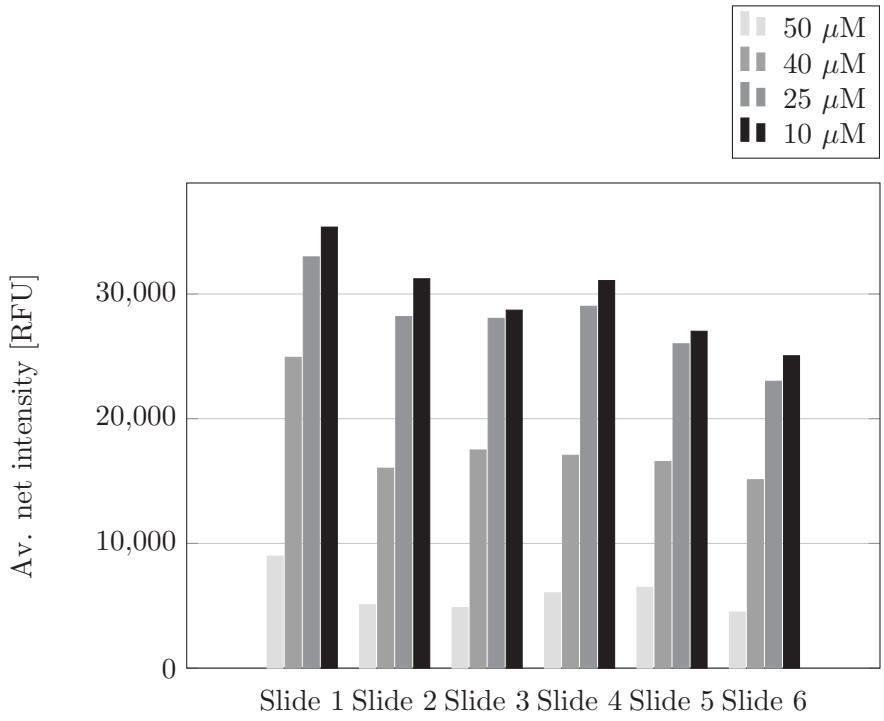

TABLE 4.32: Results APTES - Tamra-NHS

value for half of the slides. This time-dependent drop in signal disqualifies the TAMRA-NHS assay as a reliable QC routine.

4.6.2 TAMRA Cadaverine

The net intensity of the fluorescence signal of TAMRA Cadaverine was determined after washing according to the protocol described in section 3.8. Again the average net intensity within each slide was determined as well as the average over all slides for each concentration of fluorescent dye. The raw data can be found in Appendix A.

In direct comparison to the results deriving from TAMRA-NHS on APTES it could be observed that for TAMRA Cadaverine the plateau in signal was already reached for lower concentrations (table 4.33), confirming that the surface density of GLYMO is lower than for APTES.

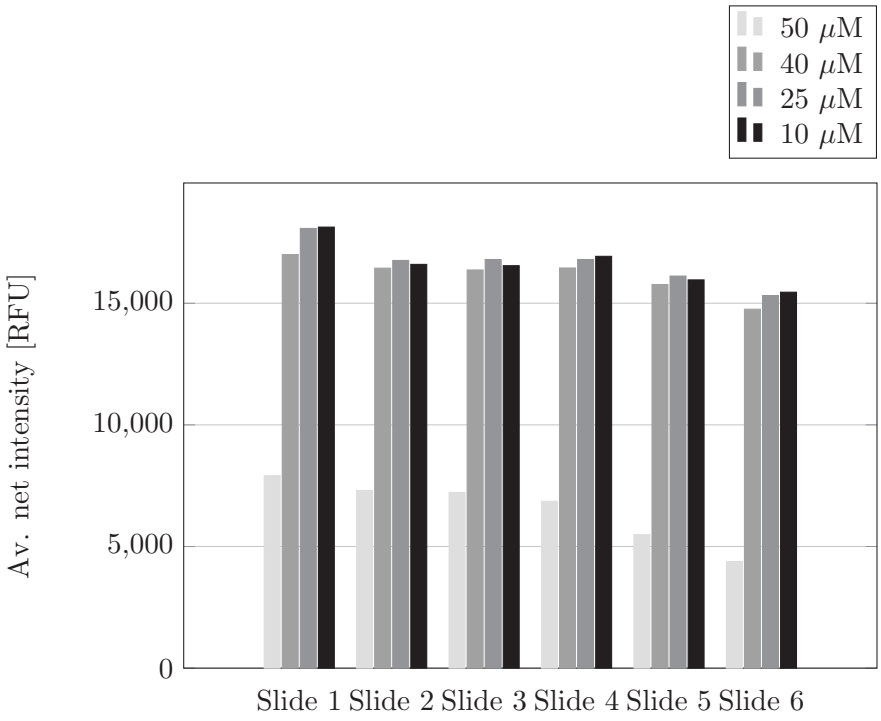

TABLE 4.33: Results GLYMO - Tamra-Cadaverine

Again for all the different concentrations one can observe a loss in signal over time. The time dependency of the signal also disqualifies the TAMRA Cadaverine assay as a reliable QC routine.

4.7 Contact angle

The contact angle was measured on MS-slides (table 4.34) with a goniometer using the sessile drop method according to section 3.10. Pictures can be found in Appendix A.

Pristine COP surfaces yielded a contact angle of 97.5 ° which has been expected given the hydrophobic nature of the material. The contact angle was significantly decreased via a Si-sputter layer. Values for the contact angle of sputtered slides ranged between 12.4 ° and 18.4 °.

A plasma treatment on either pristine COP or Si-sputtered COP decreased the contact angle to an extent that it was not possible to measure it with the given setup.

Performing APTES silanization on Si-sputtered slides resulted in a contact angle of 57.1 ° to 57.8 ° which clearly indicates the presence of aminosilane.

Sample	Treatment	Θ 1	Θ 2	Θ 3	Θ X
1	COP	97.8	97.1	97.6	97.5
2	COP	97.8	97.3	97.6	97.5
3	COP	97.7	98.1	97.8	97.8
1	COP + Plasma	x	x	x	x
2	COP + Plasma	x	x	x	x
3	COP + Plasma	x	x	x	x
1	COP + SiO_2	19.2	17.7	18.3	18.4
2	COP + SiO_2	11.4	13.6	12.4	12.4
3	COP + SiO_2	15.3	14.9	15.8	15.3
1	COP + SiO_2 + Plasma	x	x	x	x
2	COP + SiO_2 + Plasma	x	x	x	x
3	COP + SiO_2 + Plasma	x	x	x	x
1	COP + APTES	59.0	57.6	57.0	57.8
2	COP + APTES	55.2	57.5	60.1	57.6
3	COP + APTES	60.3	54.9	56.3	57.1
1	COP + SiO_2 + APTES	53.1	53.3	57.0	54.4
2	COP + SiO_2 + APTES	54.5	53.8	53.6	53.9
3	COP + SiO_2 + APTES	52.8	53.1	53.4	53.1
1	COP + GLYMO	50.1	49.7	48.1	49.3
2	COP + GLYMO	49.6	49.5	49.6	49.5
3	COP + GLYMO	49.5	48.7	49.7	49.3
1	COP + SiO_2 + GLYMO	30.8	34.7	33.5	33.0
2	COP + SiO_2 + GLYMO	31.7	29.4	33.5	31.5
3	COP + SiO_2 + GLYMO	31.7	31.1	33.18	31.9
1	COP + SiO_2 + GLYMO + Ethylenediamine 1:80	52.0	54.3	54.3	53.5
2	COP + SiO_2 + GLYMO + Ethylenediamine 1:80	53.1	50.6	52.3	52.0
3	COP + SiO_2 + GLYMO + Ethylenediamine 1:80	48.5	48.4	49.2	48.7
1	COP + SiO_2 + GLYMO + Diaminopropane 1:80	50.8	49.7	48.6	49.7
2	COP + SiO_2 + GLYMO + Diaminopropane 1:80	49.8	45.9	43.7	46.4
3	COP + SiO_2 + GLYMO + Diaminopropane 1:80	50.2	44.6	50.1	48.3

TABLE 4.34: Results Contact angle measurements on slides

Performing the same silanization procedure on non-sputtered COP yields comparable values for the contact angle of 53.1 ° to 54.4 ° suggesting that physiosorbed APTES silane is present on the surface.

For Si-sputtered COP surfaces silanized with GLYMO epoxysilane the contact angle was measured to be 31.9 ° to 33.0 ° while the same procedure resulted in 49.3 ° to 49.5 ° for slides without the sputter layer. This difference in contact angle that could not be observed during the comparable experiments with APTES aminosilane suggests that the underlying Si-sputter layer has a stronger influence on the contact angle of GLYMO silanized surfaces than it has on APTES coated surfaces.

Performing amination on Si-sputtered GLYMO silanized surfaces with ethylenediamine solution (1:80) according to section 3.6.3 lead to values for the contact angle of 48.7 ° to 53.5 °. For the amination procedure using diluted diaminopropane (1:80) the values range from 46.4 ° to 49.7 °.

The results for diaminopropane as well as for ethylenediamene treated GLYMO surfaces are comparable to the results of APTES on Si-sputtered slides which indicates that the amination procedure is working properly.

The contact angle measurements yielded distinctive results for differently treated surfaces. The contact angle can be used to differentiate between the different surface modifications and can be used for qualitative characterization of silanized slides.

4.8 Hyborg assay

The Hyborg assay was performed on GLYMO silanized Hybcell cores to qualitatively support the suitability of the Orange II colorimetric assay for the representative evaluation of high performance silane coatings.

The results for the Hyborg measurements were provided by Kristina Enzinger. For all three relevant dilutions the fluorescence signal deriving from the surface control reagents was above the required threshold (table 4.35).

All the cores fulfilled the requirement of being functionalized with a high performance epoxysilane layer.

Core	1:25 [RFU]	1:100 [RFU]	1:1400 [RFU]
1	60572	29856	7204
	60695	27489	6526
	60657	25207	5797
2	60649	22117	5131
	60126	19701	4565
	57583	16814	3938
3	54101	14681	3560
	61259	28743	6622
	60572	29856	7204
4	60695	27489	6526
	60657	25207	5797
	60649	22117	5131
5	60126	19701	4565
	57583	16814	3938
	54101	14681	3560
6	61259	28743	6622
	60572	29856	7204
	60784	24340	5569
X	**59520**	**23474**	**5522**
σ	**2294**	**5542**	**1321**
	PASSED	**PASSED**	**PASSED**

TABLE 4.35: Results COP - GLYMO + Surface control reagent - on cores

Chapter 5

Discussion

5.1 Orange II vs Coomassie brilliant blue

Initial spectrophotometric measurements of Orange II dye in acidic solution showed that it can be specifically detected and that the correlation between concentration and absorbance signal is linear in the relevant concentration range.

In an additional experiment it was determined that adjusting the pH of an Orange II solution from pH 12 to pH 3 does yield similar results as the Orange II solution that was directly prepared in pH 3. It can be concluded that the results deriving from the Orange II colorimetric assay are not affected by the adjustment of the pH in the last step of the protocol.

First results deriving from the Orange II assay on unsilanized Si-sputtered COP slides did not result in a signal that can be significantly distinguished from the noise during the spectrophotometric measurement, indicating that there is only a very low degree of non-specific binding of the dye to the unsilanized surface, but no specific interaction of the dye with the surface.

For APTES silanized surfaces on SONY MS-slides as well as on Hybcell cores the assay yielded significant values. It can be concluded that the Orange

II assay is a suitable tool for the quantification of amino groups on amino silanized substrates.

The specificity of the interaction of the Orange II dye with amino moieties was investigated via performing the Orange II assay on unsilanized as well as on epoxy silanized slides. Low values for the calculated surface density determined that for surfaces without amino groups the Orange II assay does only yield non-specific values deriving from physisorption.

According to the linearity measurements, the absorbance of Coomassie brilliant blue does not correlate linearly with the concentration in the entire range.

For APTES silanized slides the variability of the results of the CBB assay was so high that a molar extinction coefficient could not be definitely assigned in order to calculate the surface density of amino groups. The high variability of the results indicate that the assay has inherent problems rendering it unfeasible.

While the CBB assay has the advantage of a short incubation time of only 5 minutes, the non-linear behavior of concentration and absorbance during the spectrophotometric measurement and the high variability renders it useless as a tool for quality control. The toxicity of the methanol as an integral component of the wash solution would be another major hurdle for the implementation in the routine laboratory due to regulatory issues.

Based on the limitations of the CBB assay and the satisfying performance of the Orange II assay it was decided to choose the Orange II assay for establishing a standard QC-routine.

5.2 Slides VS Cores

The evaluation of vapor deposited silane coatings via the Orange II colorimetric assay was performed with the combination of SONY MS-slides and SONY 16-well upper structures, as well as with Hybcell cores. It could be

shown that both devices were compatible with the assay. In both cases there was a significant difference in the results deriving from the Orange II assay between the silanized and the unsilanized state when using APTES, while the estimated surface density of amino groups was comparable.

Due to the different ratio of elution volume and surface area, the cores yield a higher absorbance signal during the spectrophotometric assay when analyzing coatings with the same surface density of functional molecules. This is beneficial for the stability of the measurement.

Sony Biosciences plans to use one of the devices as a model system to monitor silanization processes in general. Placing such a model device in the same silanization chamber as other devices that are not compatible with the colorimetric assay themselves allows for a convenient way to evaluate the silanization performance from batch to batch. This seems to be feasible given that slides as well as cores yielded comparable results for the surface density when treated via the same silanization procedure.

The decision which of the two available devices is more suitable for routine quality control measurements mainly depends on an operational perspective. Attaching the upper structure to the MS-slide is a purely manual procedure and bears the risk that the user misaligns the two parts, or applies too little pressure. An inappropriately attached upper structure is prone to leakage which renders the whole subsequent assay useless. Even if assembled correctly, using the 16-well upper structure involves many pipetting steps during the washing procedure of the colorimetric assay. The risk of propagating contamination in the process is very high, possibly leading to distorted results during the spectrophotometric analysis. Inadequate pipetting during the assay while using the Hybcell cores may as well lead to contamination of the eluate, but the number of steps is significantly lower. In contrast to the procedure with the upper structure, no major assembly steps are required when using the Hybcell cores. Taking into consideration that the assay works properly on both devices, the cores are clearly to be favored as a model system for routine quality control.

5.3 Oxidizing agents

Oxidizing treatments are thought to improve the homogeneity of silane coatings via removal of impurities remaining on the surface and via regenerating the silanol groups on Si-sputtered substrates required for silanization.

Both for SONY MS-slides as well as for Hybcell cores it was shown that an oxygen plasma treatment increases the surface density of APTES aminosilane molecules. While the quality of vapor deposited silane layers may vary for plain Si-sputtered surfaces of different batches as impurities accumulate over time, the oxidizing treatment compensates for those differences via creating a uniform surface, independent of the age of the substrate.

For reproducible deposition of silane layers on substrates from different production runs with a high surface density it is advised to include the oxygen plasma treatment as a preparative process step.

An oxidizing treatment of Si-sputtered slides with Piranha acid yielded similar results. The time-consuming wet chemical process that requires rinsing of the slides before and after the treatment as well as an additional cleaning step in form of ultrasonication is not feasible in an industrial environment. Considering the effort while treating the slides with the Piranha acid, the risks involved while handling concentrated sulfuric acid and hydrogen peroxide and the challenge of proper disposal of the solution, it is clear that such a procedure is not suitable for the manufacturing of devices on an industrial scale.

5.4 Amination of epoxysilane coatings

Feasibility of amination of epoxy functional groups via diamines was investigated to enable the use of the Orange II assay for the evaluation of GLYMO coated surfaces. Diamines like ethylenediamine and diaminopropane are thought to aminate the surface via the reaction of one amino group with

the epoxy-functionality of the GLYMO silane. This is a reaction mechanism that is applied regularly for the cross-linking of epoxides. In this case though the second amino group of the diamine molecule remains free and is available for subsequent amine based QC routines.

For diaminopropane as well as for ethylenediamine solutions of both tested dilutions (1:8 and 1:80) it was observed that during the Orange II assay a clear signal could be observed indicating the presence of amino groups. The detected amino group surface density was in the same range regardless of the type of diamine and the concentration. Overall the surface density of silane functional groups was found to be lower than for APTES coated surfaces. This could have several reasons: GLYMO may have a larger footprint on the surface which reduces the amount of molecules within a defined area compared to APTES, the epoxy group may react before it can be aminated via a diamine molecule or because the amination reaction is incomplete and a certain amount of epoxy molecules remains undetectable by Orange II molecules.

It was further investigated if diaminopropane does adhere non-specifically to unsilanized Si-sputtered surfaces: Diaminopropane that was diluted with ethanol 1:80 does not yield a significant signal on bare COP slides indicating that non-specific adsorption of diaminopropane is negliable for low concentrations. Treating an unsilanized slide with a diaminoproane solution diluted 1:8 in ethanol leads to a clear signal during the colorimetric assay, which is significantly higher than for bare GLYMO coated surfaces. It can be therefore concluded that for concentrations of diaminopropane above a certain threshold non-specific adsorption of diamine molecules to the surface occurs even without the presence of epoxysilane.

It was therefore decided to perform the amination procedure with diamine solutions of lower concentrations. When using a diluted solution of 1:80 a similar amount of available epoxy molecules is derivatized while there is no risk of non-specifically introducing amino groups.

Deciding between ethylenediamine and diaminopropane the choice of diaminopropane was made. While both reagents yield comparable results

during the assay diaminopropane is less hazardous than ethylenediamine and therefore more suitable for QC routines in an industrial environment.

5.5 TPE

It was investigated if silanization was a suitable process for the functionalization of thermoplastic elastomer foils. The lack of available silanol groups was expected to directly translate into very low values for the surface density suggesting that silane molecules do not chemically attach to the surface during the vapor deposition. Molecules that may have attached due to physisorption were washed off during the rinsing steps of the assay, resulting in non-significant amounts of Orange II molecules remaining on the surface during the elution step. Analyzing the eluate in the spectrophotometer supported this assumption.

Overall the amount of silane molecules attached to the surface was too low, while the physisorbed molecules would most likely not show the expected and desired reactivity. Silanization is therefore not suitable for the functionalization of native TPE foils.

5.6 Fluorescence based assay

The fluorescence based assay only allows for a semi-quantitative judgement of the deposited silane layer. It indicates the presence of the silane functional group, but without having a point of reference it is not possible to determine the surface density of the silane molecules.

The assay can only be performed on SONY MS-slides due to the fluorescence scanner only being able to process microscope slides. While it might be feasible to perform the measurement on the MS-slide as a reference part undergoing the same silanization procedure as other consumables, the autofluorescence of the material strongly depends on the injection molding parameters which can vary significantly for different injection molded parts.

The baseline signal for the auto-fluorescence is therefore not comparable which might have an unfavorable impact on the outcome of the experiment.

The most critical drawback of the method is the time dependent loss in signal over time that was observed for TAMRA-NHS as well as for TAMRA Cadaverine which renders the procedure inadequate for an industrial environment where resources are shared and where spotting of the reagents and the read-out might not always happen right after each other.

Although it was not possible to determine the absolute surface density of functional groups, it was clearly observed that the surface density for GLYMO was lower relative to APTES, supporting the results from the colorimetric assay.

5.7 Contact angle

It was determined that the results deriving from the contact angle measurements clearly vary depending on the surface treatment. Contact angle measurements can be used for qualitative judgement of the success of these methods.

As expected the plasma treatment as well as the silicon sputtering significantly increased the hydrophilicity of the naturally hydrophobic COP.

While GLYMO silanized surfaces could be clearly distinguished from APTES silanized surfaces regarding the contact angle, GLYMO silanized surfaces that have been aminated via incubation in a diamine solution behave just like native APTES layers. This was observed for diaminopropane as well as for ethylenediamine. These results support the hypothesis that amination of epoxysilane layers with a diamine solution is a valid procedure to enable amine based QC-routines on epoxy silanized surfaces.

5.8 Hyborg assay

The results from the Hyborg assay determined that chemical vapor deposition of GLYMO epoxysilane according to the protocol of SONY Biosciences creates a fully functional surface for antibody immobilization. The judgement of the quality of the layer based on the results of the colorimetric assay is representative.

Chapter 6

Conclusion and outlook

According to the relevant literature, Orange II is the most appropriate dye for quantifying the extent of amine grafting via colorimetry in a reliable and specific way. Comparing the overall performance of the Orange II assay to the unreliability of the adsorption of CBB to amino groups this could be confirmed. Based on the experiments presented in this thesis, CBB could be definitely excluded from the pool of options for establishing a reliable QC protocol, while the Orange II assay appeared to be a very suitable tool.

To really confirm the performance of the assay regarding the detection of available amino groups, more sophisticated methods like XPS or atomic force microscopy need to be employed, especially for the analysis of aminated epoxy silanized surfaces. From the Orange II assay alone it could only be concluded that aminated epoxysilane layers have a lower surface density of available amino groups, which could be supported by the fluorescence based assay as well as the contact angle measurements. The reason is still unclear and needs to be further investigated.

Even though some questions remain unanswered at the moment, confidence in the Orange II assay is high enough to implement it as a quality control tool. The Hybcell cores will be implemented as a model device to evaluate silanization processes for other consumables or even in form of a DOE

assessing CVD process parameters like chamber pressure, temperature, incubation time and annealing time to further improve the procedure. The correlation with functional parameters will still be required mid-term, but in the long run the Orange II assay might be able to completely replace time consuming functional QC assays for batch to batch testing.

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Appendix A

Process flow chart

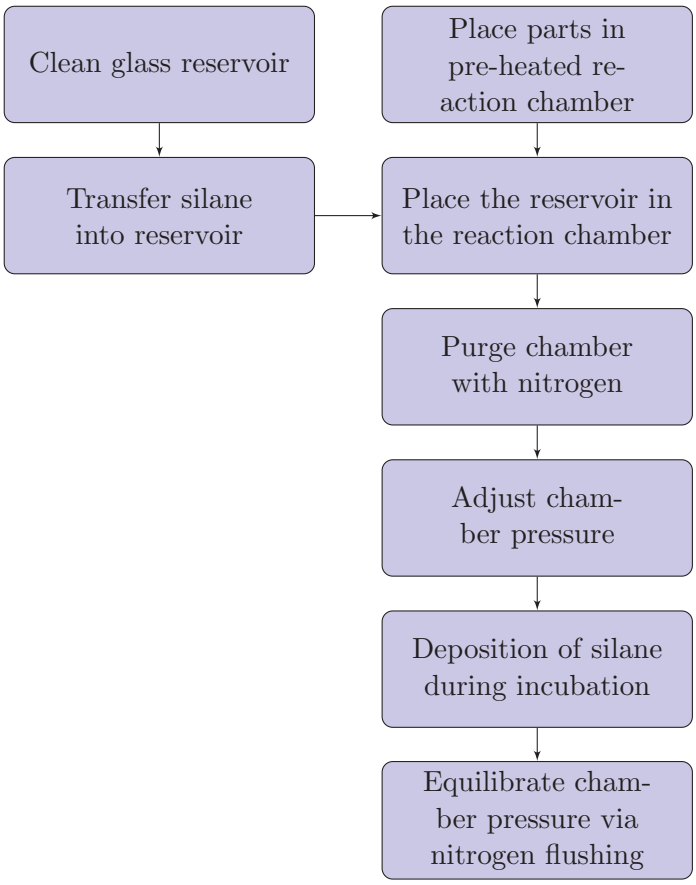

FIGURE A.1: Flow chart - Silane chemical vapor deposition

Appendix B

Extinction coefficient

Concentration [nMol/L]	Absorbance [AU]	ϵ [L Mol^{-1} cm^{-1}]
0.5	0.01065	26652.74
1.0	0.02812	28119.84
1.5	0.04320	27022.08
2.0	0.05592	27958.58
2.5	0.07130	28519.84
3.0	0.08213	29358.01
3.5	0.09677	27647.65
4.0	0.11075	27712.62
4.5	0.12552	27892.36
5.0	0.14593	28089.04
5.5	0.15350	27908.58
6.0	0.16092	26819.41
6.5	0.17440	27275.32
7.0	0.19506	27865.73
7.5	0.21242	27975.40
8.0	0.23028	28784.83
8.5	0.24423	27778.62
9.0	0.25278	28086.51
9.5	0.26613	28013.85
10.0	0.27983	28008.68

Table B.1: Results - Orange II molar extinction coefficients

Concentration [nMol/L]	Absorbance [AU]	ϵ [L Mol^{-1} cm^{-1}]
0.5	0.00909	18171.02
1.0	0.01880	18800.01
1.5	0.04420	29466.67
2.0	0.06441	32205.00
2.5	0.08925	35700.10
3.0	0.11122	37073.33
3.5	0.13517	38620.01
4.0	0.15968	39920.04
4.5	0.18740	41644.44
5.0	0.21494	42988.00
5.5	0.24948	45360.12
6.0	0.27960	46600.05
6.5	0.30246	46532.54
7.0	0.32713	46733.24
7.5	0.35650	47533.40
8.0	0.40172	50214.50
8.5	0.42682	50214.12
9.0	0.47011	52234.32
9.5	0.51417	54123.11
10.0	0.55342	55342.34

TABLE B.2: Results - Coomassie brilliant blue molar extinction coefficients

Appendix C

Colorimetric assay - Orange II

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.02013	7.22166 E-07	0.6
2	0.01994	7.15350 E-07	0.6
3	0.02097	7.52301 E-07	0.6
4	0.01877	6.73376 E-07	0.5
X	**0.01995**	**7.15798 E-07**	**0.6**

Table C.1: Results COP - unsilanized - on slides I

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.01901	6.81986 E-07	0.5
2	0.02170	7.78490 E-07	0.6
3	0.01943	6.97053 E-07	0.6
4	0.01898	6.80910 E-07	0.5
X	**0.01978**	**7.09610 E-07**	**0.6**

Table C.2: Results COP - unsilanized - on slides II

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.02437	8.74276 E-07	0.7
2	0.01892	6.78757 E-07	0.5
3	0.01954	7.01000 E-07	0.6
4	0.02123	7.61629 E-07	0.6
X	**0.02101**	**7.53915 E-07**	**0.6**

Table C.3: Results COP - unsilanized - on slides III

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.04792	2.38017 E-06	1.9
2	0.04691	2.33001 E-06	1.9
3	0.05408	2.68614 E-06	2.1
4	0.04933	2.45021 E-06	2.0
X	**0.04956**	**2.46163 E-06**	**2.0**

Table C.4: Results COP - APTES - on slides I

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.05655	2.80882 E-06	2.2
2	0.05563	2.76313 E-06	2.2
3	0.04810	2.38911 E-06	1.9
4	0.04708	2.33845 E-06	1.9
X	**0.05184**	**2.57488 E-06**	**2.1**

Table C.5: Results COP - APTES - on slides II

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.05338	2.65137 E-06	2.1
2	0.04944	2.45567 E-06	2.0
3	0.05072	2.51925 E-06	2.0
4	0.05289	2.62703 E-06	2.1
X	**0.05160**	**2.56333 E-06**	**2.0**

Table C.6: Results COP - APTES - on slides III

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.09172	4.55570 E-06	3.6
2	0.08968	4.45438 E-06	3.6
3	0.10240	5.08618 E-06	4.1
4	0.09624	4.78021 E-06	3.8
X	**0.09501**	**4.71912 E-06**	**3.8**

Table C.7: Results COP - Plasma treatment/ APTES - on slides I

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.08492	4.21795 E-06	3.4
2	0.10060	4.99677 E-06	4.0
3	0.08305	4.12507 E-06	3.3
4	0.08723	4.33269 E-06	3.5
X	**0.08895**	**4.41812 E-06**	**3.5**

Table C.8: Results COP - Plasma treatment/ APTES - on slides II

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.08610	4.27656 E-06	3.0
2	0.08209	4.07739 E-06	3.1
3	0.09715	4.82541 E-06	3.9
4	0.13710	6.80972 E-06	5.4
X	**0.10441**	**5.18601 E-06**	**4.1**

Table C.9: Results COP - Plasma treatment/ APTES - on slides III

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.3320	1.64903 E-05	13.2
2	0.4444	2.20732 E-05	17.6
3	0.3226	1.60234 E-05	12.8
4	0.3526	1.75135 E-05	14.0
X	**0.3629**	**1.80251 E-05**	**14.4**

Table C.10: Results COP - Piranha treatment/ APTES - on slides

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.0758	3.76496 E-06	3.0
2	0.0777	3.85934 E-06	3.1
3	0.0969	4.81299 E-06	3.8
4	0.1368	6.79481 E-06	5.4
5	0.1010	5.01664 E-06	4.0
X	**0.0976**	**4.84975 E-06**	**3.9**

Table C.11: Results COP - Piranha treatment + ultrasonication/ APTES - on slides

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.0828	4.11265 E-06	3.3
2	0.0478	2.37421 E-06	1.9
3	0.1498	7.44052 E-06	5.9
4	0.0469	2.32951 E-06	1.9
5	0.0698	3.46694 E-06	2.8
X	**0.0794**	**3.94477 E-06**	**3.2**

TABLE C.12: Results COP - Piranha treatment + ultrasonication/ No silane - on slides

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.01796	8.92068 E-07	0.7
2	0.01904	9.45711 E-07	0.8
3	0.01798	8.93061 E-07	0.7
4	0.01672	8.30477 E-07	0.7
5	0.01553	7.71370 E-07	0.6
X	**0.01744**	**8.66538 E-07**	**0.7**

TABLE C.13: Results COP - GLYMO - on slides

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.0398	1.97685 E-06	1.6
2	0.0386	1.91725 E-06	1.5
3	0.0342	1.69870 E-06	1.4
4	0.0406	2.01659 E-06	1.6
X	**0.0383**	**1.90235 E-06**	**1.5**

TABLE C.14: Results COP - GLYMO + Ethylenediamine 1:8 - on slides

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.0298	1.48016 E-06	1.2
2	0.0357	1.77321 E-06	1.4
3	0.0360	1.78811 E-06	1.4
4	0.0384	1.90732 E-06	1.5
X	**0.11476**	**1.7372 E-06**	**1.4**

TABLE C.15: Results COP - GLYMO + Diaminopropane 1:8 - on slides

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.02538	1.26062 E-06	1.0
2	0.01990	9.88427 E-07	0.8
3	0.01865	9.26340 E-07	0.7
X	**0.02131**	**1.05846 E-06**	**0.8**

TABLE C.16: Results TPE - APTES - on slides

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.02742	1.36194 E-06	0.7
2	0.02704	1.34307 E-06	0.6
3	0.01842	9.14916 E-07	0.4
4	0.02235	1.11012 E-06	0.5
5	0.02758	1.36989 E-06	0.7
6	0.02442	1.21293 E-06	0.6
X	**0.02453**	**1.21881 E-06**	**0.6**

Table C.17: Results COP - unsilanized - on cores I

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.02316	1.15035 E-06	0.6
2	0.02613	1.29787 E-06	0.6
3	0.02131	1.05846 E-06	0.5
4	0.02341	1.16277 E-06	0.6
5	0.02492	1.23777 E-06	0.6
6	0.02722	1.35201 E-06	0.6
X	**0.02435**	**1.20987 E-06**	**0.6**

Table C.18: Results COP - unsilanized - on cores II

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.01988	9.87434 E-07	0.5
2	0.01814	9.01008 E-07	0.4
3	0.02011	9.98858 E-07	0.5
4	0.02213	1.09919 E-06	0.5
5	0.02089	1.03760 E-06	0.5
6	0.01977	9.81970 E-07	0.5
X	**0.02015**	**1.00101 E-06**	**0.5**

Table C.19: Results COP - unsilanized - on cores III

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.11001	5.46416 E-06	2.6
2	0.11490	5.70705 E-06	2.7
3	0.12660	6.28818 E-06	3.0
4	0.09196	4.56763 E-06	2.2
5	0.13108	6.51070 E-06	2.2
6	0.12531	6.22411 E-06	2.2
X	**0.11664**	**5.79364 E-06**	**2.8**

TABLE C.20: Results COP - APTES - on cores I

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.10927	5.42741 E-06	2.6
2	0.12289	6.10391 E-06	2.9
3	0.12505	6.21120 E-06	3.0
4	0.09087	4.51349 E-06	2.2
5	0.09877	4.90588 E-06	2.4
6	0.13136	6.52461 E-06	3.1
X	**0.11303**	**5.61441 E-06**	**2.7**

TABLE C.21: Results COP - APTES - on cores II

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.11902	5.91169E-06	2.8
2	0.10249	5.09065E-06	2.4
3	0.13605	6.75756E-06	3.2
4	0.10028	4.98088E-06	2.4
5	0.09981	4.95753E-06	2.4
6	0.12093	6.00656E-06	2.9
X	**0.11309**	**5.61748 E-06**	**2.7**

TABLE C.22: Results COP - APTES - on cores III

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.18788	9.33194 E-06	4.5
2	0.18742	9.30909 E-06	4.5
3	0.16393	8.14235 E-06	3.9
4	0.20040	9.95381 E-06	4.8
5	0.19086	9.47996 E-06	4.5
6	0.19265	9.56887 E-06	4.6
X	**0.18183**	**9.03181 E-06**	**4.3**

TABLE C.23: Results COP - Plasma treatment/ APTES - on cores I

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.18890	6.89912 E-06	4.5
2	0.19060	5.99017 E-06	4.5
3	0.19513	7.69880 E-06	4.6
4	0.18410	6.66071 E-06	4.4
5	0.19073	6.66071 E-06	4.5
6	0.18921	6.66071 E-06	4.5
X	**0.18977**	**9.42623 E-06**	**4.5**

TABLE C.24: Results COP - Plasma treatment/ APTES - on cores II

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.18319	9.09899 E-06	4.4
2	0.19552	9.71142 E-06	4.7
3	0.18550	9.21373 E-06	4.4
4	0.17043	8.46521 E-06	4.1
5	0.17752	8.81736 E-06	4.2
6	0.17447	8.66587 E-06	4.2
X	**0.18110**	**8.99543 E-06**	**4.3**

TABLE C.25: Results COP - Plasma treatment/ APTES - on cores III

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.08231	4.08831 E-06	2.0
2	0.07825	3.88665 E-06	1.9
3	0.08176	4.06099 E-06	1.9
4	0.08005	3.97606 E-06	1.9
5	0.07231	3.59162 E-06	1.7
6	0.07491	3.72076 E-06	1.8
X	**0.07827**	**3.8874 E-06**	**1.9**

TABLE C.26: Results COP - GLYMO + Ethylenediamine 1:8 - on cores I

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.07732	3.84046 E-06	1.8
2	0.07982	3.96464 E-06	1.9
3	0.07101	3.52705 E-06	1.7
4	0.07392	3.67158 E-06	1.8
5	0.06998	3.47589 E-06	1.7
6	0.07770	3.85934 E-06	1.9
X	**0.07496**	**3.72316 E-06**	**1.8**

TABLE C.27: Results COP - GLYMO + Ethylenediamine 1:8 - on cores II

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.08829	4.38534 E-06	2.1
2	0.08745	4.34361 E-06	2.1
3	0.07851	3.89957 E-06	1.9
4	0.07702	3.82556 E-06	1.8
5	0.08352	4.14841 E-06	2.0
6	0.07690	3.81960 E-06	1.8
X	**0.08195**	**4.07035 E-06**	**2.0**

TABLE C.28: Results COP - GLYMO + Ethylenediamine 1:8 - on cores III

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.07943	3.94526 E-06	1.9
2	0.06745	3.35022 E-06	1.6
3	0.07021	3.48731 E-06	1.7
4	0.10930	5.42890 E-06	2.6
5	0.06378	3.16793 E-06	1.5
6	0.07045	3.49923 E-06	1.7
X	**0.07677**	**3.81314 E-06**	**1.8**

TABLE C.29: Results COP - GLYMO + Diaminopropane 1:8 - on cores I

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.07621	3.78533 E-06	1.8
2	0.06984	3.46893 E-06	1.7
3	0.07290	3.62092 E-06	1.7
4	0.08183	4.06447 E-06	1.9
5	0.07642	3.79576 E-06	1.8
6	0.06781	3.36810 E-06	1.6
X	**0.07417**	**3.68392 E-06**	**1.8**

TABLE C.30: Results COP - GLYMO + Diaminopropane 1:8 - on cores II

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.08142	4.04411 E-06	1.9
2	0.07698	3.82357 E-06	1.8
3	0.06997	3.47539 E-06	1.7
4	0.06772	3.36363 E-06	1.6
5	0.06745	3.35022 E-06	1.6
6	0.07601	3.77539 E-06	1.8
X	**0.07326**	**3.63872 E-06**	**1.7**

TABLE C.31: Results COP - GLYMO + Diaminopropane 1:8 - on cores III

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.07732	3.84046 E-06	1.8
2	0.08108	4.02722 E-06	1.9
3	0.09036	4.48815 E-06	2.2
4	0.06637	3.29658 E-06	1.6
5	0.07209	3.58069 E-06	1.7
6	0.07884	3.91596 E-06	1.9
X	**0.07767**	**3.85818 E-06**	**1.9**

Table C.32: Results COP - GLYMO + Ethylenediamine 1:80 - on cores I

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.06979	3.46645 E-06	1.7
2	0.06783	3.36910 E-06	1.6
3	0.06642	3.29906 E-06	1.6
4	0.07810	3.87920 E-06	1.9
5	0.07213	3.58268 E-06	1.7
6	0.08793	4.36746 E-06	2.1
X	**0.07370**	**3.66066 E-06**	**1.8**

Table C.33: Results COP - GLYMO + Ethylenediamine 1:80 - on cores II

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.08336	4.14047 E-06	2.0
2	0.07278	3.61496 E-06	1.7
3	0.08201	4.07341 E-06	2.0
4	0.07653	3.80122 E-06	1.8
5	0.07610	3.77986 E-06	1.8
6	0.08004	3.97556 E-06	1.9
X	**0.07847**	**3.89758 E-06**	**1.9**

Table C.34: Results COP - GLYMO + Ethylenediamine 1:80 - on cores III

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.07451	3.70089 E-06	1.8
2	0.08143	4.04460 E-06	1.9
3	0.09230	4.58451 E-06	2.2
4	0.06345	3.15154 E-06	1.5
5	0.07781	3.86480 E-06	1.9
6	0.06908	3.43118 E-06	1.6
X	**0.07643**	**3.79625 E-06**	**1.8**

Table C.35: Results COP - GLYMO + Diaminopropane 1:80 - on cores I

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.06950	3.45204 E-06	1.7
2	0.08921	4.43103 E-06	2.1
3	0.08017	3.98202 E-06	1.9
4	0.09440	4.69031 E-06	2.2
5	0.08120	4.03318 E-06	1.9
6	0.07021	3.48681 E-06	1.7
X	**0.08078**	**4.01232 E-06**	**1.9**

Table C.36: Results COP - GLYMO + Diaminopropane 1:80 - on cores II

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.08196	4.07093 E-06	2.0
2	0.07134	3.54344 E-06	1.7
3	0.08170	4.05801 E-06	1.9
4	0.06980	3.47042 E-06	1.7
5	0.06923	3.43863 E-06	1.6
6	0.08070	4.00834 E-06	1.9
X	**0.07579**	**3.76438 E-06**	**1.8**

Table C.37: Results COP - GLYMO + Diaminopropane 1:80 - on cores III

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.01617	8.03159 E-07	0.4
2	0.01139	5.65738 E-07	0.3
3	0.02271	1.12800 E-06	0.5
4	0.01501	7.45542 E-07	0.4
5	0.01488	7.39085 E-07	0.4
6	0.01224	6.07957 E-07	0.3
X	**0.01540**	**7.64913 E-07**	**0.4**

TABLE C.38: Results COP - unsilanized + Diaminopropane 1:80 - on cores I

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.01521	7.55476 E-07	0.4
2	0.01227	6.09447 E-07	0.3
3	0.01466	7.28158 E-07	0.3
4	0.01494	7.42065 E-07	0.4
5	0.01320	6.55640 E-07	0.3
6	0.01333	6.62097 E-07	0.3
X	**0.01393**	**6.92147 E-07**	**0.3**

TABLE C.39: Results COP - unsilanized + Diaminopropane 1:80 - on cores II

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.01298	6.44713 E-07	0.3
2	0.01349	6.70044 E-07	0.3
3	0.01204	5.98023 E-07	0.3
4	0.01550	7.69880 E-07	0.4
5	0.01322	6.56633 E-07	0.3
6	0.01574	7.81801 E-07	0.4
X	**0.01382**	**6.86849 E-07**	**0.3**

TABLE C.40: Results COP - unsilanized + Diaminopropane 1:80 - on cores III

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.05682	2.82223E-06	1.4
2	0.04368	2.16957E-06	1.0
3	0.04372	2.17156E-06	1.0
4	0.04750	2.35931E-06	1.1
5	0.04630	2.29971E-06	1.1
6	0.04834	2.40103E-06	1.2
X	**0.04772**	**2.37057 E-06**	**1.1**

TABLE C.41: Results COP - unsilanized + Diaminopropane 1:8 - on cores I

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.05125	2.54557 E-06	1.2
2	0.04257	2.11444 E-06	1.0
3	0.04261	2.11643 E-06	1.0
4	0.05002	2.48448 E-06	1.2
5	0.04833	2.40054 E-06	1.2
6	0.04701	2.33497 E-06	1.1
X	**0.04696**	**2.33274 E-06**	**1.1**

TABLE C.42: Results COP - unsilanized + Diaminopropane 1:8 - on cores II

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.04665	2.31709E-06	1.1
2	0.04348	2.15964E-06	1.0
3	0.04466	2.21825E-06	1.1
4	0.04634	2.30169E-06	1.1
5	0.04333	2.15219E-06	1.0
6	0.05115	2.54060E-06	1.2
X	**0.04593**	**2.28158E-06**	**1.1**

TABLE C.43: Results COP - unsilanized + Diaminopropane 1:8 - on cores III

Sample	Absorbance	Concentration [Mol/L]	Surface density [molecules/nm^2]
1	0.02893	1.43694 E-06	0.7
2	0.03014	1.49704 E-06	0.7
3	0.01919	9.53161 E-07	0.5
4	0.02697	1.33959 E-06	0.6
X	**0.02630**	**1.30669 E-06**	**0.6**

Table C.44: Results COP - GLYMO - on cores

Appendix D

Fluorescence based assay

Slide	Net intensity [RFU]	X	σ
1	9093		
	8845		
	9003	**8980**	**126**
2	5139		
	4930		
	5164	**5078**	**128**
3	4729		
	4901		
	4922	**4851**	**106**
4	5947		
	6175		
	5992	**6038**	**121**
5	6593		
	6451		
	6387	**6477**	**105**
6	4593		
	4506		
	4390	**4496**	**102**
X	**5986**		
σ	**1548**		
CV	**0.26**		

TABLE D.1: Results COP - APTES - TAMRA-NHS 10 μM

Slide	Net intensity [RFU]	X	σ
1	25123		
	24851		
	24794	**24923**	**176**
2	16188		
	15893		
	16008	**16030**	**149**
3	17374		
	17703		
	17398	**17492**	**183**
4	16830		
	17122		
	17256	**17069**	**218**
5	16449		
	16726		
	16505	**16560**	**146**
6	15238		
	14997		
	15087	**15107**	**122**
X	**17863**		
σ	**3344**		
CV	**0.19**		

TABLE D.2: Results COP - APTES - TAMRA-NHS 25 μM

Slide	Net intensity [RFU]	X	σ
1	32812		
	33104		
	33009	**32975**	**149**
2	28298		
	28144		
	28106	**28183**	**102**
3	27966		
	27990		
	28186	**28047**	**121**
4	29087		
	29123		
	28834	**29015**	**157**
5	26140		
	25977		
	25904	**26007**	**121**
6	23037		
	22865		
	23110	**23004**	**126**
X	**27871**		
σ	**3107**		
CV	**0.11**		

TABLE D.3: Results COP - APTES - TAMRA-NHS 40 μM

Slide	Net intensity [RFU]	X	σ
1	35492		
	35298		
	35302	**35364**	**111**
2	31228		
	31152		
	31279	**31220**	**64**
3	28701		
	28643		
	28768	**28704**	**63**
4	31174		
	31058		
	30996	**31076**	**90**
5	27044		
	26886		
	27099	**27010**	**111**
6	24992		
	25030		
	25117	**25046**	**64**
X	**29736**		
σ	**3417**		
CV	**0.11**		

TABLE D.4: Results COP - APTES - TAMRA-NHS 50 μM

Slide	Net intensity [RFU]	X	σ
1	23		
	30		
	12	**22**	**9**
2	37		
	42		
	29	**36**	**7**
3	21		
	44		
	39	**35**	**12**
4	20		
	14		
	11	**15**	**5**
5	30		
	32		
	18	**27**	**8**
6	33		
	21		
	19	**24**	**8**
X	**26**		
σ	**10**		
CV	**0.39**		

Table D.5: Results COP - APTES - PBS

Slide	Net intensity [RFU]	X	σ
1	7901		
	7826		
	8007	**7911**	**91**
2	7154		
	7488		
	7269	**7304**	**170**
3	7202		
	7387		
	7080	**7223**	**155**
4	6998		
	6823		
	6756	**6859**	**125**
5	5553		
	5482		
	5426	**5487**	**64**
6	4567		
	4301		
	4289	**4386**	**157**
X	**6528**		
σ	**1249**		
CV	**0.19**		

Table D.6: Results COP - GLYMO - TAMRA-Cadaverine 10 μM

Slide	Net intensity [RFU]	X	σ
1	16996		
	17112		
	16903	**17004**	**105**
2	16527		
	16352		
	16449	**16443**	**88**
3	16228		
	16512		
	16363	**16368**	**142**
4	16447		
	16509		
	16394	**16450**	**58**
5	15827		
	15796		
	15690	**15771**	**72**
6	14877		
	14685		
	14705	**14756**	**106**
X	**16131**		
σ	**736**		
CV	**0.05**		

Table D.7: Results COP - GLYMO - TAMRA-Cadaverine 25 μM

Slide	Net intensity [RFU]	X	σ
1	18231		
	18001		
	17994	**18075**	**135**
2	16859		
	16647		
	16772	**16759**	**107**
3	16801		
	16624		
	16744	**16723**	**147**
4	16689		
	16966		
	16743	**16799**	**147**
5	16247		
	16105		
	15998	**16117**	**125**
6	15423		
	15208		
	15320	**15317**	**108**
X	**16631**		
σ	**859**		
CV	**0.05**		

Table D.8: Results COP - GLYMO - TAMRA-Cadaverine 40 μM

Slide	Net intensity [RFU]	X	σ
1	18298		
	17987		
	18109	**18131**	**157**
2	16624		
	16578		
	16591	**16598**	**24**
3	16687		
	16541		
	16403	**16544**	**142**
4	17009		
	16817		
	16963	**16930**	**100**
5	16017		
	15990		
	15873	**15960**	**77**
6	15478		
	15564		
	15322	**15455**	**123**
X	**16602**		
σ	**863**		
CV	**0.06**		

TABLE D.9: Results COP - GLYMO - TAMRA-Cadaverine 50 μM

Slide	Net intensity [RFU]	X	σ
1	13		
	29		
	34	**25**	**11**
2	12		
	18		
	16	**15**	**3**
3	19		
	37		
	40	**32**	**11**
4	41		
	38		
	27	**35**	**7**
5	30		
	24		
	17	**24**	**7**
6	31		
	28		
	25	**28**	**3**
X	**26**		
σ	**10**		
CV	**0.39**		

Table D.10: Results COP - GLYMO - PBS

Appendix E

Contact Angle

FIGURE E.1: Contact angle measurement of water on COP

FIGURE E.2: Contact angle measurement of water on COP + Plasma

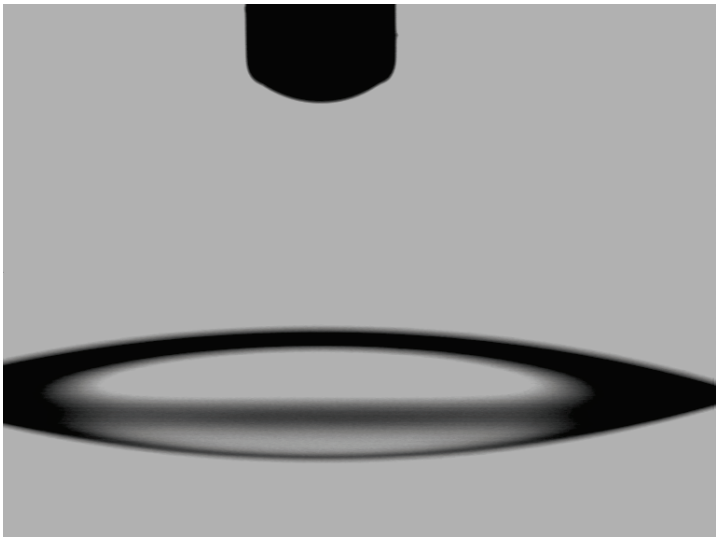

FIGURE E.3: Contact angle measurement of water on COP + SiO_2

FIGURE E.4: Contact angle measurement of water on COP + SiO_2 + Plasma

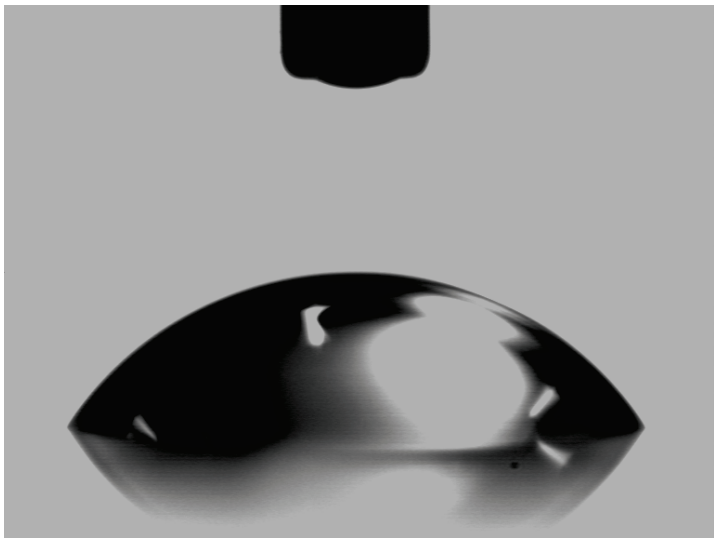

FIGURE E.5: Contact angle measurement of water on COP + APTES

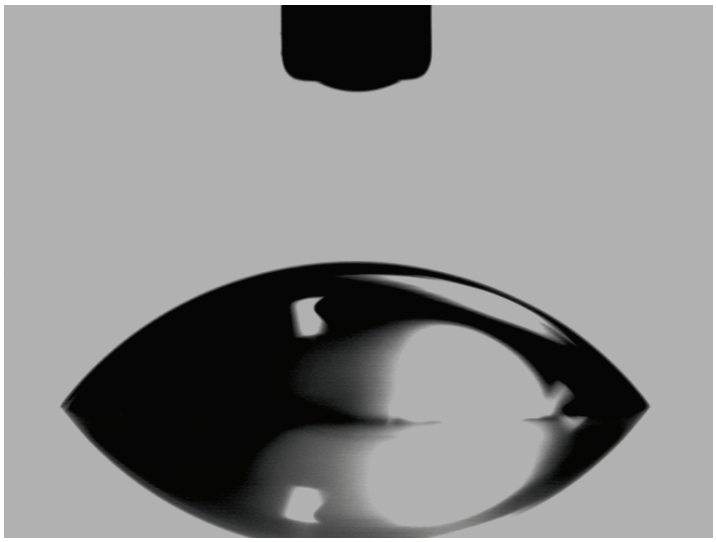

FIGURE E.6: Contact angle measurement of water on COP + SiO_2 + APTES

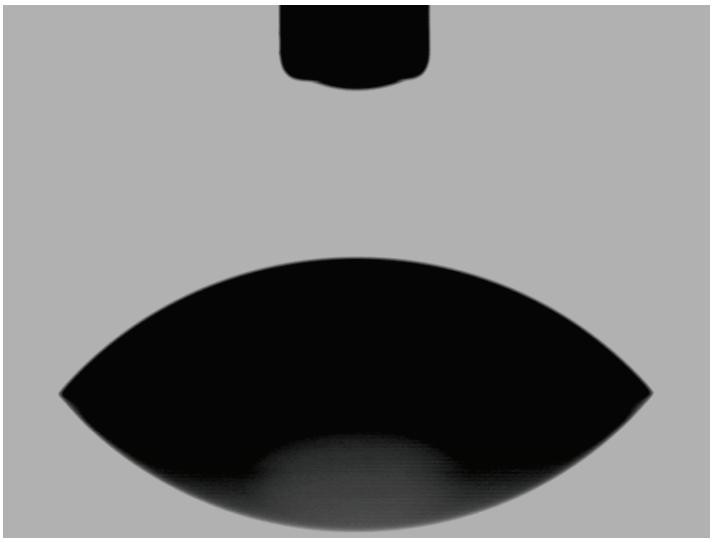

FIGURE E.7: Contact angle measurement of water on COP + GLYMO

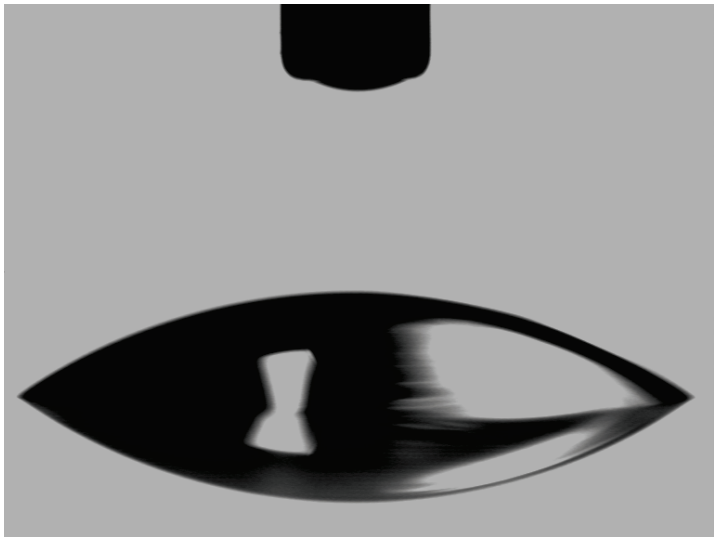

FIGURE E.8: Contact angle measurement of water on COP + SiO_2 + GLYMO

FIGURE E.9: Contact angle measurement of water on COP + SiO_2 + GLYMO + Ethylenediamine (1:80)

FIGURE E.10: Contact angle measurement of water on COP + SiO_2 + GLYMO + Diaminopropane (1:80)

Bibliography

[1] G.S. Fiorini and D.T. Chiu. Disposable microfluidic devices: fabrication, function, and application. *BioTechniques*, 38:429–446, March 2005.

[2] D. Kim and A.E. Herr. Protein immobilization techniques for microfluidic assays. *Biomicrofluidics*, (7), July 2013.

[3] F.F. Bier, E. Förster-Ehrentreich, and S. Schumacher. Biosensoren der Zukunft: in vitro-Diagnostik im Point-of-Care-Format für die personalisierte Medizin. *Deutsche Zeitschrift für klinische Forschung*, (24), September 2011.

[4] K.G. Lee, T.J. Lee, S.W. Jeong, H.W. Choi, N.S. Heo, J.Y. Park, T.J. Park, and S.J. Lee. Development of a plastic-based microfluidic immunosensor chip for detection of H1N1 influenza. *Sensors*, (12):10810–10819, 2012.

[5] G.A. Diaz-Quijada, R.Peytavi, A. Nantel, E. Roy, and M.G. Bergeron. Surface modification of thermoplastics towards the plastic biochip for high throughput screening devices. *Lab Chip*, 7:856–862, June 2007.

[6] J.C. McDonald, D.C. Duffy, J.R. Anderson, D.T. Chiu, H. Wu, O.J.A. Schueller, and G.M. Whitesides. Fabrication of microfluidic systems in poly(dimethylsiloxane). *Electrophoresis*, 21:27–40, 2000.

[7] H. Becker and L.E. Locascio. Commercialization of microfluidic point-of-care diagnostic devices. *Talanta*, 56:267–287, 2002.

[8] D.J Harrison, K.Fluri, K. Seiler, Z. Fan, C.S. Effenhauser, and A. Manz. Micromachining a miniaturized capillary electrophoresis-based chemical analysis system on a chip. *Science*, 261(5123):895–897, August 1993.

[9] EN ISO 12781-1. Geometrical product specifications (GPS) Flatness, 2011.

[10] C.D. Chin, V. Linder, and S.K. Sia. Commercialization of microfluidic point-of-care diagnostic devices. *Lab on a Chip*, 12:2118–2134, January 2012.

[11] X. Illa, O. Ordeig, D. Snakenborg, A. Romano-Rodriguez, R.G. Compton, and J.P. Kutter. A cyclo olefin polymer microfluidic chip with integrated gold microelectrodes for aqueous and non-aqueous electrochemistry. *Lab on a Chip*, 10:1254–1261, March 2010.

[12] E.J. Wong. Modeling and control of rapid cure in polydimethysiloxane (PDMS) for microfluidic device applications). *Unpublished PhD thesis. Massachusetts Institute of Technology*, 2010.

[13] C. Jönsson, M. Aronsson, G. Rundström, C. Pettersson, I. Mendel-Hartvig, J. Bakker, E. Martinsson, B. Liedberg, B. MacCraith, O. Öhman, and J. Melin. Silane-dextrane chemistry on lateral flow polymer chips for immunoassays. *Lab on a Chip*, 8:1191–1197, May 2008.

[14] C.W. Kan, A.J. Rivnak, T.G. Campbell, T. Piech, D.M. Rissin, M. Mösl, A. Peterca, H.P. Niederberger, K.A. Minnehan, P.P. Patel, E.P. Ferrell, R.E. Meyer, L. Chang, D.H. Wilson, D.R. Fourniera, and D.C. Duffy. Isolation and detection of single molecules on paramagnetic beads using sequential fluid flows in microfabricated polymer array assemblies. *Lab Chip*, 12:977–985, 2012.

[15] J. Raj, G. Herzog, M. Manning, C. Volcke, B.D. MacCraith, S. Ballantyne, M. Thompson, and D.W.M. Arrigan. Surface immobilisation of antibody on cyclic olefin copolymer for sandwich immunoassay. *Biosensors and Bioelectronics*, 24(24):2654–2658, January 2009.

[16] S. Noel, B. Liberelle, L. Robitaille, and L. De Crescenzo. Quantification of primary amine groups available for subsequent biofunctinalization of polymer surfaces. *Bioconjugate Chemistry*, 22(8):1690–1699, July 2007.

[17] U. Jönsson, G. Olofsson, M. Malmqvist, and I. Rönnberg. Chemical vapor deposition of silanes. *Thin Solid Films*, 124:117–123, September 1985.

[18] N.R. Glass, R. Tjeung, P. Chan, L.Y. Yeo, and J.R. Friend. Organosilane deposition for microfluidic applications. *Biomicrofluidics*, 5, August 2011.

[19] S. Ek, E.I. Iiskola, and L. Niinistö. Gas-phase deposition of aminopropylalkoxysilanes on porous silica. *Langmuir*, 19:3461–3471, January 2003.

[20] Z. Guo, R.A. Guilfoyle, A.J. Thiel, R. Weng, and L.M. Smith. Direct fluorescence analysis of genetic polymorphisms by hybridization with oligonucleotide arrays on glass supports. *Nucleic Acids Research*, 22 (24):5456–5465, October 1994.

[21] K. Lindroos, U. Liljedahl, M. Raito, and A.C. Syvänen. Minisequencing on oligonucleotide microarrays: Comparison of immobilisation chemistries. *Nucleic Acids Research*, 29(13), February 2001.

[22] V.G. Janolino and H.E. Swaisgood. A spectrophotometric assay for solid phase primary amino groups. *Applied Biochemistry and Biotechnology*, 1(36):81–85, 1992.

[23] J.R. Benson and P.E. Hare. O-phtaldehyde: Fluorogenic detection of primary amines in the picomole range. Comparison with fluorescamine and ninhydrin. *Proceedings of the National Academy of Sciences*, 72(2): 619–622, February 1992.

[24] M. Torimura, S. Kurata, K. Yamada, T. Yokomaku, Y. Kamagata, T. Kanagawa, and R. Kurane. Fluorescence-quenching phenomenon by photoinduced electron transfer between a fluorescent dye and a nucleotide base. *Analytical Sciences*, 2(17):155–160, January 2001.

[25] J.Y. Shin, J.Y. Park, C. Liu, J. He, and S.C. Kim. Chemical structure and physical properties of cyclic olefin copolymers. *Pure Applied Chemistry*, 77(5):801–814, 2005.

[26] T. Voutsas, H. Nishiki, M. Atkinson, J. Hartzell, and Y. Nakata. Sputtering technology of Si films for low-temperature poly-Si TFTs. *SHARP Technical Journal*, 1(3), 2001.

[27] C.M. Halliwell and A.E.G. Cass. A factorial analysis of silanization conditions for the immobilization of oligonucleotides on glass surfaces. *Analytical Chemistry*, 73(11):2476–2483, June 2001.

[28] E.T. Vandenberg, L. Bertilsson, B. Liedberg, K. Uvdal, R. Erlandsson, H. Elwing, and I. Lundström. Structre of 3-aminopropyl triethoxy silane on silicon oxide. *Journal of Colloid and Interface Science*, 147(1):103–118, November 1991.

[29] H. Juvaste, E.I. Iiskola, and T.T. Pakkanen. Preparation of new modified catalyst carriers. *Journal of Molecular Catalysis A: Chemical*, 150 (1):1–9, December 1999.

[30] H. Juvaste, E.I. Iiskola, and T.T. Pakkanen. Aminosilane as a coupling agent for cyclopentadienyl ligands on silica. *Journal of Organometallic Chemistry*, 587(1):38–45, September 1999.

[31] K.M.R. Kallury, U. Krull, and M. Thompson. Chemical structure and physical properties of cyclic olefin copolymers. *Analytical Chemistry*, 60:169–172, 1988.

[32] A. Carré, W. Birch, and V. Lacarrière. Glass substrates modified with organosilanes for DNA immobilization. *Silanes and Other Coupling Agents*, 4:1–14, 2007.

[33] G.T. Hermanson, editor. *Bioconjugate Techniques*. Academic Press, 2008.

[34] T. Goldmann and J.S. Gonzalez. DNA-printing: utilization of a standard inkjet printer for the transfer of nucleic acids to solid supports. *Journal of Biochemical and Biophysical Methods*, 1(42):105–110, 2000.

[35] P. Koltay. Non-contact micro dispensing technologies for science and industry. *Actuator 2012, Messe Bremen*, 2012.

[36] Y.B. Ham, W.S. Seo, S.J. Oh, J.H. Park, and S.N. Yun. A piezoelectric-driven single-nozzle dispenser for liquid droplet jetting. *Journal of the Korean Physical Society*, 57(4):877–881, October 2010.

[37] R. Mannhold, H. Kubinyi, and G. Folkers. High throughput-screening in drug discovery. *Methods and Principles in Medicinal Chemistry*, 35: 48, October 2006.

[38] M.G. Gore, editor. *Spectrophotometry and spectrofluorimetry: A practical approach*. Oxford University Press, 2000.

[39] D.P. Subedi. Contact angle measurement for the surface characterization of solids. *The Himalayan Physics*, 2, May 2011.

[40] A.W. Neumann and R.J. Good. Techniques of measuring contact angles. *Surface and Colloid Science*, 11:31–91, J 1979.