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Surface-immobilized Hairpin DNA Sensors
for Direct and Specific Detection
of Target DNA

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A thesis submitted in complete fulfillment of the requirements
for the degree of Doctor of Philosophy,
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Abstract

Gene-sensors show great promise as tools for various applications, such as clinical diagnosis, reliable forensic analysis, environmental monitoring, and biological research. There is a great demand for DNA sensors that are able to detect single-base mismatches, which are the most common genetic defects that need to be discriminated for medical diagnostic purposes. The development of label free, direct, and fast sensors can be essential in meeting that requirement. Current gene-sensing technology relies heavily on fluorescent labeling of samples but this approach suffers from the disadvantage of time-consuming, expensive and multi-stepped procedures, especially during the sample preparation stage. Although the use of fluorescent labels has overcome the hazards involved with radioactive markers, development of alternative approaches to the traditional assays is still vital to advance the area of DNA sensors.

The aim of this research was to develop a one-step sensor, offering direct and specific detection of a target DNA. Nanostructured materials, such as quantum dots (QDs) and self-assembled monolayers, together with hairpin structured DNA probes were applied and investigated for optical as well as electrochemical sensors. The properties of inorganic quantum dots (or nanoparticles), such as narrow and intensive emission spectra, resistance to photobleaching and a wide range of possible surface functionalities, give QDs a great potential as labels in biological sensing applications. Self-assembled monolayers provide well established and versatile platforms for biosensors and hairpin probes, which are able to discriminate single-base mismatches in the target sequences, are ideal components for DNA sensors.

Generally, the electrochemical sensors demonstrated a superior response compared to the optical sensors. The best prepared sensor showed sensitivity down to 4.7 fM of target and was capable of detecting single-base mismatches, fulfilling the requirements for a high-quality DNA sensor.
Declaration

This is to certify that:

1) This thesis comprises only the authors original work, except where indicated below;

2) Due acknowledgment to all other material used has been made in the main text of the thesis.

My overall contribution to the work presented in this thesis is approximately 95%, based on the following:

Chapter 4

95 % The models for fitting of the acquired neutron reflectometry data were developed by Dr Ducncan McGillivray, from the Department of Chemistry, The University of Auckland. Dr McGillivray also carried out the polarized neutron reflectometry experiments at the NIST Center for Neutron Research, USA.
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<td>θ</td>
<td>Angle</td>
</tr>
<tr>
<td>( \bar{P} )</td>
<td>Average height</td>
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<tr>
<td>α</td>
<td>Charge transfer coefficient</td>
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<tr>
<td>ε</td>
<td>Extinction coefficient</td>
</tr>
<tr>
<td>ω</td>
<td>Frequency</td>
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<tr>
<td>μ</td>
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<tr>
<td>Ω</td>
<td>Ohm</td>
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<tr>
<td>K</td>
<td>Radiation intensity</td>
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<td>Surface roughness</td>
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<tr>
<td>ν</td>
<td>Sweep rate</td>
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<tr>
<td>λ</td>
<td>Wavelength</td>
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<td>13C NMR</td>
<td>Carbon nuclear magnetic resonance spectroscopy</td>
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</tr>
<tr>
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<tr>
<td>A</td>
<td>Absorbance / Area</td>
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<tr>
<td>A</td>
<td>Adenine</td>
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<td>Å</td>
<td>Ångström</td>
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<tr>
<td>a</td>
<td>Atto, ( 10^{-18} )</td>
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<tr>
<td>( a )</td>
<td>Chemical activity coefficient</td>
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<tr>
<td>a.u.</td>
<td>Arbitrary unit</td>
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<tr>
<td>AC</td>
<td>Alternating current</td>
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<td>ACCV</td>
<td>Alternating current cyclic voltammetry</td>
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<tr>
<td>AdTSV</td>
<td>Adsorptive transfer stripping voltammetry</td>
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<tr>
<td>AFM</td>
<td>Atomic Force Microscopy</td>
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ANSTO  Australian Nuclear Science and Technology Organisation
ATR-FTIR  Attenuated total reflection fourier transform infrared
\( b_i \)  Scattering length of nucleus i
\( c \)  Complementary
\( C \)  Concentration
\( C \)  Cytosine
\( c \)  Speed of light in vacuum
CCD  Charge-coupled device
\( C_d \)  Double layer capacitance
\( \text{cm} \)  Centimeter
CM  Contrast matching
Compound A  Biotin N-hydroxysuccinimide ester
Compound B  N-(13-Amino-4,7,10-trio- atridecanyl) biotinamide
Compound C  11-mercaptoundecanoyl- N-hydroxysuccinimide ester
\( C_p \)  Heat capacity
CPE  Constant phase element
CV  Cyclic voltammetry
Cy3  Indocarboxycyanine
\( D \)  Diffusion constant
\( d \)  Thickness
\( \text{Da} \)  Dalton (mass unit)
DC  Direct current
DHLA  Dihydrolipoic acid
DMF  N,N-dimethylformamide
DNA  Deoxyribonucleic acid
\( d_p \)  Depth of penetration
DPV  Differential pulse voltammetry
ds  Double-stranded
DSBA/  \((5-(6,8-Diaza-7-oxo-3-Ligand 2 \  \text{thiabicyclo}[3.3.0]oct-2-yl)-N[7-(3-\{2-\text{N}[7-[5-(6,8-diaza-7-oxo-3- \  \text{thiabicyclo}[3.3.0]oct-2-yl]pentanoylamino] \  \text{heptyl}]\text{-arabamoyl} [\text{ethyl}] \text{disulfonyl} [\text{propanoyl- amino} \text{heptyl}] \text{pentanamide}])\)
DSC  Differential scanning calorimetry
dT  Thymine deoxyribonucleotide
\( E \)  Energy
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>mSAM</td>
<td>Mixed self-assembled monolayer</td>
</tr>
<tr>
<td>MUA</td>
<td>11-Mercaptoundecanoic acid</td>
</tr>
<tr>
<td>$n$</td>
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<tr>
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<td>$N_A$</td>
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<td>Non-complementary</td>
</tr>
<tr>
<td>NCNR</td>
<td>NIST Center for Neutron Research</td>
</tr>
<tr>
<td>NDL</td>
<td>National Device Laboratories</td>
</tr>
<tr>
<td>NIST</td>
<td>National Institute of Standards and Technology</td>
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<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>NN</td>
<td>Nearest-neighbor</td>
</tr>
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<td>Nanoparticle</td>
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<tr>
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<td>Oligonucleotide</td>
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<td>$P$</td>
<td>Power of transmitted radiation</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>m-PEG</td>
<td>methyl-Poly(ethylene glycol)</td>
</tr>
<tr>
<td>PEO</td>
<td>Poly(ethylene oxide)</td>
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<td>$P_i$</td>
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<td>Mass density</td>
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<tr>
<td>PL</td>
<td>Photoluminescence</td>
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<tr>
<td>PNR</td>
<td>Polarized neutron reflectometry</td>
</tr>
<tr>
<td>pPy</td>
<td>PolyPyrrole</td>
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<tr>
<td>q</td>
<td>Quadruplet</td>
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<tr>
<td>QD</td>
<td>Quantum dot</td>
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<tr>
<td>$Q_z$</td>
<td>Momentum transfer</td>
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<td>$R$</td>
<td>Resistance</td>
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<td>R</td>
<td>The universal gas constant</td>
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<td>$R_a$</td>
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<td>$R_f$</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>RMS</td>
<td>Root mean square</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>$R_s$</td>
<td>Solution resistance</td>
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<td>Singlet</td>
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<tr>
<td>SAM</td>
<td>Self-assembled monolayer</td>
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<td>Scanning electron microscopy</td>
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<td>SLD</td>
<td>Scattering length density</td>
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<td>Single-stranded</td>
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<td>Saline-sodium citrate</td>
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<td>Scanning tunneling microscopy</td>
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<tr>
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<td>Tapping-mode</td>
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Chapter 1 – General introduction

DNA sensors have been the focus of research for several decades. The prospect of diagnosing diseases has been a major motivation for the development of gene sensors.\textsuperscript{1, 2} The earlier sensors were primarily based on radio labeled probes\textsuperscript{3} but today most sensors rely heavily on chemiluminescent or fluorescent markers. When the polymerase chain reaction (PCR) was introduced\textsuperscript{4} significant improvement in gene analysis was achieved and to this day great efforts have been made to develop sensors that can rival the PCR-based procedures. In general, a DNA sensor is based on the detection of hybridization between a single-stranded oligonucleotide (ODN) probe and its complementary target, a process that is described in more detail in Section 1.2. Briefly, when a labeled target ODN is introduced to the probe strand, which is of a known sequence, Watson-Crick base-pairing of complementary sequences occur. The formed dsODN can be detected by eg. fluorescence or chemiluminescence from the labeled probe/target complex.\textsuperscript{5} In the continuous evolution of DNA sensors a new generation of tags that have emerged are the quantum dots (QDs). QDs exhibit interesting attributes, such that the wavelength of their luminescence is size dependent, and they show greater photostability than organic dyes.\textsuperscript{6, 7} This special characteristic opens up the possibility for multi-labeled probe arrays that are able to detect different targets simultaneously\textsuperscript{8} and more efficiently compared to dye based assays.\textsuperscript{9} QDs and their properties will be discussed further in Section 1.5. Although QDs have proven to be valuable tags for DNA detection, label free sensor are still preferable in terms of simplicity, down-scaling, cost effectiveness and possible manufacturability. Other nanoparticles (NPs), such as gold, have also been utilized for characterization of DNA sequences.\textsuperscript{10} In the literature there is some inconsistency in what is referred to as label free sensor. Traditionally, the target sequence was labeled and thus designs that rely on labeled probes, but not labelled targets, are now frequently described as label free. The sensors presented in this thesis are, however,
referred to as labeled even if it is only the probe that is tagged and as label free when no label is attached to either probes or targets.

1.1 Objectives

The research presented in this thesis was conducted with the aim to develop a one-step label-free sensor, offering direct and specific detection of target DNA. A surface-immobilization approach was chosen, based on mixed self-assembled monolayers (SAMs), and quantum dot (QD)-labelled and completely label-free sensor designs were investigated. Following an introduction to the main building blocks used in the sensor design in this chapter, the experimental methods are presented in Chapter two. Chapter three describes preliminary studies of the quenching efficiency of gold on the fluorescence of an organic dye and functionalized QDs as well as initial efforts to characterize the mixed self-assembled monolayer (mSAM) used as the sensing platform. In Chapter four, the results of a detailed investigation of the mSAMs, preformed with neutron reflectometry (NR), are presented. Valuable information about the probe density was obtained with this technique and efforts to clarify the melting behaviour of the surface-attached hairpin probes (HPPs) were made. The response of a QD-labelled sensor was detected with optical- as well as electrochemical methods and the results are presented in Chapter five. Chapter six contains a thorough examination of a label-free DNA sensor, which shows high sensitivity and single-base selectivity towards its target sequence. Finally, Chapter seven provides a general summary of the work that has been carried out and an outlook for future work.
1.2 Deoxyribonucleic acid (DNA)

DNA is a long polymer, which consist of deoxyribonucleotide units. The nucleotides are made up by a nitrogen-containing base (derivative of either purine or pyrimidine), a sugar (deoxyribose) and one or several phosphate groups. The structures of the purines - adenine (A) and guanine (G), and the pyrimidines - cytosine (C) and thymine (T), are shown in Scheme 1.1. The bases carry the genetic information, whereas the sugar and the phosphate groups are relevant to the DNA structure.¹¹

![Scheme 1.1. The purines in DNA are adenine (A) and guanine (G) and the pyrimidines are cytosine (C) and thymine (T).]¹¹

The backbone of DNA consists of deoxyriboses linked by phosphate groups. The 3’ hydroxyl of the sugar of deoxyribonucleotide is connected to the 5’ hydroxyl of the neighboring sugar through a phosphodiester bridge and the part that varies in the DNA composition is the sequence of the four bases. A part of a DNA structure, is shown in Scheme 1.1, with the continuous phosphate-sugar backbone linked to the bases.¹¹ Two complementary single-stranded DNA (ssDNA) sequences (as exemplified in Scheme 1.2) will spontaneously form a double stranded DNA (dsDNA) helix and this process is called hybridization.
The structural part of a fragment of a DNA chain. At the top is the phosphate-sugar backbone, connected to the bases shown underneath. The 5’ and 3’ positions of the linking hydroxyl groups are marked in blue and red, respectively.\(^\text{11}\)

The two strands can easily dissociate if the hydrogen bonds between the bases are disrupted and the stability of the hybridized dsDNA is dependent on the nucleotide sequences of both strands and the identity of the bases present. Even a one base mismatch in the sequences will decreases the stability of the duplex and the more mismatches that are found, the less stable will the helix be. The helix can be completely dissociated by a change in pH, as the bases get ionized, or by heating to “melt” the duplex. The melting temperature (\(T_m\)) of DNA is defined as the temperature at which half the helical structure is lost. The helix will, however, spontaneously re-associate whenever possible.\(^\text{11}\)

A simplified diagram of a more complete structure of DNA is presented in Scheme 1.3. The backbone is drawn in black and the hydrogen bonds (dashed lines) between the bases (in color) are holding the helix together. The dissociation and re-association of double- versus single stranded DNA is at the heart of DNA replication, where a single stranded sequence acts as a template for duplication of the genetic information stored in the molecule.
The three-dimensional structure of DNA (exemplified in Figure 1.1) was first reported in 1953, by Watson and Crick. Their observation was based on analysis of x-ray diffraction photographs, taken by Rosalind Franklin and Maurice Wilkins, of a DNA fiber. The structure that Watson and Crick proposed has since been proven to be, in essence, correct. The most significant features in their model are listed below:

1. Two helical polynucleotide chains, running in opposite directions, are coiled around a shared axis. The bases are situated on the inside of the helix, in a plane perpendicular to the helix axis. The phosphate and sugar units are on the outside of the helix, at almost right angles to the bases.
II. The diameter of the helix is 20 Å and neighboring bases have a 3.4 Å distance between them, along the helix axis, but are still related to each other through a 36 degree rotation. Thus the helical structure is repeated every tenth unit.

III. Base pairs in the two chains are linked by hydrogen (H) bonds; adenine is always paired with thymine (two H-bonds) and guanine with cytosine (three H-bonds).

IV. The particular sequence of bases holds the genetic information and is not restricted in any way.

Figure 1.1. A three-dimensional DNA double helix, courtesy of Bill Frymire.

The most important feature of the DNA double helix is the specificity in the base pairing. As stated above, adenine preferentially pairs with thymine and guanine with cytosine, due to both steric and hydrogen bond-related constraints. The steric constraints arise from the helical nature of the phosphate-sugar backbone, which has a space within the helix that exactly fits a purine-pyrimidine base pair. There is not enough space for two purines, whereas two pyrimidines would be too far apart to form any hydrogen bonds. Additionally some hydrogen-bonding requirements further restrict the base pairing. The hydrogen atoms are not randomly
positioned in the bases and if there is not a hydrogen atom in the appropriate bonding position the bases cannot pair up properly. For example, guanine cannot easily pair with thymine because there would be two hydrogen atoms close to one binding position and none at the other. The hydrogen bonds between adenine and thymine and guanine and cytosine, however, are positioned in such an orientation, and at such a distance, that strong attraction between the bases is attained and stable bonds are formed.\textsuperscript{11} Pairing between mismatched bases can, however, occur if all surrounding base pairs match. Such a bond is weaker and thermodynamically less favorable than a bond between perfectly matched bases.

\section*{1.3 DNA sensors}

\subsection*{1.3.1 Optical DNA sensors based on molecular beacons}

Radio labeled sensor arrays encompass certain disadvantages, which mainly stems from the use of hazardous label materials. Fluorescent tags have offered an alternative to radioactive labels providing a range of successful sensor designs, particularly when molecular beacons (MBs) have been employed as probes. The first MBs developed for detection of hybridization were presented by Tyagi and Kramer\textsuperscript{13} in 1996. This landmark paper resulted in a cascade of research on MBs both in solution assays and in various surface-attached schemes for recognition of specific nucleic acids.\textsuperscript{8, 9, 14-17} MBs are single-stranded ODN probes that possess a stem and loop-structure, also called hairpin structure. The stem is composed of two arm sequences on either side of the loop and often contains 5 – 6 complementary base pairs that form a stable structure in absence of target. The sequence of the arms is preferably unrelated to the target but can also be designed differently. A fluorescent marker is covalently attached to one end of the hairpin and a non-fluorescent quencher moiety is attached to the other end.\textsuperscript{13} The loop part of the probe contains the predetermined target sequence, usually 15 – 30 nucleotides long.
The sequence and the length of the loop can be tailored as required with the aim that a stable hybrid is formed with the target. The working principle of a MB is shown in Scheme 1.4.

The stem keeps the fluorescent marker and the quencher in close proximity to each other and thus the fluorescence from the marker is quenched and no signal is obtained. Introduction of the target DNA induces a thermodynamically driven hybridization of the MB and a rigid probe-target hybrid, which is longer and more stable than the hairpin, is formed. The arm sequences are forced apart due to the spontaneous conformational reorganization and the fluorophore and the quencher move away from each other, which restores the fluorescence of the marker and a signal is attained.\textsuperscript{13}

![Scheme 1.4. Working principle of a molecular beacon DNA sensor.\textsuperscript{8,13}](image)

High selectivity for the target in question is a crucial characteristic of any DNA sensor and one of the most important properties of the MBs is their ability to identify single base mismatches. The structural constraints imposed by the hairpin conformation needs to be overcome and, as a fully complementary target is most likely to prevail, the excellent selectivity has been attributed to the presence of the hairpin structure.\textsuperscript{8,13} In the literature there is a great variety of detection schemes involving MBs and a rough division into solution based- and surface-attached assays can be made. Solution based assays are useful when it is not possible or desirable to isolate the probe-target from an excess of the probes, for example
when biological processes are studied in real time and in vivo (PCR or detection of RNA in living cells). There is no need for labelled targets and the method is highly sensitive and selective, especially when stringency conditions (reactions conditions that are unfavorable for mismatches) are applied.\textsuperscript{8, 9, 13, 18}

Fang et al.\textsuperscript{17} were among the first to report on MBs in surface-attached sensing schemes. The MBs were immobilized on an avidin-conjugated glass surface to which a biotinylated ssDNA MB was attached as visualized in Scheme 1.5. The sensor showed sensitivity for its target sequence, with a detection limit below nmol l\textsuperscript{-1}. This nanometer-scale optical biosensor technology has been used for the development of DNA sensors for single living cell monitoring and DNA molecular dynamic interaction studies.\textsuperscript{17}

![Scheme 1.5. Schematic of a biotin-avidin binding surface-attached DNA sensor.\textsuperscript{17}](image)

Another approach, which also utilized the strong affinity between avidin and biotin for surface immobilization of MBs was presented by Piestert et al.\textsuperscript{19} The authors were able to detect target sequences down to concentrations of $10^{-13}$ M utilizing the low oxidation potential of the DNA base guanine for efficient quenching of the dye, via photoinduced electron transfer. A 20-fold increase in fluorescence intensity upon specific binding of the target sequence was detected.\textsuperscript{19} The avidin-biotin bond was also employed by Yao et al.\textsuperscript{20} to attach
MBs to quartz glass slides. The MBs were excited with an evanescent wave field, produced by a quartz prism, while the cDNA solution was introduced.

Time-lapse fluorescence images of the surface hybridization progression were obtained by a fluorescence microscope equipped with an intensified charge couple device (ICCD). The surface-immobilized MBs showed two major types of kinetics during their hybridization: fast dynamics for 87.5% of the MBs and slow dynamics for 12.5% of the MB probes. The authors also found that the fluorophores in newly formed double-stranded hybrids were much more stable optically than those of the denatured single-stranded molecular beacon DNA probes. Wang et al. chose to study the influence of different substrates, glass slides and agarose films, on the performance of their MB-based DNA sensor. The annealing and hybridization processes of the MBs were investigated and it was concluded that the agarose film provided an ideal substrate as there was low fluorescence background and a high mismatch discrimination ratio.

More recently gold has been used as a substrate for immobilization of MBs. Du et al. demonstrated that fluorophore-tagged DNA hairpins attached to gold films (Scheme 1.6) preformed well as MBs. Two DNA hairpins were attached to a gold substrate through a thiol-gold bond and when the probe was exposed to the complementary target, a considerable increase in fluorescence intensity was measured. The sensor was shown to be unresponsive to nonspecific targets, but discrimination against single-base mismatches was not evaluated. A more thorough investigation of the sensitivity and selectivity of a surface-immobilized MB DNA sensor, where the gold substrate was actively involved in the sensor, acting as the quencher for the fluorescent marker was published a couple of years later. The hybridization efficiency was found to be sensitive to the secondary structure of the hairpin, as well as to the surface distribution of DNA hairpins on the substrate. The identity of the bases used in the
hairpin stem and the overall loop length significantly affected the sensitivity and selectivity of
the sensor\textsuperscript{15} and carefully designed, the surface-immobilized hairpins were able to
discriminate between single base-pair mismatched sequences with high sensitivity.\textsuperscript{15}

![Scheme 1.6. Schematic of a MB sensor immobilized on a gold substrate.\textsuperscript{14}](image)

There is great potential for use of MBs in chip-based arrays and different types of biosensors,
especially with gold as the substrate. Not only does gold have the ability to quench
fluorescence, it is also commonly used as an electrode material for electrochemical sensing,
which will be discussed in the next section.

\textbf{1.3.2 Electrochemical DNA sensors}

The process involved in the operation of any DNA hybridization sensor can be
generalized into 3 steps: 1) base-pair recognition, 2) signal transduction and 3) readout\textsuperscript{22} as
shown in Scheme 1.7. Electrochemical DNA sensors combine an ODN layer with an
electrochemical transducer to provide fast, simple and cost-effective ways of highly sensitive
target detection. Minimal power requirements and compatibility with modern fabrication
technologies are other features that make electrochemical transducers attractive for DNA
sensor designs.\textsuperscript{22}
Scheme 1.7. The process involved in the operation of a DNA hybridization sensor: base-pair recognition, signal transduction and readout. Reproduced from Wang et al.22

Electrical biosensors measures changes in current (amperometric sensors), voltage (voltammetric sensors) and/or impedance (impedometric sensors) to detect the hybridization event.23-25 In both voltammetric and amperometric sensors the current is monitored at an electrode as the potential is varied.26 An impedance sensor, on the other hand, relies on a current-voltage ratio for the readout.27

In 2003 a comprehensive review on electrochemical DNA sensors was presented by Drummond et al.24 Five categories of sensors were identified, depending on what component provides the electrochemical transduction, as follows: 1) direct DNA electrochemistry, 2) indirect DNA electrochemistry, 3) DNA-specific redox indicator detection, 4) nanoparticle based electrochemical amplification and 5) DNA-mediated charge transport.24 All of these sensing schemes show target sensitivity at femtomol levels, with even atto- or zeptomoles have been detected by indirect or nanoparticle-amplification methods (2 and 4). The main advantage of using the intrinsic electroactivity of DNA or DNA-mediated charge transport design (1 and 5) is that no labels are required.

Direct DNA electrochemical detection relies either on the electroactivity of the nucleic acids (oxidation of the purine bases in the DNA chain) or changes in electronic or interfacial properties induced by hybridization, for signal transduction.22 In 1988 Palecek et al. presented
a method to distinguish between single- and double stranded DNA immobilized on a hanging mercury drop electrode, with adsorptive transfer stripping voltammetry (AdTSV). Another direct electrochemical detection of DNA was accomplished by Singhal et al., using sinusoidal voltammetric detection at copper microelectrodes. In this case the detection relied on the electrocatalytic oxidation of sugars and amines at copper surfaces. As all DNA molecules contain a sugar backbone and primary amines the different bases of any kind of oligonucleotide can be detected by this method. The sensitivity increases with the length of the oligonucleotide and both ssDNA and dsDNA were detected in the pM concentration range. An electrochemical sensor, which used the oxidation signal of guanine for the detection of an allele-specific factor V Leiden mutation from PCR products, was developed by Ozkan et al. Differential pulse voltammetry (DPV) was employed to monitor the guanine signal, as a result of the specific hybridization between the probe and the PCR product at a carbon paste electrode. No labeling was necessary and the assay offered fast and simple detection of the factor V Leiden mutation. More recently, Komarova et al. developed a direct DNA sensor based on thin polypyrrole films, doped only with an oligonucleotide. The sensor design is presented in Scheme 1.8 The sensitivity of the sensor was on the verge of pmol l^{-1} of target, when chronoamperometry was used. No label was needed and the sensor was reusable and highly stable.
In addition to the direct electrochemical DNA sensor, Komarova et al. investigated an *indirect electrochemical sensor*, utilizing a horseradish peroxidase-labeled probe, as shown in Scheme 1.9.\textsuperscript{31} A sandwich approach was used, where a target ODN was hybridized to the dopant-ODN in the pPy film, followed by a second hybridization between the previous target and a horseradish peroxidase (HRP)-labelled probe. Another indirect approach is to oxidize target DNA through electrochemical mediators. A polypyridyl complex of Ru(II) and Os(II) has been used to mediate the oxidation of guanine in order to detect trinucleotide repeat expansions.\textsuperscript{32} Guanine residues were immobilized within the target sequence, providing catalytic currents and a linear dependence on the repeat number was observed.\textsuperscript{32}

Scheme 1.8. Schematic of direct electrochemical detection of DNA hybridization using polypyrrole-(pPy) coated electrode.\textsuperscript{31}
DNA sequences labeled with *redox-active reporter molecules* have also been investigated in different sensor designs. The hybridization is detected based on the electrochemical response from the marker. Hashimoto et al. presented a voltammetric sensor, using Hoechst 33258, which is a DNA minor groove binder and an electrochemically active dye, in 1994. The use of Hoechst 33258 resulted in a sequence-specific detection of 1.6 μM of target DNA.\(^1\) Kelley et al. employed different redox-active intercalators, such as Ru(NH\(_3\))\(_6\)\(^{3+}\) and Fe(CN)\(_6\)\(^{4-}\) for
electrochemical detection of DNA. The DNA-mediated electron transfer served as sensitive means of detection as various mismatches could be detected. The assay developed by Kelley et al. was also able to detect mutations within a duplex, thus providing an option to hybridization-based assays. More recently a sensitive, reagentless and reusable electrochemical DNA sensor, based on a surface-attached, molecular beacon-like DNA probe was described. The probe was labeled with an electroactive reporter, which recognized the hybridization event. The distance between the label and the electrode was significantly changed upon hybridization, resulting in a large and readily measurable signal.

Nanoparticl (NP)-based sensors have been successfully employed in optical DNA sensors and Willner et al. managed to combine the means for both optical fluorescence and electronic (photoelectrochemical) transduction of a DNA-sensing events in a sensor. Double-stranded DNA was cross-linked with CdS NPs in arrays on electrode supports and photocurrent was generated upon irradiation of these arrays. Wang et al. employed NPs with different redox potentials as labels in a DNA sensor. The probes were immobilized on magnetic beads, which after hybridization with target DNA, were magnetically separated from the bulk solution and re-hybridized with a NP-modified reporter sequence. The products were then separated, the NPs dissolved and analyzed by anodic stripping voltammetry. Simultaneous detection of three different targets, labeled with different NPs was also achieved electrochemically. A different approach was used by Ozsoz et al., who monitored the direct oxidation of gold NP labels upon hybridization. The probe DNA was covalently attached to a graphite electrode when hybridized with the tagged target DNA.

Sensors based on DNA-mediated charge transport generally contain a redox-active reporter molecule that is intrinsically, but non-covalently, related with the double helix. The first to explore the area of electrostatic probe molecules were Millan et al. In their design, DNA
was covalently immobilized onto carboxylic acid groups on the surfaces of oxidized glassy carbon electrodes. Hybridization with complementary target induced a preconcentration of cobalt complexes near the electrode surface due to the higher negative charge density at the hybridized electrode. The response was detected with cyclic voltammetry, which was sensitive for the characteristic redox reactions of the cobalt complexes. Steel et al. reported on a DNA sensor based on a self-assembled monolayer of thiolated DNA probes on gold. Ru(NH₃)₆³⁺ was employed as a charge-compensating redox marker and was thus accumulated at the electrode surface as the hybridization reaction proceeded. This redox reporter provided qualitative results, with high resolution, in a simple and effective fashion.

1.4 Self-assembled monolayers (SAMs) on gold

Self-assembled monolayers (SAMs) are two-dimensional nano-assemblies, which have become the foundation for many nanostructured systems. The formation of these single-molecule thin layers is spontaneous when the substrate is exposed to a solution containing the surfactant, and does not require any external input. The first spontaneous formation of a monolayer, alkyl amines on a platinum surface, was observed by Bigelow et al. in 1946. Almost forty years later di-n-alkyl disulfides were found to form ordered layers on gold, resulting in rapid developments in the field. As it is possible to accurately control formation of these layers, SAMs have been frequently used as platforms in DNA sensing.

Gold is a commonly used substrate for self-assembly of thiolated species for a number of reasons. Thin gold films are easily made by thermal evaporation or sputtering techniques and a variety of supports can be used to grow gold films. Additionally, no stable oxide of gold is formed at room temperature and none of the regularly present atmospheric gases chemisorbs on gold. Cleaning procedures are straightforward, such as oxidation of carbonaceous species
with piranha solution (a 1:3 mixture of 30 % H₂O₂ and concentrated H₂SO₄) or repeated voltammetric cycling between -0.3 V and +1.5 V versus Ag/AgCl. Often, merely repeated washing with common solvents is sufficient to clean the Au surface. Although there are several different kinds of monolayers the focus here will be solely on SAMs formed on gold.

SAM preparation is simply achieved by dipping substrates into a mM-solution of the surfactant or by adding a droplet of the surfactant solution on a surface. Firstly the assembled molecules attach to the surface in a fast reaction step, which is then followed by a slow reorganization step. The self-assembly process generally takes between 12 and 24 h if a well-ordered and dense layer is desired. Mixed monolayers can be formed through ligand exchange by using a solution that already contains both components. For example, Herne et al. showed how 6-mercapto-1-hexanol was able to disrupt nonspecific interactions between a gold surface and the backbone of a thiol-modified single-stranded DNA. This resulted in a mixed self-assembled monolayer (mSAM), where the DNA probes were attached to the gold primarily through its sulfur group, which was believed to have enhanced the efficiency of the sensor. One of the disadvantages of SAMs, especially when used for sensor applications, is that two SAMs will never be arranged in exactly the same way. Depending on the conditions used the stability of SAMs can vary for months to days. Horn et al. observed changes in the C-H stretching bands of three straight-chain alkanethiol SAMS over almost 6 months, although the major changes seemed to occur after 20 days. Temperature, concentration, type of solvent and substrate used are all parameters that can be varied, which also impact on SAM layer formation and stability. Herne et al. heated their SAM sensor platform to 70 °C in order to regenerate the sensor and no loss of probes was observed when the sensor was re-hybridized. It is thus reasonable to assume that thiolated SAMs show the required stability and robustness needed as DNA sensing platforms.
1.5 Quantum Dots (QDs)

Quantum dots (QDs) belong to a class of materials that are referred to as nanoparticles (NPs) but while NPs includes semiconductor, metal, insulator, organic and other particles, the term QDs is only used for semiconducting particles. Consequently, QDs are defined as inorganic, colloidal, semiconducting nanocrystals composed of elements from the periodic groups II-VI, III-V or IV-VI. They are typically roughly spherical with sizes ranging from 1 - 12 nm and as the radius of the particles approaches the Bohr radius of an exciton, quantum size effects manifest themselves as characteristic properties of the QDs. The quantum size effects mainly involve confinement of the electronic states, i.e. the widening of the highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) gap with decreasing size of the particles. A direct consequence is that both the optical absorption and emission shifts to the blue (higher energies) as the size of the particle decreases as shown in Figure 1.2, where different sized CdTe QDs are excited at 360 nm.

![Figure 1.2. CdTe QDs of various size excited at the same wavelength, 360 nm.](image)

The properties of the QDs also depend on their elemental composition, which together with the tuneable optical characteristics via particle size, offers a substantial control of the QD features. Figure 1.3 shows representative QD core materials in relation to their emission wavelengths. If Figure 1.2 and 1.3 are compared, there is some disagreement between the figures regarding CdTe QDs. In Figure 1.3 the CdTe QDs do not exhibit emission wavelengths in the blue region of the spectrum, while a blue colour is clearly visible in Figure
1.2. The reason for this disagreement is the continual development in the field, CdTe QDs small enough to emit in the blue region have now been successfully synthesized, although they were not available when Figure 1.3 was created.

![Figure 1.3. Representative QD core materials scaled as a function of their emission wavelength.](image)

Broad absorption with narrow photoluminescence (PL) spectra over a wide range of wavelengths, a high quantum yield together as well as high resistance to photobleaching and degradation are properties that have made it possible for the QDs to rival organic dyes in many applications.\(^5\)\(^2\)

In order to stabilize and protect the QDs, the core material can be capped with another inorganic material that has a wider bandgap than the core. This means that the energy difference between the valence and the conducting band in the shell needs to be greater than that in the core. A core-shell structure passivates nonradiative recombination sites on the surface, which results in a highly improved PL efficiency. In core-shell QDs the electrons and holes are most likely to be found in the same section (either in the core or in the shell), but the carriers can also be spatially separated, i.e. the electrons and the holes are located in different sections of the structure.\(^5\)\(^5\) This spatial separation of the carriers results in absorption and emission of at lower energies than the bandgaps of the individual materials exhibit. Particles that are passivated with shell structures are also more robust than organically passivated dots.
and can be more easily processed.\textsuperscript{55} Additionally, the shell protects the particle from oxidation and prevents leakage of the core crystal into the surrounding solution.\textsuperscript{40, 56, 57} A rough schematic of a core/shell CdSe/ZnS QD is presented in Scheme 1.10.

Scheme 1.10. A schematic of a core/shell QD structure.

Traditionally the QD synthesis pathways produced hydrophobic QDs,\textsuperscript{56, 58-60} which needed to be modified to become both water-soluble and biocompatible for use as biological labels.\textsuperscript{57, 61-63}

Substitution of the native tri-n-octylphosphine (TOP) and tri-n-octylphosphine oxide (TOPO)\textsuperscript{56, 58, 59} with bifunctional ligands, e.g. mercaptoacetic\textsuperscript{62} or lipid acid\textsuperscript{63} are common procedures to produce water-soluble QDs. Encapsulation of the original QDs in a layer of silica\textsuperscript{61} or polymers\textsuperscript{64, 65} has also been employed. Scheme 1.11 shows a sketch of the functionalization process of QDs, from a QD as synthesized (A) through the hydrophobic step (B) and to the final hydrophilic QD (C).
Scheme 1.11. A rough schematic of the functionalization-stages of quantum dots: A) a QD as synthesized, B) a hydrophobic QD (soluble in hexane, toluene and chloroform) and C) a hydrophilic QDs with an excess cap that makes it soluble in buffers.

Presently, QDs are employed in various applications, such as biological labelling, photovoltaics, electrochromic devices or photocatalysis. They complement the use of organic dyes, especially in the biological field but have also paved the way for other inorganic-biological hybrid materials. An interdisciplinary approach is important to maximize the benefits of the special properties that QDs possess and to develop new areas where they can be utilized.
Chapter 2 - Materials and methodology

This chapter describes the experimental details and the methodologies used in this work. As the techniques, which have been utilized for characterization purposes or to detect the sensor response, are extensively described in the literature only the basic principles for each of the techniques are presented here.

2.1 Synthesis

2.1.1 Quantum Dots (QDs)

2.1.1.1 CdSe/ZnS QDs

The synthesis of CdSe/ZnS core/shell QDs was done according to the literature.\textsuperscript{57, 59} Briefly, CdO was added to mixture of n-dodecylphosphonic acid, trioctylphosphine oxide (TOPO) hexadecylamine (HDA), degassed and heated to 300 °C under argon flow, followed by stabilization at 270 °C. A solution of Se in triocetylphosphine (TOP) was quickly injected to the mixture. The resulting CdSe nanocrystals were redispersed in heptane with subsequent addition of TOPO and HDA, followed by heating to 200 °C under argon flow. A mixture of zinc ethylxanthate in dioctylamine, and zinc stearate dispersed in octadecene, was injected at a rate of 4.8 ml h\textsuperscript{-1} to create the ZnS shell around the core crystals.

2.1.1.2 CdTe QDs

The synthesis of CdTe QDs was performed according to the procedure developed by Rogach et al.,\textsuperscript{66} with some modifications. First, NaHTe was prepared by adding 40 mg NaBH\textsubscript{4} to a flask containing 46 mg tellurium powder and 2 ml Milli-Q water under nitrogen atmosphere. The reaction was kept on for several hours until all tellurium powder was dissolved. 0.092 g (0.5 mmol) of CdCl\textsubscript{2} and 0.092 mg (1 mmol) of thioglycolic acid were dissolved in 100 ml Milli-Q water, followed by adjusting pH to 8.2 by addition of 1 M NaOH
solution. The mixture was deaerated by N₂ bubbling for 30 min. Then NaTeH solution (0.062 mmol) was quickly injected into the mixture under vigorous stirring, followed by refluxing the mixture for 10 min under open-air conditions. 400 ml of 2-propanol was added to as-prepared CdTe QDs colloid solution to precipitate the CdTe QDs, which were collected by centrifugation. The obtained CdTe QDs were dried at room temperature under vacuum, dissolved in 10 ml of Milli-Q water and then used as prepared for the subsequent experiments.

2.1.2 Ligands for functionalization of the CdSe/ZnS QDs

Ligand 1 (8-thio-3,6-dioxaoctanol) and 2 (DSBA, (5-(6,8-diaza-7-oxo-3-thiabicyclo[3.3.0]oct-2-yl)-N[7-(3-[2-(N[7-(6,8-diaza-7-oxo-3-thiabicyclo[3.3.0]oct-2-yl)pentanoylamino]heptyl]-carbamoyl)ethyl]disulfonyl]propanoylamino)heptyl]pentanamide)), which were used for the functionalization of the CdSe/ZnS QDs, were synthesized according to Charvet et al., with some modifications, following the pathways visualized in Schemes 2.1 and 2.2, respectively.

2.1.2.1 Ligand 1 (8-thio-3,6-dioxaoctanol)

Ligand 1 was obtained by dissolving 29.6 mmol of 2-(2-(2-chloroethoxy)ethoxy)ethanol and 39 mmol of thiourea in 15 ml of milli-Q water and stirring under reflux for 2.5 h. A solution of 2.5 g sodium hydroxide dissolved in 20 ml of milli-Q water was added and the reflux was continued at 95 °C for another 2.5 h. After cooling the mixture, 6 ml of concentrated sulfuric acid in 4 ml of milli-Q water was used for acidification and 5×25 ml of ethyl acetate was used for the extraction of the aqueous phase. The collected organic phases were dried over sodium sulfate, filtered, evaporated under reduced pressure and purified on silica (chloroform/methanol 8:1 and ethylacetate/hexane 2:1) to yield Ligand 1, as a colourless oil.
2.1.2.2 Ligand 2 (DSBA)

Ligand 2 was synthesized from the precursors A, B and C (biotin N-hydroxysuccinimide ester, N-(13-amino-4,7,10-trioatridecanyl) biotinamide and 11-mercaptoundecanoyl-N-hydroxysuccinimide ester, respectively) as shown in Scheme 2.2.

Scheme 2.2. Synthetic pathway for Ligand 2, DBSA ((5-(6,8-Diaza-7-oxo-3-thiabicyclo[3.3.0]oct-2-yl)-N[7-[3-[[2-(N[7-[5-(6,8-diaza-7-oxo-3-thiabicyclo[3.3.0]oct-2-yl]pentanoylamino)heptyl]-carbamoyl]ethyl]disulfonyl]propanoylamino)heptyl]pentanamide))
Biotin N-hydroxysuccinimide ester, compound A

Biotin N-hydroxysuccinimide ester was synthesized by adding 24.44 mmol of 1,3-dicyclohexylcarbodiimide to a stirred solution of equimolar amounts (20.47 mmol) of biotin and N-hydroxysuccinimide in 150 ml of anhydrous N,N-dimethylformamide (DMF) according to Bayer et al. The mixture was stirred at room temperature for 48 h. The formed dicyclohexylurea was filtered off and the solvent was evaporated under reflux. 500 ml of diethyl ether was added to the residue and the solution was stirred for 2 h to yield a white precipitate, which was filtered under reduced pressure and recrystallized in isopropanol. The final product, 4.2 g of a white powder, was allowed to dry in a vacuum oven at 50 °C for 3 - 4 hours before further use.

N-(13-Amino-4,7,10-trioxatridecanyl) biotinamide, compound B

The procedure on preparation of N-(13-Amino-4,7,10-trioxatridecanyl) biotinamide was reported by Wilbur et al. 4.2 g of biotin N-hydroxysuccinimide ester, A, was dissolved in 100 ml of dry DMF. Under inert atmosphere, this solution was added dropwise within 1 h to a solution of 13.56 g of 4,7,10-trioxa-1,13-tridecanediamine in 4 mL of triethylamine. The reaction mixture was stirred at room temperature for 65 h, after which the solid that was formed was filtered off. Evaporation of DMF from the solid was carried out under reduced pressure. The resulting oil was added dropwise, and under continuous stirring, to 850 ml of hexane and a white precipitate was formed. The solution was stirred for several hours followed by recrystallization in isopropanol for 2 h at reflux and then for 12 h without heating or stirring. After drying the product in a vacuum oven at 50 °C a final yield of 5.7 g of white crystals was obtained.
11-mercaptoundecanoyl-N-hydroxysuccinimide ester, compound C

Connolly et al.⁷⁰ has published the preparation procedure for 11-mercaptoundecanoyl-N-hydroxysuccinimide ester as follows: 1.04 g of N-hydroxysuccinimide in 500 ml of dichloromethane was stirred for 30 min followed by addition of 1.88 g of 11-mercaptoundecanoic acid (MUA), dissolved in 10 ml of dichloromethane. 1.95 g of 1,3-dicyclohexylcarbodiimide was dissolved in 50 ml of dichloromethane and added dropwise, within 30 min, to the reaction mixture. The mixture was then stirred for 26 h and after filtration and evaporation of the solvent the residue was purified with flash chromatography (hexane/diethylether, 1:1) and approximately 1.2 g of 11-mercaptoundecanoyl-N-hydroxysuccinimide ester was obtained.

Ligand 2

Once 11-mercaptoundecanoyl-N-hydroxysuccinimide ester, C, is obtained it can be reacted with N-(13-amino-4,7,10-trioxatridecanyl) biotinamide, B, to form ligand 2.⁷⁰ 894 mg of B was added to a solution of 630 mg C, in 100 ml of dry chloroform and 200 ml of triethylamine. The reaction mixture was stirred at room temperature for 4 h under inert atmosphere. Chloroform was evaporated under reduced pressure and the residue was purified by flash chromatography (dichloromethane/methanol, 9:1, ethylacetate/methanol 20:1, ethylacetate/methanol 5:1 and methanol). 1.1 g of yellowish oil was obtained as the final product.⁷⁰
2.1.3 Functionalization of CdSe/ZnS QDs

2.1.3.1 Ligand 1 and 2

In order to obtain water-soluble CdSe/ZnS QDs, ligands 1 and 2 were used for functionalization (biotinylation) of the synthesized QDs. This was done according to the procedure described by Charvet et al.,\textsuperscript{57} with some modifications. 21.7 mg of ligand 1 and 4.9 mg of ligand 2 dispersed in 20 µl of dry chloroform was mixed with 500 µl of a 8 g l\textsuperscript{-1} QD-solution. The mixture was stirred under inert atmosphere, at room temperature and covered to prevent photodegradation for 48 h. Then 500 µl of milli-Q water and 100 µl of acetone were added and the mixture was stirred for 1 h. Another 100 µl of water was added and the stirring continued for another 10 min. After separation of the aqueous layer the solution was centrifuged at 1320 rpm for 5 min. No precipitate was obtained and thus the amount of solvent was reduced by heating the solution under nitrogen flow. Again, 100 µl of water was added to the sample, followed by centrifugation. This procedure was repeated two times before the QD precipitated. The supernatant was removed, followed by evaporation to dryness to yield a colored solid, which was redispersed in water for further use.

2.1.3.2 Thioglycolic- and dihydrolipoic acid (TGA and DHLA)

Thioglycolic acid (TGA) and dihydrolipoic acid (DHLA) were also used for the replacement of the TOPO-ligands on the hydrophobic QDs. The procedure reported by Clapp et al.\textsuperscript{71} was followed with some modifications. 100 µl of 0.28 M DHLA or 1 ml of 1 M TGA was mixed together with 200 µl of 0.26 M CdSe/ZnS QDs and added to 1 ml of milli-Q water. A further 800 µl of chloroform was added and the mixture was shaken at 1000 rpm at room temperature for 18 h (DHLA) or 90 h (TGA). After it could be visibly established that the QDs had transferred to the aqueous phase the chloroform was removed. The remaining
suspension was centrifuged through a micro centrifuge filter, with a nominal molecular weight limit of 100,000 Da, at 2200 rpm for 4 min and used as prepared.

2.2 Materials and Chemicals

2.2.1 Oligonucleotides (ODNs)

The DNA oligonucleotide (ODN) sequences were synthesized by Alpha DNA (Quebec, Canada) and the sequences used in the sensor designs are listed in Table 2.1. Table 2.2 contains additional sequences, used for characterization of the HPP and its duplex formation with different targets in solution (Section 3.3).

<table>
<thead>
<tr>
<th>Oligonucleotide sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotin-modified probe, HPP-1</td>
</tr>
<tr>
<td>Cy3-modified probe, HPP-2</td>
</tr>
<tr>
<td>Amine-modified probe, HPP-3</td>
</tr>
<tr>
<td>Un-modified probe, HPP-4</td>
</tr>
<tr>
<td>Short Cy3-probe, Cy3-P</td>
</tr>
<tr>
<td>Complementary target, cDNA</td>
</tr>
<tr>
<td>One-point mismatch target, 1MM (std)</td>
</tr>
<tr>
<td>Non-complementary target, NC</td>
</tr>
</tbody>
</table>

Table 2.1. Summary of the synthesized ODN sequences used in the sensor designs.
Table 2.2. Summary of the ODN sequences used for the characterization of the melting behavior of the HPP and its targets.

<table>
<thead>
<tr>
<th>Oligonucleotide sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Longer complementary target, long cDNA</td>
</tr>
<tr>
<td>One-point mismatch target, 1MM (center)</td>
</tr>
<tr>
<td>One-point mismatch target, 1MM (end)</td>
</tr>
<tr>
<td>2nd non-complementary target, NC-2</td>
</tr>
</tbody>
</table>

2.2.2 Spacer molecules

Thiolated m-PEG [H₃C-(CH₂CH₂O)₆CH₂CH₂SH], molecular weight 356.5 g mol⁻¹, was purchased from Polypure (Norway). Thioglycolic acid (TGA), also called mercaptoacetic acid (MAA), (≥ 98 %) was obtained from Sigma-Aldrich.

2.2.3 Buffers

SSC buffer (10 mM NaCl and 1 mM sodium citrate) and PBS buffer (0.01 M) was prepared using milli-Q water (18.2 Ω cm resistivity).

2.2.4 Gold substrates and electrodes

An Au foil, with a purity of 99.99 % and thickness of 0.050 mm, was obtained from Advent Research Materials Ltd. For the ATR-FTIR, quenching study and the optical sensor experiments, a circular piece, with a diameter of 15 mm, was used.
Four different gold substrates were used as substrates for the characterization of the mSAM by AFM: 100 nm Au on 50 nm Ti on glass (Au 1), thin Au film on glass (Au 2), 10 nm of Au sputtered on silica (NDL-Au), provided by the National Nano Device Laboratories, Hsinchu, Taiwan, and 150 nm of evaporated Au on freshly cleaved mica (Au-Mica) from Structure Probe Inc.

The substrates used for neutron reflectometry were obtained from the Centre for Neutron Research (NCNR) at the National Institute of Standards and Technology (NIST), USA. Silicon wafers, with a diameter of 75 mm and a thickness of 5 mm, were coated with approximately 30 Å of Cr (NR) or Fe (Polarized Neutron Reflectometry, PNR) and 150 Å of gold in a DC Magnetron Sputtering chamber (Auto A306; BOC Edwards, UK).\textsuperscript{72-74}

The gold working electrodes (with a diameter of 1.6 mm) the Ag/AgCl reference electrode and a platinum wire counter electrode were obtained from Bioanalytical Systems Inc. and used for all the electrochemical measurements.

2.3 \textbf{Formation of self-assembled monolayers (SAMs) as sensor platforms}

The prepared mSAMs were always stored in buffered solution between measurements to ensure the stability of the layers. As it had been previously shown that major changes occur in SAMs of alkanethiols assembled on gold after a period of twenty days\textsuperscript{75}, the mSAM prepared in this work were kept and used for a maximum of fourteen days. During this time period no loss of material on the gold surfaces was observed.
2.3.1 Pretreatment of gold substrates and monolayer preparation for SAM characterization by Attenuated Total Reflection-FTIR (ATR-FTIR)

A circular piece of gold foil, with a purity of 99.99 %, a thickness of 0.050 mm and a diameter of 15 mm was fitted in a Teflon cell and used as the substrate for the ATR-FTIR experiments. The Au foil was cleaned with piranha solution and ultra-sonicated in methanol for 15 min before usage. The mSAM was formed by simultaneous immobilization of HPP-1 and m-PEG onto the Au surface. All ODN-, dye- and m-PEG-solutions were prepared with PBS buffer, unless otherwise specified. The Au foil was incubated with 100 µl of a 4.65 µM HPP-1 and 100 µl of a 4.65 µM m-PEG solution for 90 min, followed by washing with PBS buffer (pH 7.4) to remove any unattached molecules. Avidin was attached by placing a 100 µl drop of a 1.89 µM solution on the SAM for 1 h. After rinsing with PBS buffer 100 µl of 4.65 µM Cy3 or CdSe/ZnS QD solutions were added and incubated for 1 h followed by extensive rinsing. All immobilization and attachment steps were carried out at room temperature.

ATR-FTIR spectra were collected between every immobilization step and after hybridization. Spectra were also measured for samples containing: 1) only m-PEG, 2) only Cy3-probe, 3) only cODN (100 µl of the relevant 4.65 µM sample solution immobilized in room temperature for 1 h), and 4) only CdSe/ZnS QDs (20 µl of 0.37 M QD solution immobilized in room temperature for 1 h). Prepared mSAMs were always stored in buffered solutions between measurements to ensure the stability of the thiol linkages.
2.3.2 Pretreatment of gold substrates and monolayer preparation for SAM characterization by Atomic Force Microscopy (AFM)

The four gold substrates, Au 1, Au 2, NDL Au and Mica Au, mentioned in section 2.2.4 were used as substrates for the characterization of the mSAM by AFM. High resolution AFM images were collected and analyzed with the software Nanoscope from Digital Instruments.

 Depending on the substrate used, due to size variations, different amounts of solution were placed on the gold to create the mSAMs (80 or 200 µl for the NDL Au substrates and 20 or 30 µl for the Mica Au). Au 1 and Au 2 were not used as substrates for characterization of the mSAMs due to the high surface roughness of the bare substrates. The NDL Au and Mica Au substrates were used to investigate SAMs with m-PEG only, HPPs only (HPP-4) and m-PEG:HPP-4 molar ratios of 2:1, 10:1 and 25:1. Immobilization of the mSAMs was done at room temperature for 1 h and the samples were rinsed with milli-Q water and dried with N₂ (g) before AFM images were collected.

2.3.3 Pretreatment of gold substrates, monolayer preparation and hybridization used for neutron reflectometry (NR)

2.3.3.1 Experimental details for NR performed at ANSTO, Australia

Gold-coated silicon wafers,\textsuperscript{72-74} as described in Section 2.2.4, were used as substrates for the NR measurements. The wafers were rinsed with ethanol prior to SAM formation. Two immobilization strategies were used: (1) immobilization of the gold surface with HPPs, followed by addition of m-PEG molecules and (2) simultaneous self-assembly of probes and m-PEG from a mixture containing a predetermined molar ratio of the two components. The self-assembly of probes and/or m-PEG on the gold surface was done by flowing 3 ml, of a solution with a predetermined probe and/or m-PEG concentration, through the assembled cell.
For immobilization strategy (1), the probe (HPP-4) solution (4.65 μM) was pumped first through the cell at a rate of 3 ml h\(^{-1}\) during 1 h, at room temperature. The surface was rinsed thoroughly with PBS buffer to remove any unattached HPPs, followed by back-filling with m-PEG, deposited in the same manner (flow rate and time) as the probe but with a m-PEG concentration of 46.5 μM. The surface was then thoroughly rinsed again with PBS buffer to remove any remaining unattached m-PEG molecules. A similar deposition procedure was used for immobilization strategy (2) but molar ratios of m-PEG:HPP (2:1 and 10:1) were used for simultaneous immobilization during 1 h at room temperature.

Hybridization was carried out by flowing 6 ml of a 4.65 µM complementary target solution through the assembled cell at a flow rate of 5 ml h\(^{-1}\) using a syringe pump. The temperature was kept at 37 °C. After hybridization the surface was thoroughly rinsed with PBS buffer to remove any non-hybridized target DNA.

2.3.3.2 Experimental details for polarized neutron reflectometry (PNR), preformed at NIST Centre for Neutron Research, USA

Gold-coated silicon wafers,\(^{2-74}\) as described in Section 2.2.4, were used as substrates for the PNR experiments. The gold-coated wafers were rinsed with ethanol prior to SAM formation. HPP-4 was initially immobilized for 1 h but after the first measurement the immobilization was continued for an additional hour to enhance the density of the probes. After the mSAM was formed and PNR data had been collected at 22 °C, the temperature was raised stepwise and data were acquired at selected temperatures of 35, 45, 55 and 65 °C. The temperature was allowed to stabilize for at least 10 min before each measurement.

Hybridization was carried out by flowing 3 ml of a 4.65 μM complementary target solution through the assembled cell over an hour. The temperature was kept at 35 °C. After
hybridization the surface was thoroughly rinsed with PBS buffer to remove any non-hybridized target DNA and PNR data was collected at 35 °C.

2.3.4 Pretreatment of gold substrates, monolayer preparation and hybridization for optical signal detection

The experimental procedure for the study of quenching of an indocarbocyanine dye and functionalized CdSe/ZnS quantum dots by gold surfaces was carried out as follows. A circular piece of gold foil, with a diameter of 15 mm and fitted in a Teflon cell, was used as the substrate for the optical detection experiments. The Au foil was cleaned with piranha solution and ultra-sonicated in acetone for 15 min before usage. The SAM of m-PEG onto the Au surface was formed by incubating the Au foil with 80 µl of a 4.65 µM m-PEG solution for 90 min, followed by washing with PBS buffer (pH 7.4) to remove any unattached m-PEG. 60 µl of 38.75 µM Cy3 and CdSe/ZnS QD solutions were drop-cast and dried on both the m-PEG-modified and the unmodified Au foil surface, at 40 °C for 1 h. Following the initial measurements, the surface was rinsed twice with an excess of PBS buffer and emission spectra were collected after each rinsing on air dried surface.

The Teflon cell, with the fitted Au foil, was also used for optical detection of the Cy3- or QD-labeled hairpin DNA sensors. The self-assembly of probes and/or m-PEG on the gold surface was done by placing an 80 µl droplet, with a predetermined probe and/or m-PEG concentration on the electrode surface. For immobilization strategy (1), initially the probe solution, with a concentration of 2.33 µM (HPP-1), was kept at the electrode surface for 30 min in room temperature. The electrode was rinsed thoroughly with SSC buffer to remove any unattached HPP, followed by back-filling with TGA or m-PEG, deposited in the same manner as the probe, but with concentration of 46.5 µM and 1 h immobilization time.
The substrate was then thoroughly rinsed again with PBS buffer to remove any remaining unattached TGA or m-PEG molecules. A similar deposition procedure was used for immobilization strategy (2) in which TGA and HPP or m-PEG and HPP (with molar ratios of TGA or m-PEG to HPP of 10:1) was used for simultaneous immobilization for 1 h.

After the SAM formation 100 µl of a 100 mg l⁻¹ streptavidin solution was placed on the substrate for 20 min in room temperature to attach streptavidin to the biotin-modified 3’-end of the HPP. The Au foil was rinsed thoroughly with PBS buffer to remove any unattached streptavidin. Finally a short biotin-modified and Cy3-labelled short probe (Cy3-P) or QDs were conjugated to the HPP/streptavidin complex, following the above mentioned procedure: 80 µl of a 2.33 µM Cy3-probe was put on the SAM for 30 min in room temperature to allow for the attachment, followed by thorough rinsing with PBS.

Hybridization was carried out by depositing a 100 µl droplet, with a target concentration of 2.33 µM on the Au surface and kept at 40 ºC for 1 h. After hybridization the Au foil was thoroughly rinsed with PBS buffer to remove any non-hybridized target ODN and kept in the SSC-buffer for a minimum of 5 min prior to the measurement.

2.3.5 Pretreatment of gold substrates, monolayer preparation and hybridization for electrochemical signal detection

For the experiments based on electrochemical detection, a gold working electrode was used. Prior to SAM formation the electrode was treated with piranha solution (H₂SO₄/H₂O₂ 70/30), rinsed with milli-Q water, and cycled 100 times between -1.2 and -1.8 V (vs. Ag/AgCl) in standard PBS solution to obtain a clean gold surface. Although a larger potential window is commonly used to oxidize Au when cleaning Au electrodes, the narrower range applied here has been shown to efficiently desorp thiolate layers bound to
Au and was anticipated to cause less wear on the gold electrodes. The electrode was then rinsed with milli-Q water and dried in air. Again, the two different immobilization strategies (1 and 2) were used. The self-assembly of probes and/or m-PEG on the gold surface was carried out by placing a 30 μl droplet, with a predetermined probe and/or m-PEG concentration on the electrode surface. For immobilization strategy (1), initially the probe solution, with a concentration of 4.65 μM, was kept at the electrode surface for 30 min at room temperature. The electrode was then rinsed thoroughly with SSC buffer to remove any unattached HPPs, followed by back-filling with m-PEG, deposited in the same manner as the probe, but with a m-PEG concentration of 46.5 μM and 1 h immobilization time. The electrode was then thoroughly rinsed again with SSC buffer to remove any remaining unattached m-PEG molecules and kept in the SSC buffer for 5 min prior to the measurement. A similar deposition procedure was used for immobilization strategy (2) but molar ratios of m-PEG to HPP of 2:1, 10:1 and 25:1 were used for simultaneous immobilization for 1 h. The immobilization time was kept at a minimum to ensure a swift preparation procedure of the sensor, as this is considered important in sensing application.

Covalent attachment of the CdTe QDs to a NH2-modified HPP (HPP-4) (applicable for the CdTe QD-modified electrochemical hairpin sensor in chapter 5.3) was achieved by placing 40 μl of 4.65 μM CdTe solution and 40 μl of EDC, in molar excess, on the immobilized SAM for 2 h at 44 °C for the spontaneous formation of a covalent peptide bond between the –COOH groups at the QD surface and the NH2-modified HPP-4.

Hybridization was carried out by depositing a 30 μl droplet, with different concentrations (ranging between 4.65 fM and 4.65 μM) of target ODN, on the electrode surface and kept at 37 °C or 44 °C for 1 h. After hybridization the electrode was thoroughly rinsed with SSC buffer to remove any non-hybridized target ODN and kept in the SSC buffer for a minimum
of 5 min prior to the measurement. For the sensitivity measurements increasing concentrations of targets were used for hybridization, while the mSAM sensing platform was kept intact. After mSAM formation hybridization was carried out with the lowest target concentration and electrochemical response was measured as impedance. Subsequent hybridizations were performed with a continuous ten-fold increase of the target concentration and the impedance was recorded with no explicit re-generation of the mSAM between the steps.

2.4 Characterization techniques

2.4.1 UV-vis spectroscopy

2.4.1.1 Introduction

Ultraviolet-visible spectroscopy (UV-vis) is a technique which relies on the absorption of radiation by matter and can be used for either qualitative or quantitative applications. Molecules absorb radiation in certain spectral regions, where the radiation gives rise to some excited state in the molecules. The radiant power of the incident beam is proportional to the number of photons per time unit. An absorption spectrum (absorption vs. wavelength) of a given molecule species thus contain information that is specific to that molecule and can serve as a “fingerprint” for identification purposes. In the UV-spectrometer the sample absorbs a part of the incident radiation and the remainder is transmitted to a detector. The detector then converts the transmitted radiation to an electrical signal, which is usually amplified before it is displayed.26

Quantitative analysis in UV-vis spectroscopy is based on the direct relationship between absorbance at specific wavelengths and concentration; known as the Lambert-Beer law (Equation 2.1).
\[ A = \log \frac{P_0}{P} = \varepsilon b C \]  
(Eq. 2.1)

where \( A \) is the absorbance, \( P_0 \) is the power of the incident radiation, \( P \) is the power of the transmitted radiation, \( \varepsilon \) is the extinction coefficient in units of liter mol\(^{-1}\) cm\(^{-1}\), \( b \) is the path length of the beam in units of cm and \( C \) is the concentration of the absorbing species in units of mol l\(^{-1}\). For the law to hold, the light source need to supply a sufficient amount of monochromatic light to ensure a well defined value for the extinction coefficient, \( \varepsilon \).\(^{26}\)

When performing quantitative analysis with UV-vis spectroscopy, the preferred absorption band has to be chosen from the whole spectrum. This can be done either experimentally, preferably through a wavelength scan, or by consulting available literature. A collected UV-vis spectrum contains information about the whole structure of the sample, not only about specific bonds, as rotational and vibrational modes as well as electronic transitions are represented in it. It is not possible to obtain a purely electronic fingerprint of a sample in the UV region but combined with other spectroscopic methods the identity of the sample species and a more general fingerprint can be determined.\(^{26}\)

2.4.1.2 Characterization of the synthesized QDs

The absorption of the all synthesized QDs was measured in a 3-Q-10 mm rectangular quartz cell, using a UV-1700 Pharmaspec spectrometer from Shimadzu. 20 µl of the original QD solution was added to 2 ml of chloroform (CdSe/ZnS) or milli-Q water (CdTe) to measure the absorbance of the QDs. The absorbance wavelength is related to the size of the QDs and also to the concentration of the QD-solution, which can be determined theoretically based on the first absorption peak observed.\(^{78}\)
2.4.1.3 Melting Profiles of HPPs and probe/target duplex ODNs

The UV-vis spectrometer and quartz cell mentioned in section 2.4.1.2 were used for the melting profile measurements. The melting profiles for the HPPs and the various duplexes (HPP: cDNA, HPP: 1MM-DNA, HPP: NC-DNA etc.) were measured in standard PBS buffer, in which hybridization was also carried out. The UV-vis spectrometer was connected to a temperature controller and the temperature was raised from 20 °C to 80 °C with a ramp rate of 1 °C min$^{-1}$. The absorbance was measured once every degree, at 260 nm. The data was fitted to a sigmoid Boltzmann equation to obtain the melting temperature of the HPP. The melting profile of the buffer alone was subtracted from the raw absorbance versus temperature curves of the HPP. Simulated melting behavior of the HPPs and of the duplexes it formed with complementary, one-base mismatch and non-complementary target ODN was calculated using the DINAMelt server, powered by “UNAFold”.79, 80

2.4.2 Fluorescence spectroscopy

2.4.2.1 Introduction

Photoluminescence is a process where a molecule absorbs photons, which raises it to an excited state, and then re-emits photons when returning to its ground state. Fluorescence and phosphorescence are included in photoluminescence, with the difference that energy transitions involve a change in electron spin (the transition occurs through a triplet state) in phosphorescence but not in fluorescence. Thus fluorescence is a faster process compared to phosphorescence. The absorption of photon almost always involves excitation from the lowest vibrational level of the ground state of a molecule to a higher vibrational level of the first excited singlet state. When the molecule relaxes back to its ground state luminescence may occur (Scheme 2.3) but also non-radiative relaxation through dissipation of heat or through interaction with a second molecule through fluorescence quenching are possible pathways.26
Scheme 2.3. Schematic of the photoluminescence processes, where $S_0$ is the ground state, $S_1$ is the first excited state and $S_2$ and $S_3$ are higher energy singlet states. Reproduced from the Jablonski diagram.\textsuperscript{81, 82}

2.4.2.2 Characterization of the synthesized QDs

Fluorescence spectra were measured using a Perkin Elmer LS 55 spectrophotometer. A 3-Q-10 mm rectangular quartz cell was used for the measurements in solution. Various concentrations of original QD-solutions (20 – 100 µl) was added to 2 ml of chloroform (for the CdSe/ZnS QDs) or milli-Q water (for the CdTe QDs) to measure the fluorescence of the QDs. The functionalized CdSe/ZnS QDs were excited at 360/400 nm and the CdTe (aq) at 360 nm. All emission spectra were collected at room temperature.

2.4.2.3 Optical sensor response

The spectrophotometer mentioned in section 2.4.2.3 was also used for detection of the optical sensor response. A Teflon cell, in which the Au substrate was fitted, was used for all measurements. Where Cy3-modifed probes (HPP-2) were used the excitation wavelength was
520 nm. The excitation wavelengths for the QDs were 360 nm. All measurements were done at room temperature.

### 2.4.3 Scanning– and Transmission Electron Microscopy (SEM and TEM)

#### 2.4.3.1 Introduction

In both scanning- and transmission electron microscopy (SEM and TEM, respectively) an electron beam is directed on the sample and the wave nature of the electrons is used to obtain an image. The electron beam passes through three electromagnetic lenses: 1) a condenser lens to focus the beam on the sample, 2) an objective lens for magnification of the image and 3) a projector lens and is focused onto a plane, which can be photographed or stored. As the electron beam interacts with the sample various particle (X-ray, ion/atom) or photon (primary-, backscattered-, Auger- and secondary electron) emission processes occur. The emitted electrons are scattered, in two possible ways: elastically and in-elastically. Elastic scattering involves no energy losses and can be both coherent and incoherent, depending on whether it is phase related or not. Well-ordered atoms in crystalline samples exhibit elastic scattering, observed as spot patterns in the obtained image. Inelastic scattering gives rise to regular patterns, characteristic for absorption or emission, specific for the sample. In an energy-dispersive spectrometry (EDS) SEM all the photons emitted by the sample are collected and measured. Thus the obtained signal is proportional to both the energy and the intensity of the electron beam. The spatial resolution, i.e. the ability to distinguish between two closely spaced objects, of the current state-of-the-art SEM instruments is in the order of 50 Å.

In TEM the transmitted electrons are detected to provide the resulting image of the sample. Two main mechanisms of contrast are found in a TEM image. A phase contrast image is
obtained when the amplitude and the phase of the transmitted and the scattered beam recombine at the image plane, whereas the diffracted beam has been eliminated in an amplitude contrast image. The formed TEM image can either be photographed or captured by a digital charge-coupled device (CCD). The resolution of the TEM instrument is superior to the SEM and is presently in the vicinity of 1 Å.\textsuperscript{40}

### 2.4.3.2 Characterization of Au foils used as substrates in the optical sensor design

A Philips XL30S Field Emission Gun (FEG) Scanning Electron Micrograph (The Netherlands) with a Si Li EDS detector was used to acquire the SEM images of the clean Au foil (see section 2.3.4 for cleaning procedure). The sample was kept flat or tilted at a 60° angle and the instrument was operated at 5 keV.

### 2.4.3.3 Characterization of the synthesized QDs

Transmission Electron Micrographs (TEMs) were acquired by David Flynn and Dr Richard Tilley at the University of Victoria, Wellington, New Zealand. A JEOL 2011-instrument, operated at 200 keV, was used to determine the diameter of the synthesized CdTe QDs.

### 2.4.4 Nuclear Magnetic Resonance Spectroscopy (NMR)

#### 2.4.4.1 Introduction

Nuclear Magnetic Resonance Spectroscopy is based on a similar phenomenon as visible spectroscopic techniques. When a photon of light is absorbed, a transition to a higher energetic state from the ground state occurs. In visible techniques an electron absorbs the photon whereas NMR relies on the induced transition of nuclear spin.\textsuperscript{83} Certain spinning nuclei show a characteristic absorption of energy (transition from one alignment to another in...
the applied field) in the presence of a strong external magnetic field, perpendicular to the applied magnetic field.\textsuperscript{26} The ground and excited NMR states are generated in the external magnetic field and the characteristic frequencies of the transitions can be altered by changing the field strength of the applied magnetic field. Factors such as field strength, electronic configuration around the nucleus, anisotropy, type of molecule and intermolecular interactions determine the amount of energy needed to induce realignment in a particular nucleus. The obtained spectra contain information on the identity, location and quantity of not only particular nuclei, but also about its neighboring molecules.\textsuperscript{26} The most commonly investigated nuclei in NMR spectroscopy are \textsuperscript{1}H, \textsuperscript{13}C, \textsuperscript{15}N.

\textbf{2.4.4.2 Characterization of the synthesized ligands}

\textsuperscript{1}H spectra were acquired for the ligands and their precursors. All the experiments were performed on a 300 or 400 MHz Bruker NMR spectrometer. The NMR results were similar or in accordance with the reference data.\textsuperscript{57}

\textbf{Ligand 1:} \textsuperscript{1}H NMR (CDCl\textsubscript{3} / ppm) 4.30 (1H, m); 3.11 (1H, m); 2.89 (1H, m); 2.81 (5H, s); 2.67 (2H, m); 1.36-1.75 (6H, m).

\textbf{Compound B:} \textsuperscript{1}H NMR (CDCl\textsubscript{3} / ppm) 4.37 (1H,m); 4.00 (1H, m); 3.57-3.40 (12H, m); 3.24-3.10 (3H, m); 2.89-2.80 (2H, m); 2.65 (2H, m); 1.75-1.49 (8H, m).

\textbf{Compound C:} \textsuperscript{1}H NMR (CDCl\textsubscript{3} / ppm) 2.83 (4H, s); 2.60 (2H, t); 2.53 (2H, q); 1.74 (2H, m); 1.60 (2H, m); 1.40-1.28 (12H, m).

\textbf{Ligand 2:} \textsuperscript{1}H NMR (CDCl\textsubscript{3} / ppm) 6.77, 6.59 (2H, m); 4.51 (1H, m); 4.34 (1H, m); 3.70-3.50 (12H, m); 3.39-3.30 (4H, m); 3.16 (1H, m); 2.96-2.88 (2H, m); 2.53 (2H, m); 2.17 (2H, m); 1.86-1.51 (14 H, m); 1.51-1.21 (12 H, m).
2.4.5 Attenuated Total Reflection Fourier Transformed Infrared (ATR-FTIR) Spectroscopy

2.4.5.1 Introduction

The electromagnetic spectrum contains a wide range of radiation, where the region extends from the red end of the visible spectrum to the microwave range.\textsuperscript{26} This radiant energy can be considered as a wave or as a particle traveling at the speed of light, with a certain length and frequency. The frequency, $\nu$, which is the number of wave cycles that pass through a point in one second, is measured in Hz, where $1 \text{ Hz} = 1 \text{ cycle sec}^{-1}$. The wavelength, $\lambda$, is the length of one complete wave cycle and is often measured in cm. There is an inverse relationship between the wavelength and the frequency

$$\nu = \frac{c}{\lambda} \quad \text{(Eq. 2.2)}$$

where $c$ is the speed of light in vacuum. The energy, radiation in this case, is related to the wavelength as follows

$$E = h\nu = \frac{hc}{\lambda} \quad \text{(Eq. 2.3)}$$

where $h$ is Planck’s constant. In an infrared (IR) spectrum wavelengths are often expressed as wavenumbers in cm$^{-1}$, although $\mu$m is also occasionally used. The IR region includes frequencies from 14 000 to 20 cm$^{-1}$ (0.7 to 500 $\mu$m), where the mid-IR part, between 4000 and 200 cm$^{-1}$ is the most commonly used.\textsuperscript{26} IR is sensitive to the twists, bends, rotations and vibrations that occur in the atoms of a molecule. When a molecule is ir-radiated with IR-light a part of the incident beam is absorbed at specific wavelengths affecting the motions within the molecule. A complex spectrum, which is very characteristic of the functional groups within the molecule and also of the general configuration of the molecule, is generated.\textsuperscript{26}
Attenuated total reflection (also called internal reflection) FTIR is more versatile than traditional IR. The incident beam enters a crystal (prism) or a plate, which is surrounded or immersed in the sample, and is then reflected internally between the sample and the crystal if the angle of incidence is greater than the critical angle, related to the refractive index of the material. All energy is reflected when internal reflection occurs, although the beam appears to penetrate beyond the reflecting surface before it returns as shown in scheme 2.4. The depth of this penetration can be expressed as

\[
d_p = \frac{\lambda}{2\pi \sin^2 \theta - \eta_{sp}^2}^{\frac{1}{2}}
\]  

(Eq. 2.4)

where \(d_p\) is the depth of penetration (cm\(^{-1}\)), \(\lambda\) is the wavelength of the radiation in the crystal, \(\eta_{sp} = \eta_s / \eta_p\), where \(\eta_s\) is the refractive index of the sample, and \(\theta\) is the incident angle of the beam. The angle, \(\theta\), must be greater than the critical angle \(\theta_c\), which is given by

\[
\theta_c = \sin^{-1} \left( \frac{\eta_2}{\eta_1} \right)
\]  

(Eq. 2.5)

where \(\eta_2\) and \(\eta_1\) are the refractive indexes of the sample and the crystal, respectively. When the sample is placed in contact with the crystal the incident beam loses energy at the wavelengths where the sample absorbs. The obtained absorption spectrum, where the attenuated radiation is plotted as a function of wavelength is characteristic for the sample and similar to a normal transmission IR spectrum.\(^{26}\)

Scheme 2.4. Schematic of the working principle of an ATR-FTIR instrument.
2.4.5.2 Characterization of the mSAMs

ATR-FTIR spectra were collected on a Nicolet 8700 FTIR, with a diamond ATR crystal. Six different spots were measured for each sample to obtain representative results. All experiments were performed in room temperature and the sample preparation procedure is outlined in Section 2.3.1.

2.4.6 Atomic Force Microscopy (AFM)

2.4.6.1 Introduction

Atomic force microscopy (AFM) is a mechanical imaging technique based on the measurement of various forces between a sharp tip and the sample. The imaging results not only in a three dimensional topographic map of the sample but also provides information of the physical properties of the sample surface. Different forces, such as van der Vaals, magnetic, electrostatic, attractive and repulsive, are part of the force field with which the tips interacts. Essentially the signal is generated based on short range, repulsive forces between atoms and the deflection of a soft cantilever, which is raster-scanned across a sample surface, providing a measure of the interaction forces. The total force experienced by the tip is made up of both short- and long range forces but only the very short range forces can provide high resolution. Long range forces, such as Coulomb forces between charges, dipole-dipole interactions, polarization and capillary forces, are likely to deteriorate the experimental conditions due to unwanted attraction of the tip towards the sample surface or increase in the local repulsive force. The sample surface can be severely damaged if the tip scratches across it and thus the long-range forces need to be minimized, especially when soft materials are being investigated.84
Most AFM-instruments have the possibility of using either contact- or tapping mode, the two primary scanning modes. Contact mode can be used with hard samples, where the risk of scratching or removing loosely bound sample from the surface is negligible. In tapping-mode (Scheme 2.5) the cantilever is driven close to its resonance frequency by a piezo oscillator and there is only intermittent contact between the tip and the sample. Thus tapping-mode offers a gentle way to image the topography of soft or fragile samples.\textsuperscript{84}

Scheme 2.5. The principle of a tapping-mode atomic force microscope (AFM), reproduced from Friedbacher et al.\textsuperscript{84} The sample, symbolized as circles, is scanned by a piezoelectric translator.

2.4.6.2 Characterization of the mSAMs

AFM imaging was done in collaboration with Dr. Mao-Nan Chang from Nano Device Laboratories (NDL), Hsinchu, Taiwan on a high-resolution Nanoscope AFM instrument. The results were analyzed primarily with the software Nanoscope and secondarily with Image J.\textsuperscript{85} The sample preparation procedure is outlined in section 2.3.2.
2.4.7 Neutron Reflectometry (NR)

2.4.7.1 Introduction

Neutron reflectometry (NR) is a relatively new technique with the bulk of reports on its use emerging in the late 1980’s. The theory of neutron reflection can be approached in two ways, either by demonstrating that neutrons either behave in the same way as light (applying standard optics) or by treating them as particles (applying the Schrödinger equation):

\[
-\frac{\hbar^2}{8\pi^2 m} \nabla^2 \Psi + V\Psi = E\Psi
\]  
(Eq. 2.6)

where \( \hbar \) is Planck’s constant, \( m \) is the neutron mass, \( V \) is the potential applied to the neutron and \( E \) is the energy of the neutron. In the medium, through which the neutron moves, the net effects of the interactions between the neutron and the scatterers are represented by \( V \), approximately given by

\[
V = \frac{\hbar^2}{2\pi m} \rho
\]  
(Eq. 2.7)

where \( \rho \) is the scattering length density (SLD) defined as

\[
\rho = \sum_i b_i n_i
\]  
(Eq. 2.8)

where \( b_i \) is the scattering length of nucleus \( i \) and \( n_i \) is the number of nuclei per unit volume. Variations in the potential, \( V \), give rise to scattering of the neutrons. The motion of the neutrons through a planar boundary between air (\( V \sim 0 \)) and a uniform medium is thus dependent only on the potential normal to the interface and the \( b_i \)-value is experimentally known for most nuclei. Various isotopes of the same element can have significantly different scattering lengths, resulting in different SLD-values. Hydrogen and deuterium have SLD-values of \(-0.57\times10^{-6} \, \text{Å}^2 \) and \( 6.35\times10^{-6} \, \text{Å}^2 \), respectively. The SLD of hydrogen is the same order of magnitude as for many other elements, which means that hydrogen is very
visible to a neutron beam. The large difference in SLD between hydrogen and deuterium enable isotopic substitution within a sample and the variation in SLD can be detected with neutron reflectometry. This is called contrast variation and can be used to highlight specific areas in a sample as well as camouflage others and thus solve the structure of the sample. It is also possible to make sub-phases from mixtures of H$_2$O and D$_2$O to match the SLD of secondary components in the system, such as the solvent or the substrates, thus isolating the scattering arising only from the density variation of the sample by providing an additional contrast variation.$^{74}$ A contrast matched interface, where the protonated and deuterated material in the sample is mixed correctly, can thus be invisible to the neutron beam.$^{74}$

The neutron scattering length density (nSLD) of a material is shown in equation 2.9

$$nSLD = N_A \sum_i \frac{p_i A_i}{b_i}$$

(Eq. 2.9)

where $N_A$ is Avogadro’s number, $p_i$ the mass density, $A_i$ the atomic weight and $b_i$ the nuclear scattering length of component $i$. $^{74}$ Comparison between theoretical and experimentally obtained and fitted nSLD-values provides the means to calculate the surface coverage of the sample.$^{74}$

However, a more convenient way to understand neutron reflection instead of focusing on the Schrödinger equation is to consider the momentum transfer perpendicular to the interface, $Q_z$. The application of standard optics is particularly advantageous in the case of specular reflection, where the angle of incidence is equal to the angle of the reflected beam (see the scheme in Figure 2.6). The momentum transfer is given by the change in the neutron wave vector upon reflection at the boundary and the scattering vector consists only of its $z$-component ($Q_z$).
\[ Q_z = \frac{4\pi}{\lambda} \sin \theta \]  

(Eq. 2.9)

where \( \lambda \) is the wavelength of the incident beam and \( \theta \) is the incident angle of the beam with the interface,

\(^{88}\) under the assumption that the reflection is purely elastic and that there is no absorption of neutrons by the medium.\(^ {89}\)

Scheme 2.6. The geometry of specular reflection, where \( \theta_i \) is the angle of incidence, \( \theta_r \) is the angle of reflection; \( K_i, K_r \) and \( K_t \) are the intensities of the incident, reflected and transmitted radiation, respectively.

By analysis of the reflected beam and its variation as a function of \( Q_z \), valuable information about the sample composition on the surface can be obtained, as the momentum transfer is entirely in the \( z \)-direction. The intensity of the reflected beam is related to the depth dependence of the index of refraction (related to the SLD) averaged over the horizontal dimensions of the interface.\(^ {90}\) The measured reflectivity cannot be inverted to give the interaction profile of a surface directly due to the loss of phase information. Instead, data is
compared to a matching model, based on calculated reflectivity from model surfaces so that useful information (such as surface coverage and density) can be extracted.

2.4.7.2 Instrumentation and NR- Measurements

The basic requirements of a neutron reflection experiment is 1) a neutron source (either a nuclear reactor or a spallation source), 2) a method for wavelength selection, 3) a collimation system, 4) a sample stage and 5) a detector system.\textsuperscript{88} For example, the Platypus, at the Australian Nuclear Science and Technology Organisation (ANSTO) facilities in Sydney, Australia is a time-of-flight reflectometer, which uses a reactor as its neutron source.\textsuperscript{91} A continuous beam of high-energy neutrons is obtained through nuclear fission and directed into a cold source, where the neutrons are scattered until a sufficient amount of their energy has been lost and a thermal equilibrium has been reached.\textsuperscript{88} This reduction in neutron energy is required to give neutrons with a longer wavelength appropriate to measure the molecular dimensions required.

Reflectometry experiments can be performed in two ways, either the 1) \textit{angular dispersive} method, where a monochromatic neutron wavelength is used and $\theta$ is varied or with the 2) \textit{energy dispersive} method, where a broad range of wavelengths is used to achieve variation in $Q_z$ and only a few distinctive angles. These two techniques, known as monochromatic and time-of-flight reflectometry, respectively, provide a slightly different balance between the intensity of the incident beam and instrumental resolution. This balance is important and should ideally be customized for each experiment. Time-of-flight reflectometry is better suited for the study of thin films at the air-water interface, such as SAMs and proteins, since a range of resolutions can be accessed with a pair of chopper discs.\textsuperscript{91} In time-of-flight NR studies the instrument operates at a fixed $\theta$ acquiring a range of q-values resulting from the various wavelengths and determined from the time of arrival of each neutron as a function of
its wavelength. Every pulse of neutrons gives information about the whole $Q$-range, as the frequency of the chopper discs is set so that the slowest neutrons (longest wavelengths) have all reached the detector before the arrival of the fast neutrons (shortest wavelengths) from the next pulse.\textsuperscript{88, 91} For high $Q$-values the signal-to noise ratio grows weaker due to detection of neutrons from off-specular- as well as specular reflection. Isotropic scattering also contributes to the background noise, which needs to be measured and subtracted from the acquired profile before data analysis. The background is particularly high for solid-liquid samples due to incoherent scattering of the liquid and additional scattering from the sample cell.\textsuperscript{88}

In order to analyze specular reflection data a model of the studied interface needs to be constructed. The most common approach is to use a model in which the interface is described as constructed. The thickness, $d$ (Å), a Gaussian interfacial roughness, $\sigma$ (Å), and the nSLD, $\rho$ (Å$^{-2}$) are the three parameters that describe each slab or layer of the interface.\textsuperscript{74} Two (or more when available) data sets were always superimposed and fitted simultaneously during the analysis. Data analysis was done with the program ga_refl\textsuperscript{92} based on an optical matrix method,\textsuperscript{93} which assumes that the interface can be described as a series of slabs where there is change in refractive index of the neutrons at each slab. The calculated profile is then compared to the measured profile and the quality of fit is evaluated (typically through a standard chi-square test). The SLD value and the thickness of the calculated model are variables which can be changed in the model to obtain an optimal fit. When a model of a solid-liquid interface is constructed and optimized, the thickness of the solid medium and the liquid solvent is considered infinite and their SLD are fixed. This is where the benefits of contrast variation come into play, as the reflectivity profile of a system changes substantially depending on whether hydrogenated or deuterated materials are used but the chemical structure of the sample at the interface remains unchanged.\textsuperscript{88}
2.4.7.3 Polarized neutron reflectometry (PNR)

Polarized neutron reflectometry (PNR) provides an alternative to conventional NR and the magnetization profile near the surfaces of thin films and multilayers can be studied. The theory of PNR, the instrumentation required and the corresponding analysis and spin-dependent data has been well described presented by Majkrzak\textsuperscript{94} and Fitzsimmons and Majkrzak.\textsuperscript{90} In PNR an internal reference layer, incorporated in the sample, can be used to achieve contrast variation. Polarized, up- or down-spin, neutrons offer two independent datasets (one with up-spin and another with down-spin polarized neutrons) in a single measurement of a composite system, which consists of a buried, saturated ferromagnetic layer and the unknown surface of interest. By combining the two datasets, the reflection amplitude for the unknown segment alone can be determined to be further converted into the corresponding SLD depth profile.\textsuperscript{74, 90} The greatest advantage of magnetic contrast over isotopic variation is the simplicity of the approach, involving less sample preparation and movement of the sample. An appropriate metallic binder, such as nickel or iron, has to be chosen as the magnetic layer and incorporated in the standard substrate. An external magnetic field is then applied to control the direction of the magnetization in the reference layer (see Scheme 4.1 in Chapter 4.4 for details). The incident neutron beam is then polarized so that the neutron spin is either parallel or anti-parallel to the direction of the magnetization layer, thus giving rise to the two independent datasets for the two polarizations states. The two sets of data are then modeled simultaneously to fit a model and very accurate system models, even for quite complex systems, can be solved with good fits based on magnetic contrast variation in PNR.\textsuperscript{73, 74}

2.4.7.4 Characterization of the mSAMs and detection of the sensor response

Neutron reflectometry was carried out on the Platypus reflectometer, at the Australian Nuclear Science and Technology Organisation (ANSTO) facilities in Sydney, Australia. Data
was collected from the reflected beam at three different angles of incidence: 0.5, 2.0 and 6.0 ° up to a momentum transfer of $Q_z = 0.4 \ \text{Å}^{-1} \ (4 \ \text{nm}^{-1})$ and a minimum of two contrasts were used (H$_2$O-, and D$_2$O-based 0.01 M PBS buffer with a pH of 7.4) for all samples, with the addition of the sub-phase CM 2.5 (1:1 H$_2$O:D$_2$O/PBS buffer) when possible within the experimental timeframe. Two (or three when available) data sets were always superimposed and fitted simultaneously during the analysis. Analysis and fittings of the data was done with the software Igor Pro 6.04 (Wavemetrics) and with the program ga_refl. The sample preparation procedure is outlined in section 2.3.3.1 and a more detailed description of the data analysis is presented in section 4.2.

2.4.7.4 Characterization of the melting behavior of the surface-attached HPPs in the mSAM by polarized neutron reflectometry (PNR)

Polarized neutron reflectometry was carried out at the NG-1 reflectometer at NIST Center for Neutron Research. The NCNR reactor is a 10 MW reactor and a cold source produces neutrons of long wavelengths for the NG-1 reflectometer. A supermirror polarizes the neutron beam by reflecting from the incident beam one spin state out of the plane of the neutron beam. The incident, polarized neutron beam then passes through a spin-flipper, which can flip the neutrons to the opposite spin if activated. Neutrons with spins that are parallel to the applied magnetic fields are named “spin-up” and those that are anti-parallel are named “spin-down”. Data were collected up to a maximum momentum transfer of $Q_z = 0.3 \ \text{Å}^{-1} \ (3 \ \text{nm}^{-1})$ for the two magnetic contrasts (from an embedded Fe layer) mainly in D$_2$O/PBS buffer but also in H$_2$O/PBS buffer (0.01M, pH 7.4). Two (or more when available) data sets were always superimposed and fitted simultaneously during the analysis. Data analysis was done with the program ga_refl based on an optical matrix method, as described above. The sample preparation procedure is outlined in section 2.3.3.2 and a more detailed description of the data analysis is presented in section 4.2.
2.4.8 Cyclic voltammetry (CV)

2.4.8.1 Introduction

Potential sweep methods are commonly used to study electrode processes. A continuous, time-varying potential is applied to the working electrode and the oxidation and/or reduction of the electroactive species in the solution, possible absorption of species (depending on the potential used) and a capacitive current are observed in a double layer at the electrode surface.

The total current is defined as

\[ I_{\text{tot}} = I_f + I_c = I_f + vC_d \]  

(Eq. 2.10)

where \( I_{\text{tot}} \) is the total current in the system, \( I_c \) is the Faradaic- and \( I_c \) is the capacitive component, \( C_d \) is the double layer capacitance and \( v \) is the sweep rate. The \( I_c \) contribution to the total current increases with increasing sweep rate, \( v \). The Faradaic current observed in a system depends on the kinetics and transport by diffusion of the electroactive species in solution. Nernst equation (Eq. 2.11) describes the fundamental relationship between a potential applied to an electrode and the concentration of the electroactive species in the immediate vicinity of the electrode surface.

\[ E = E^\circ - \frac{RT}{nF} \ln \frac{a_{\text{red}}}{a_{\text{ox}}} = E^\circ - \frac{2.3026RT}{nF} \log \frac{a_{\text{red}}}{a_{\text{ox}}} \]  

(Eq. 2.11)

where \( E^\circ \) is the standard potential of the electrode, \( R \) is the universal gas constant, \( T \) is the absolute temperature, \( F \) is Faraday’s constant, \( n \) is the number of transferred electrons and \( a_{\text{red}} \) and \( a_{\text{ox}} \) are the chemical activities of the reduced and oxidized species, respectively. The chemical activity of a species is defined as the activity coefficient multiplied with the concentration and since activity coefficients tend to 1 at low concentrations, the activities in the Nernst equation are often simply replaced with concentrations.\(^{26,27}\)
In cyclic voltammetry (CV) the potential of the working electrode is swept back and forth across the formal potential of the analyte. As the analyte is repeatedly reduced and oxidized alternating cathodic and anodic currents flow at the electrode. The current is measured as a function of the applied potential, and plotted in the voltammogram as shown in scheme 2.7. Two asymmetric peaks, one cathodic and the other anodic, are characteristic for regular voltammograms, although theoretically the peaks could be symmetric for completely reversible processes (as detailed in Table 2.3).\textsuperscript{26, 27}

![Scheme 2.7](image)

Scheme 2.7. A general cyclic voltammogram, where $E_{p,c}$ and $E_{p,a}$ are the cathodic and anodic peak potential and $I_{p,c}$ and $I_{p,a}$ are the cathodic and anodic peak currents, respectively.\textsuperscript{26}

The parameter of primary interest is the peak height, $I_p$ as it is directly proportional to the analyte concentration as described by the Randles-Sevčík equation (Eq. 2.12).
\[ I_p = -0.4463nF \left( \frac{nF}{RT} \right)^{1/2} c^{\infty}_o D^{1/2} \nu^{1/2} \]  
(Eq. 2.12)

where \( n \) is the number of transferred electrons, \( F \) is the Faraday constant, \( R \) is the universal gas constant, \( T \) is the absolute temperature, \( c \) is the analyte concentration, \( D \) is the diffusion coefficient and \( \nu \) is the scan rate. At 25 °C equation 2.12 can be rewritten as

\[ I_p = -(2.69 \cdot 10^{-5})n^{3/2} c^{\infty}_o D^{1/2} \nu^{1/2} \]  
(Eq. 2.13)

For a reversible system it can thus be concluded that: \( I_p \) is directly proportional to \( \nu \) and that \( E_{p,a/c} \) is independent of \( \nu \), c) \( |E_{p,a/c} - E_{p/2,a/c}| = 57/n \text{ mV} \), d) \( E_{p,a} - E_{p,c} = 57/n \text{ mV} \) and e) \( I_{p,a}/I_{p,c} = 1 \).26-27 For a quasi-reversible redox reaction the shape of the voltammogram is similar to that of a reversible reaction, there is however a broadening of the peaks and a larger peak separation. The various conditions for the important parameters defining reversible, quasi-reversible and irreversible reactions are listed in table 2.3.27

<table>
<thead>
<tr>
<th>A reversible reaction</th>
<th>A Quasi-reversible reaction</th>
<th>An irreversible reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \Delta E = E_{p,a} - E_{p,c} = \frac{57}{n} \text{ mV} )</td>
<td>(</td>
<td>I_p</td>
</tr>
<tr>
<td>(</td>
<td>E_p - E_{p/2}</td>
<td>= \frac{57}{n} \text{ mV} )</td>
</tr>
<tr>
<td>(</td>
<td>I_{p,a} / I_{p,c}</td>
<td>= 1 )</td>
</tr>
<tr>
<td>( I_p \propto \nu^{1/2} )</td>
<td>( E_{p,c} ) moves towards negative potentials with increasing ( \nu )</td>
<td>(</td>
</tr>
<tr>
<td>( E_p ) is independent of ( \nu )</td>
<td>When the potential ( &gt; E_p, I^{-2} \propto t )</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.3. Summary of the conditions determining reversible, quasi-reversible and irreversible reaction probed by cyclic voltammetry.27
In alternating current (AC) cyclic voltammetry a sinusoidal potential, of small amplitude, is superimposed on the slowly changing direct current (DC) potential. A phase-sensitive detector has to be incorporated into the instrumentation if for the detection of the AC current. It is possible to detect only the AC component and thus separate the Faradaic- from the capacitive current due to the phase difference between them. The total AC current is the vector sum of the Faradaic component and the charging current, arising from the capacitance in the double layer. Normally, the Faradaic current has a phase angle of 45°, while the capacitive current is 90° out of phase with respect to the applied AC potential. If phase-sensitive lock-in amplifiers are used either of the two AC components can be selected and the other rejected. AC techniques are especially useful when kinetics or adsorption is studied.26

2.4.8.2 Characterization of the mSAMs and detection of the sensor response

AC cyclic voltammograms were recorded before the SAM formation as well as before and after hybridization using a CH Instruments electrochemical workstation, Model 650C (CH Instruments, USA). All spectra were measured in SSC buffer containing 5.0 mM K$_4$Fe(CN)$_6$/K$_3$Fe(CN)$_6$ (1:1 molar ratio) as a redox couple at room temperature. A conventional three-electrode cell with a gold (Au) working electrode (1.6 mm in diameter), an Ag/AgCl (3 M KCl) reference electrode and a platinum (Pt) wire counter electrode, were used. The sample preparation procedure is outlined in section 2.3.5.

2.4.9 Electrical Impedance Spectroscopy (EIS)

2.4.9.1 Introduction

Electrical resistance is the ability of a circuit element to resist the flow of electrical current and is defined by Ohm's law.
\[ R = \frac{E}{I} \]  
(Eq. 2.14)

where \( R \) is the resistance, \( E \) is the potential and \( I \) is the current. This relationship is true only for an ideal resistor, which has certain simplified properties: 1) Ohm's Law is obeyed at all current and voltage levels, 2) the resistance is independent of the frequency, and 3) the alternating current (AC) and voltage signals though a resistor are in phase with each other.

Ideal resistors are rarely encountered in practical applications, where the more complex behavior needs to be represented by other circuit elements. Impedance can be defined as a measure of the ability of a circuit to resist the flow of electrical current, which will reveal information about the electrode process and about the kinetics of the whole electrochemical system. Electrochemical impedance, \( Z \), is generally measured by applying an AC potential to an electrochemical cell and measuring the current through the cell.\(^{27,95,96}\) A small amplitude, alternating sinusoidal potential is applied to the system, at equilibrium or steady-state, resulting in an AC current signal. The response can be analyzed as a sum of sinusoidal functions and is considered to be linear as long the applied amplitude is small. Then equation 2.14 can be written as

\[ I = \frac{E}{R_f} \]  
(Eq. 2.15)

where \( R_f \) is the Faradaic impedance, inversely proportional to the rate of electron transfer.\(^{27,95}\)

Presentation of impedance data is complicated by the fact that there is a phase difference between the applied potential and the current response. The total impedance, \( Z \), can be expressed through the real impedance, \( Z' \) (the impedance in-phase with the applied potential), and the imaginary impedance, \( Z'' \) (the impedance 90° out of phase with the applied potential).

The relationship between these and \( \theta \) (the phase angle) vary with frequency \( (\omega) \) (Scheme 2.8).
Scheme 2.8. A vector diagram describing the relationship between total- \((Z)\), real- \((Z')\) and imaginary- \((Z'')\) impedance.

Equivalent circuits are commonly used as representations of the electrochemical cell. Even if the equivalent circuit does not truly describe the electrochemical phenomena themselves, it can provide insight to the interfacial processes at the electrode surface and can also offer the means to manage the obtained data in a meaningful way. Based on equation 2.15 at least three components must be included in any equivalent circuit model: 1) the double layer capacitance, 2) the Faradaic impedance, and 3) the uncompensated resistance (usually the solution resistance between the working and the reference electrodes). All processes that inhibit or impede the applied AC potential at the electrode surface can be described as various components in the equivalent circuit. Scheme 2.9 shows a circuit, which includes the solution resistance, \(R_s\), in series with a parallel circuit containing a constant phase element, CPE, the charge transfer resistance, \(R_{ct}\), and the Faradaic impedance, \(W_o\), also referred to as Warburg impedance.\(^{27,95}\)
Scheme 2.9. An equivalent circuit model that includes a solution resistance \( R_s \) in series with a parallel circuit containing a constant phase element \( (CPE) \), the charge transfer resistance \( (R_{ct}) \) and Warburg impedance \( (W_o) \).

Nyquist plots are commonly used to present impedance data and typically consist of a semi-circle region and a straight line of unity slope as shown in Scheme 2.10. The semi-circle region describes processes that are controlled by the rate of electron transfer; usually at high frequencies. In the region represented by the straight line, at low frequencies, diffusion-controlled processed are dominating. The drawback with the Nyquist plot is that the frequency dependence of the system is not clearly displayed.\(^{27}\)

Scheme 2.10. An example of a Nyquist plot, with the imaginary impedance plotted against the real impedance, for a simple electrochemical system. \( \omega \) represents the frequency.
Nyquist plots are commonly for presentation of impedance data but other methods are also available. If there are several rate-determining processes in the system the Bode plots have better resolution than the Nyquist plot as they provide information about the frequency dependence of the system (e.g. impedance or phase angle vs. frequency). The data can also be displayed as the inverse of impedance, i.e. admittance, $Y$. Admittance plots are especially useful when elements in parallel are added to the equivalent circuit.\textsuperscript{27, 95}

2.4.9.2 Characterization of the mSAMs and detection of the sensor response

AC impedance spectra were recorded before the monolayer was formed as well as before and after hybridization using a CH Instruments electrochemical workstation, Model 650C (CH Instruments, USA) or a EG&G Instrument potentiostat, Model 283 connected to a Model 1025 Frequency Response Detector (Princeton Applied Research, USA). All spectra were measured in SSC buffer containing 5.0 mM K$_4$Fe(CN)$_6$/K$_3$Fe(CN)$_6$ (1:1 molar ratio) as a redox couple at room temperature. The same cell and electrodes, as mentioned in section 2.4.8.2 were used. The AC impedance measurements were run at an applied bias potential of 230 mV with a 5 mV sinusoidal excitation amplitude. The data were collected for harmonic frequencies from 0.1 Hz to 0.1 MHz at 1 step per decade and analyzed using ZView software (version 2.80, Scribner Associates Inc. North Carolina). All experiments have been performed at least 2 times, of which some are explicitly shown in the thesis, to ensure the consistency of the response trend. It was found that the trends in the sensor responses were reliable but the absolute values of the responses varied slightly. The surface cleaning procedures and the freshly assembled monolayer for each sensor are considered to be a likely cause of the observed variations. Therefore, a representative sensor response for each case is presented.
Chapter 3 – Characterization of the components of the general DNA sensor design

In the development of a sensitive and selective DNA sensor it is of great importance that the various components of the sensing platform are well characterized and understood. Although the sensor design was based on well-understood principles, some initial investigations were carried out to ensure that the individual parts of the systems preformed in the way they were expected to. The quenching ability of the gold substrate of the fluorescence of the utilized label was vital to obtain a high signal-to-noise ratio for the optical DNA sensor and thus an evaluation of the quenching ability of gold was conducted. Characterization of the sensing monolayer (mSAM), consisting of hairpin probes (HPPs) and methyl poly(ethylene glycol) (m-PEG) molecules, was conducted to obtain information about the monolayer formation and the distribution of the two components in the layer. Furthermore, a study of the thermodynamic behavior of the active constituent in the mSAM (the HPPs) was done in solution to ensure the functionality of the probe and also to confirm its interaction with various target ODNs (such as fully complementary, single-base mismatch and non-complementary sequences).

3.1 Investigation of the quenching ability of Au and monolayered m-PEG of the fluorescence of Cy3 dye and CdSe/ZnS quantum dots

As mentioned in section 1.5, fluorescent quantum dots (QDs), have become an attractive alternative to organic dye molecules, especially in a variety of biological applications, due to their characteristic properties (such as a broad excitation band, narrow bandwidth emission, photochemical stability). The synthesis, modification and applicability of high quality nanocrystals, in particular CdSe/ZnS QDs, have been widely investigated and reviewed.
Quenching of the fluorescence from CdSe/ZnS QDs by Au surfaces is similar to the quenching of organic dye molecules by smooth, metallic surfaces\textsuperscript{100} and can be incorporated as a functional part of a sensor design.

Self-assembly of alkanethiols, with mixed as well as with homogenous layers\textsuperscript{101} of poly(ethylene oxide) (PEO)\textsuperscript{102} and poly(ethylene glycol) (m-PEG), has been used on various surfaces to make them more resistant to protein binding.\textsuperscript{103} m-PEG is known for its ability to make surfaces more biocompatible and is therefore of particular interest for further studies.\textsuperscript{103} To investigate how an unmodified, versus a m-PEG-modified, Au surface would affect the luminescence of fluorescent molecules, the emission spectra for an indocarbocyanine (Cy3) dye was compared to that of functionalized CdSe/ZnS QDs. It was also further investigated whether the Au surface is able to quench the fluorescence from the dye and the QDs while keeping other interactions, such as adhesion between them, to a minimum. Both these characteristics are important for biosensing applications. The results show that the quenching properties of Au vary, depending on the modification of the surface and the fluorescent molecule used.

### 3.1.1 Quenching of the Cy3 fluorescence

Smooth, metallic surfaces are known to quench the fluorescence of various organic dye molecules and similar behavior has also been observed for CdSe/ZnS quantum dots.\textsuperscript{100} Schemes 3.1.A and B show the steps for investigating the quenching of a Cy3-dye by an unmodified and a m-PEG-modified Au surface, whereas 3.1.C and D describe the steps for the corresponding study of the quenching of CdSe/ZnS quantum dots.
Scheme 3.1. Steps for investigating the quenching of a Cy3-dye and CdSe/ZnS quantum dots by an unmodified and a m-PEG-modified Au surface.

The emission maximum of Cy3 is at 570 nm when illuminated at 520 nm (Figure 3.1.A). As shown in Figure 3.2.A and B, both the unmodified and the m-PEG-modified Au surface can quench the emission from Cy3 (compared to the emission of Cy3 drop-cast on quartz glass - Figure 3.1.A).

It was visually confirmed that Cy3 was present on the surface prior to any rinsing. After the first rinsing with PBS buffer it is likely that Cy3 is completely dissolved and washed away due to its good solubility (Figure 3.2.A.b and c). The PBS solution, which was used for rinsing, was collected and fluorescence measurements were performed. The obtained spectra (results not shown) indicated that all the dye was present in the washing solution and it was no longer present on the surface.
In the case of the m-PEG–modified Au substrate a high density of m-PEG monolayer was ensured by using a high concentration of m-PEG during the substrate preparation. The properties of interest here were the quenching ability of the m-PEG-modified Au substrate and the adhesion of Cy3 to that substrate. As shown in Figure 3.2.B, the background emission from the dye is very similar to the corresponding emission background for an unmodified Au surface (Figure 3.2.A). No significant fluorescence emission from the Cy3 that had been dried on the m-PEG modified Au surface was detected. Therefore, the quenching of Cy3 was not affected by the presence of the SAM of m-PEG. After rinsing (Figure 3.2.B.b and c) there is a loss of background intensity, presumably reflecting a successive removal of Cy3 with each rinsing.
Figure 3.2. Quenching of the fluorescence of the Cy3 dye by an unmodified (A) and a m-PEG-modified Au surface (B): a) after no rinsing, b) rinsing once and c) rinsing twice with an excess of PBS buffer.

3.1.2 Quenching of the QDs fluorescence

Commonly, colloidal QDs are obtained via organometallic routes using hydrophobic trioctylphosphine (TOP) or trioctylphosphine oxide (TOPO) ligands. In order to make the QDs hydrophilic and compatible for use as biological labels, additional surface functionalization is essential.\textsuperscript{6, 57} In this study functionalization was achieved by ligand exchange with 8-thio-3,6-dioxa-octanol and DSBA, that is an alkylthiol substituted biotin molecule (Scheme 2.1 and 2.2. in section 2.1.2.1 and 2.1.2.2, respectively).\textsuperscript{57} The emission maximum of the QDs is at 572 nm (Figure 3.1.B). Following the ligand exchange, the QDs would be able to bind selectively to streptavidin\textsuperscript{57}, allowing, for example, attachment of end-modified DNA probe sequences for further biosensing applications.

The quenching of functionalized CdSe/ZnS QDs by an unmodified and a m-PEG-modified Au surface was investigated (Scheme 3.1.C and D). Both Au surfaces were capable of
significantly quenching the fluorescence from the functionalized CdSe/ZnS QDs as can be seen by comparing Figure 3.4.A. and B with Figure 3.1.B. The unmodified Au surface can, however, quench the QDs more readily than the modified surface (Figure 3.3.A vs. B). It is believed that this is due to the fact that the m-PEG monolayer increases the distance between the QDs and the Au, thereby effecting the quenching. Additionally, the functionalized CdSe/ZnS QDs are not fully dissolved in water and retain some of their original hydrophobic character, even after the ligand exchange. m-PEG has a moderate hydrophilic character and weak repulsive interactions between the QDs and the m-PEG layer can be expected to contribute in keeping of the QDs further away from the Au. Schimizu et al. have reported increasing emission from CdSe/ZnS QDs with increasing roughness of an Au surface. The Au foil, which was used as a substrate in this study, cannot be considered to have a completely smooth surface, as was also confirmed by Scanning Electron Microscope experiments (Figure 3.3). The emission seen in Figure 3.4.A and B can partly be attributed to the roughness of the Au surface.

Figure 3.3. SEM image of the Au foil used as the substrate. The image was collected at tilt angle of 60° and the magnification 100 000 times.
Figure 3.4. Quenching of the fluorescence of the functionalized CdSe/ZnS QDs by an unmodified (A) and a m-PEG-modified Au surface (B): a) after no rinsing, b) rinsing once and c) rinsing twice with an excess of PBS buffer.

There is a clear adhesion of the QDs to the unmodified Au surface, since after rinsing with an excess of PBS buffer some fluorescence can still be detected (Figure 3.4.A.b) and the presence of QDs was visually identified as colored spots on the Au surface. The rinsing partly removes the QDs from the surface, as seen by a decrease in the emission intensity (Figure 3.4.A.b). Increasing the number of rinsing steps does not further affect the quenching or the adhesion of the QDs to the surface (Figure 3.4.A.c). The QDs were functionalized with a mixture of Ligand 1 and 2 and the observed adhesion is probably due to interactions between the sulfur in the biotin–functionalized ligand (Ligand 2) of the QDs and the Au surface. On the other hand, some adhesion was also observed in the case of a m-PEG-modified surface (Figure 3.4.B). In this case rinsing with PBS has no great effect, seen as a low, steady fluorescence emission (Figure 3.4.B b) and c) even after two rinsing steps.
Although PEG-layers have been show to prevents interactions between proteins and gold\textsuperscript{101-103} the m-PEG layer still allows for some contact between the functionalized QDs and the Au surface.

### 3.1.3 Conclusions

Both an unmodified and a m-PEG-modified Au surface show the ability to effectively quench the fluorescence of the organic dye Cy3 as well as the fluorescence of functionalized CdSe/ZnS quantum dots. A monolayer of m-PEG allows the fluorescent molecules to still get sufficiently close to the Au surface for their emission to be readily quenched. A low background emission is obtained, which appeared dim enough to not interfere with detecting a significant increase in the fluorescence emission. The QDs show adhesion to the Au surface (probably due to the sulfur atoms in the QD shell and in the ligands), which is also seen after modification of the Au surface with -m-PEG. The m-PEG modified Au surface was still considered the most suitable for further applications where its efficient quenching can be utilized.

### 3.2 Characterization of the mixed SAMs

#### 3.2.1 Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR)

IR-radiation is absorbed by a sample at specific wavelengths, which are characteristic of the functional groups within it.\textsuperscript{26} Not only the composition of the sample, but also the configuration of the molecules within it, can be elucidated from an IR spectrum. ATR-FTIR is more versatile than conventional IR and well suited for studies of nucleic acids and proteins\textsuperscript{105} as it is a non-destructive and fast technique, which requires small amounts of sample.\textsuperscript{106} Data analysis is mainly done through comparison of the obtained spectra to existing libraries with known, characteristic wavelengths for the specific functional groups. An extensive library of
IR bands characteristic for nucleic acids in various conformations in aqueous solutions can be found in the review by Banyay et al.\textsuperscript{106}. Single-stranded\textsuperscript{107}, double-stranded\textsuperscript{47} and triple-stranded\textsuperscript{108,109} DNA has been studied with IR and the most interesting region, with respect to nucleic acids in aqueous system, is the mid-IR range (1800 – 800 cm\(^{-1}\)). H\(_2\)O and CO\(_2\) will cause interfering vibrations (at 1645 and 1700 cm\(^{-1}\), respectively) that need to be taken into account when the spectra are analyzed. The vibrations from H\(_2\)O can be eliminated using deuterated samples, however, D\(_2\)O also has interfering vibrations in the mid-IR range (1450 cm\(^{-1}\)) and thus a spectrum should preferably be recorded in both solvents when possible.\textsuperscript{106}

Petrovykh et al.\textsuperscript{107} successfully managed to quantitatively determine the amount of DNA immobilized on gold using a combination of FT-IR and X-ray photoelectron spectroscopy (XPS). The authors were able to determine both the absolute and the relative coverage. For these reasons ATR-FTIR was chosen as a method for characterization of the mSAMs. Thymine deoxyribonucleotide (dT) was used as a marker to study the evolution of its characteristic absorbance peak, from the carbonyl groups in the thymine ring, at 1714 cm\(^{-1}\).\textsuperscript{107}

Recognition and detection of dsDNA has been developed at a thionalid self-assembled monolayer modified gold electrode by Wang et al.\textsuperscript{47}

### 3.2.1.1 Characterization of the mSAM by ATR-FTIR

We conducted ATR-FTIR measurements to investigate the structure of the generated SAMs and to verify that import functional groups were present on the Au surface. Figure 3.5 shows an ATR-FTIR spectrum of a mixed monolayer of hairpin probes (HPP-1) and m-PEG immobilized on an Au foil. HPP-1 has a biotin unit attached to its 3’end, which would be expected to influence the spectrum of the mSAM. The peaks labelled in Figure 3.5 were assigned to functional groups in the HPP-1 as listed in Table 3.1.
Figure 3.5. ATR-FTIR spectra of a mixed SAM of m-PEG and the biotinylated probe, HPP-1, recorded from three different spots, blue (dotted line), red (dash-dotted line) and green (solid line), on the same sample. The peak assignments are listed in Table 3.1.

Indication of DNA backbone stretching can be seen at 962 cm\(^{-1}\), in the region where sugar/sugar-phosphate vibrations also occur. In the region between 1000 and 1250 cm\(^{-1}\), the signals are due to vibrations along the sugar-phosphate chain, whereas the bands appearing from 1250 to 1500 cm\(^{-1}\) are specific for the base-sugar interactions. In the 1500 to 1800 cm\(^{-1}\) region the recorded peaks originate from the base vibrations of the nucleic acids.\(^{106}\) Spectra were recorded at different locations (as represented by the blue, red and green curve in Figure 3.5) on the same sample to get representative information. The sampled surface was equivalent to the area of the top surface of the crystal, approx. 600 µm in diameter. The differences between the blue (dotted line), red (dash-dotted line) and green (solid line) curve in Figure 3.5 can be explained by the heterogeneous nature of the monolayer. It is to be expected that HPPs are not evenly spread throughout the layer as the self-assembly of m-PEG
and HPPs on the Au surface is a mixed process. Whether the heterogeneities in the layer can be observed or not would very much depend upon the scale on which they occur. The variations seen argue for heterogeneities equal or larger to the spot size. As the samples were dried before the ATR-FTIR measurements were carried out, some locations were exposed to air for longer periods of time than others, thus creating further variations between the sample spots. Characterization of mSAM should preferably be carried out in a liquid, buffered environment, which would resemble the immobilization- and hybridization conditions more accurately.

<table>
<thead>
<tr>
<th>Wavenumber (cm$^{-1}$)</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>962</td>
<td>CC stretching of the DNA backbone</td>
</tr>
<tr>
<td>1067</td>
<td>Symmetric PO$_2$- stretching</td>
</tr>
<tr>
<td>1087</td>
<td>Symmetric PO$_2$- stretching</td>
</tr>
<tr>
<td>1132</td>
<td>Anti-symmetric PO$_2$- stretching</td>
</tr>
<tr>
<td>1486</td>
<td>Cytosine</td>
</tr>
<tr>
<td>1605</td>
<td>Adenine</td>
</tr>
<tr>
<td>1655</td>
<td>Thymine</td>
</tr>
<tr>
<td>1680</td>
<td>Guanine</td>
</tr>
<tr>
<td>1680-1710</td>
<td>C=O stretching of the cyclic urea in biotin</td>
</tr>
<tr>
<td>1742</td>
<td>C=O stretching of biotin carboxylic acid</td>
</tr>
</tbody>
</table>

Table 3.1. Peak assignments for the mSAM sample, containing m-PEG and the biotinylated probe, HPP-1.$^{106}$

HPP-1 was used in the mSAM studied and thus some vibrations arising from the biotin unit on the probe can be found in the spectra. Between 1680 and 1710 cm$^{-1}$ the C=O stretching of the cyclic urea of biotin can be observed and another peak at 1742 cm$^{-1}$ indicates C=O stretching of the biotin carboxylic acid.$^{104}$ Generally, signals around 1400 cm$^{-1}$ also arise due to carboxylic groups,$^{104,110}$ confirming the presence of biotin on the surface.
3.2.1.2 Conclusions

The ATR-FTIR-technique was found to be useful for characterization of the mSAMs. However, since no particular marker was used that could have been easily monitored, the recorded spectra were difficult to analyze due to the abundant number of peaks. The sensor design, for which this sensing platform was intended, required additional attachment of QDs to the HPP-1 through biotin-streptavidin binding. It was concluded that peak assignment would be further complicated for the complete sensing platform due to the vast number of vibrations involved in the large structures of the molecules involved (particularly the streptavidin and the probe itself). This also excluded the possibility of quantitative characterization of the mSAM with ATR-FTR for this system. Therefore, alternative methods (such as Atomic Force Microscopy) to characterize the surfaces of the mSAMs were explored.

3.2.2 Atomic Force Microscopy (AFM)

To further enhance the understanding about the sensing platform and the sensing mechanism at work, Atomic Force Microscopy- (AFM) measurements were conducted. Previous studies suggest that tapping-mode (TM) is the preferable mode for investigating soft biomolecule samples because of the smaller lateral forces compared to e.g. contact mode AFM.111, 112 TM-AFM can image the topography of the surface and also produce phase images that show the surface as a function of the mechanical and adhesion properties of the immobilized molecules.112 Contact mode has however also been successfully used in the study of monolayers of DNA on various Si-10, 113, thin oxide-114 and Au-modified surfaces.112, 115 Erts et al. conducted a study of spatially distributed DNA on a gold surface investigating the distribution and mechanical properties of individual molecules. In the study mercaptohexanol (MCH) was adsorbed to the Au to produce an ordered monolayer. TM-AFM
was then used to visualize individual DNA molecules, attached on top of the self-assembled MCH-monolayer, and to determine the distribution of DNA oligomers on the surface. The authors found that the surface density of DNA molecules increased nonlinearly with increasing oligonucleotide concentration and that the interactions between the tip and the monolayers could be attractive or repulsive depending on what scanning parameters were used. Generally, the heights of the immobilized ODN structures were found to be shorter and broader than expected, possibly due to tip mechanical interaction between the tip and the monolayer. Short single stranded probes (ssDNA), 25 base pairs, and their hybridization with complementary target have also been detected with AFM. Holmberg et al. employed a similar approach, with a mSAM composed of ssDNA and MCH, but performed the measurements in a liquid cell, preserving the natural environment of the DNA. An increase in the smoothness and the thickness of the monolayer was interpreted as the result of hybridization between the probe and the complementary target DNA.

To improve the visibility of the DNA probes and/or the formed duplexes, Au NPs have been used as labels for signal enhancement. Jin et al. developed a method for characterization of two different sequences of DNA immobilized on a Si substrate. The complementary sequences were modified with two sizes of Au NPs to provide distinctive topography for each of the hybridized duplexes. The lateral distributions and densities of the two DNA sequences were revealed on a sub-micrometer scale using this method. An alternative approach is to use Au NPs as size enhancers in the DNA hybridization reaction, as described in the work by Bui et al. The complementary target was added to the two sets of Au NP-functionalized DNA probes in sandwich hybridization. Aggregation of the Au NPs was then observed upon hybridization and the mean diameter of the NPs was correlated with the target DNA concentration to achieve detection of target concentrations down to 10 pM. Hairpin structured DNA probes have also been labelled with Au NPs for AFM imaging, as
first reported by Lavalle et al. HPPs were modified by very small Au NPs (1.4 nm) and grafted on two kinds of thin oxide films: antimony doped tin oxide (Sb doped SnO\textsubscript{2}) film and silicon oxide (SiO\textsubscript{2}) film. After immobilization of the HPP the two films exhibited different features in terms of surface roughness: an increase was observed for the smooth SiO\textsubscript{2}-film but a decrease was measured for the rougher SnO\textsubscript{2}-film. After hybridization a smoother surface was obtained in both cases.

### 3.2.2.1 Characterization of the mSAM by AFM

In this work AFM was used to characterize the mixed monolayer of m-PEG and an unmodified hairpin probe (HPP-4) on a gold surface and also to study the effect of the hybridization event on the thickness of the monolayer. Attachment of NPs, or a protein such as streptavidin, to the probe was expected to alter the dynamics on the surface and was considered likely also to affect the hybridization event and was therefore not attempted in this study. Another main objective for conducting high-resolution AFM measurements was to obtain information about the distribution and density of HPPs and m-PEG in the mSAMs. It is highly unlikely that the HPPs would be homogenously immobilized and evenly spread on the substrate, even in the presence of m-PEG, with great implications on the future sensor performance.

The quality of the substrate is crucial in high resolution AFM-imaging. The smoothness of a surface can be evaluated by calculating the roughness average, $R_a$, or the root mean square value (RMS) from the obtained AFM images. $R_a$ is defined by

$$ R_a = \frac{1}{n} \sum_{i} |P_i - \bar{P}| $$  

(Eq. 3.1)
where $P_i$ is height at each measured point, $\bar{P}$ is average height and $n$ is number of measured points. $R_a$ represents the mean height of the surface profile (peaks and inverted valleys) whereas $RMS$ is a statistical measure of the magnitude of a varying quantity, i.e. the surface profile in this case. $RMS$ is a more sensitive measure and can be written as

$$RMS = \sqrt{\frac{\sum_{i} (P_i - \bar{P})^2}{n}}$$

(Eq. 3.2)

The $R_a$ and $RMS$ values for four different substrates (listed in Section 2.2.4) were characterized to determine which was most suitable for further use. As shown in Figure 3.6 the values of the $R_a$ and the $RMS$ show the same pattern for all the examined surfaces. Generally $RMS$ values are more sensitive to the morphology of the surface and the focus here will therefore be on the obtained $RMS$ values. Au 1, which consists of 100 nm of Au sputtered on 50 nm of Ti on glass showed the highest surface roughness with an $RMS$ value of 3.6 nm and was therefore considered to be the least suitable for further use. Au sputtered directly on glass (Au 2) and Au sputtered on silica, (NDL-Au), showed $RMS$ values of 1.1 and 1.9 nm, respectively. Although Au 2 and NDL-Au exhibited significantly smoother surfaces compared to Au 1, it was difficult to distinguish HPPs (~ 5 nm) and m-PEG (~2.5 nm). However, the fourth substrate, Au-mica, showed a considerably smoother surface with a $RMS$ value of only 0.28 nm, which is significantly smaller than the estimated heights of the HPP and m-PEG, providing a suitable starting point for imaging the mSAMs.
Figure 3.6. Root mean square (RMS) and roughness average (R_a) values for a range of Au substrates. The RMS was found to be: 3.6 nm for Au 1, 1.1 nm, for Au 2, 1.9 nm for NDL-Au and 0.28 nm for Au-mica.

Figure 3.7 shows the AFM-images for: A) a bare Au-mica substrate, after immobilization of B) only m-PEG, C) only HPP, D) m-PEG:HPP 25:1, E) m-PEG:HPP 10:1 and F) m-PEG:HPP 2:1. The preparation of the samples is outlined in section 2.3.2. As seen in Figure 3.7.A the bare Au substrate shows an unknown contamination, with a similar height as the HPPs. This provides a problem for analysis of the images of the SAMs as it is difficult to distinguish HPPs from the contaminant to obtain reliable results. The image of only m-PEG (Figure 3.7.B) is very similar to that of the bare Au substrate (Figure 3.7.A) and no clear differences can be observed. For the SAM build up by only HPPs (Figure 3.7.C), an indication of a network pattern can be made out, which suggests HPP-assembly on the surface. The samples containing various m-PEG:HPP densities show a tendency towards a denser network pattern when the ratio of the HPP is increased (Figure 3.7.D-F).
Analysis of the observed network pattern, to give useful topographical information about the SAMs, was hindered by the presence of contaminants.

Figure 3.7. AFM image of A) a bare Au-mica substrate, after immobilization of B) only m-PEG, C) only HPP, D) m-PEG:HPP 25:1, E) m-PEG:HPP 10:1 and F) m-PEG:HPP 2:1.

Some conclusions can, however, be drawn from the $RMS$ values of the various samples. Figure 3.8 compares the $RMS$ values calculated based on the data obtained from the images in Figure 3.7 for the studied SAMs. The $RMS$ values show that the m-PEG:HPP 10:1 layer has the highest $RMS$ value, indicating the roughest surface of the SAMs examined. The m-PEG-modified Au surface shows a decrease in $RMS$, demonstrating the ability of the m-PEG to assemble into dense, smooth layers. This has also been shown with neutron reflectometry, which will be discussed in Chapter 4. The $RMS$ for the HPP-only layer is very similar to the $RMS$ for the bare Au, with a difference of only 0.14 nm.
Figure 3.8. A comparison of root mean square (RMS) and roughness average (Ra) values for a bare and modified Au-mica substrate.

The RMS for the various ratios of m-PEG:HPP are higher than for either the bare Au or the m-PEG- or HPP-only modified samples. This is expected for when molecules of different size are used. The RMS value for m-PEG:HPP 10:1 is the highest, an indication of the formed heterogeneous layer. Although the m-PEG:HPP 25:1 and 2:1 layers are unlikely to be homogeneous, they still exhibit fewer variations in height compared to m-PEG:HPP 10:1.

Another scanning probe technique, which potentially could give more useful information about the mSAMs, is scanning tunnel microscopy (STM). In 1999 Zhao et al. characterized DNA-modified gold electrodes by STM. The authors were able to distinguish differences in how ss vs. dsDNA adsorbed on a gold electrode based on the collected STM images. SsDNA formed a disordered pattern on the surface, whereas dsDNA adsorbed on the surface in an ordered form.
The mechanism of DNA immobilization and the structure and stability of mixed self-assembled monolayers have also been successfully studied by STM, which indicates that this technique could also be utilized to study the mSAMs used in this work.

3.2.2.2 Conclusions

TM-AFM was used for characterization of the mSAMs and evidence of monolayer formation was obtained. Some insight in the composition and morphology of the SAM was gained and the importance of a smooth substrate cannot be stressed enough to be able to use TM-AFM to its full abilities. Also, some differences in surface roughness between the different layers were observed. m-PEG molecules formed a relatively dense and smooth SAM, whereas the mSAMs showed increased surface roughness. However, the detailed distribution of the two components in the mSAMs, or the densities of the various layers, could not be clearly elucidated within the timeframe of the experiments, which were performed during a visit at NDL.

3.3 Characterization of the HPPs

The melting temperature, T_m, of DNA is typically defined as the temperature at which half of the base pairs, which normally holds the double stranded helix together, are disrupted. High acidity can also cause the dissociation of the complementary strands in the double helix into two single strands. When an aqueous solution of DNA is heated at 100°C or exposed to a very high pH (pH ≥ 13), the complementary base pairs that normally hold the two strands of the double helix together are disrupted and the double helix rapidly dissociates into two single strands. It provides a measure of the stability of the duplex and is dependent on the base composition, sequence length, and the solution conditions (such as ionic strength, pH, and buffer composition). GC base pair rich sequences have a higher T_m compared to AT rich ones.
because the GC bases are held together by three hydrogen bonds instead of two hydrogen bonds as in AT pairs. High ionic strength stabilizes the duplexes in solution (increasing the $T_{m}$) due to screening of electrostatic repulsion of the negatively charged phosphate groups in the DNA backbone. Certain single-stranded DNA molecules form stem-loop secondary structures, also called ‘hairpin’ structures, which can exist in a variety of conformations. Simply, all of the conformations can be divided into two states: the open and the closed state. Low enthalpy, due to base pairing in the stem region, is characteristic for the closed state, whereas high entropy, due to the large number of configurations that a single-stranded DNA chain can form, is distinctive for the open state. The transition from the closed to the open state requires a change in energy, sufficient enough to unzip all the base pairs in the stem. The closing of the loop involves a collision between the two arms of the hairpin, which sets of the nucleation and the propagation of a base-paired stretch.

The melting temperature of a dimer (duplex), or of a single stranded, folded monomers can be measured by calorimetric or spectroscopic methods. UV-vis spectroscopy is often used, as it offers a convenient, fast and simple way to measure a melting profile of a sample in solution. Differential scanning calorimetry (DSC) also provides detailed information about the melting behaviour and is often used either as a stand-alone technique or to confirm the results from spectroscopic measurements or predicted values. Most thermodynamic studies have been carried out in solution but there are also a few available on the melting behaviour of surface-attached sequences. In 1998 Bonnet et al. investigated the kinetics of the conformational changes in HPPs. In this study the dependence of closing and opening rates on such parameters as the length and composition of the loop sequence and salt concentrations over a wide range of temperatures were presented. It was found that the characteristics of the loop had a larger influence on the closing rate of the HPP than on the opening rate, which has been an important consideration in the design of HPP-based DNA sensors. A year later it
was reported that hairpin-structured probes were capable of a very high specificity, distinguishing targets that differed by only a single nucleotide. This excellent specificity was attributed to the constrained secondary structure of the HPP. 123

3.3.1 Predicted melting profiles for the basic hairpin structure and its probe/target duplexes

The first efforts to develop methods for prediction of the stability of nucleic acid duplexes were made in the early 1960’s by Tinoco and Zimm et al.128,129 They built up a method using a nearest-neighbour (NN) model for helix propagation, where the stability of a given base pair is assumed to be dependent on the identity and orientation of neighbouring base pairs.130 Several nearest-neighbour parameter sets for predicting DNA duplex stability are now available in the literature.131-135 Further studies on duplex stability were conducted and SantaLucia et al. presented improved prediction parameters for the NN-model in 1996 based on a thermodynamic study of 26 oligonucleotides of varying length (4 to 16 base pairs). The data for 23 of the sequences was compared with data for 21 sequences, found in the literature and improved NN-parameters, able to predict the stabilities of dimers within the limits of the NN-model, were produced.136 Hall et al. showed that the flanking sequence context, beyond nearest-neighbours, influences the thermodynamic stability of a single-base mismatch in a short (31 base pair) linear ODN duplex.137 A model was later developed by Markham et al.,79 building mainly on the parameters obtained by SantaLucia. Markham et al. aimed to obtain a model, which was able to not only compute the melting temperature of a DNA duplexes and stemmed probes, but also calculate the changes in the free energy, ($\Delta G$), enthalpy ($\Delta H$) and entropy ($\Delta S$) associated with the transition from a hybridized state, at temperature $T$, to a random coil formation. $\Delta G$, $\Delta H$ and $\Delta S$ are related as follows

$$\Delta G = \Delta H - T\Delta S$$

(Eq. 3.1)
Both the free energy and the enthalpy are computed using published nearest neighbour coefficients and the parameters of SantaLucia.\textsuperscript{136, 138}

The most common method in use for predicting melting temperatures for duplexes or for single-stranded, folded monomers is based on a two-state model, where two molecules, A and B, are either hybridized or not. The non-hybridized state (random coil) for each molecule represents a single reference state. A usual assumption is that A and B are fully complementary sequences but one or more mismatches can sometimes be permitted in the calculations. In the case of a single, folded molecule, a simple stem–loop structure is used as the default formation.\textsuperscript{79} In the model developed by Markham et al. the melting temperature, $T_m$, for a simple stem–loop structure (a hairpin) is computed as $T_m = 1000 \times \Delta H / \Delta S$, where the factor of 1000 converts from e.u. (entropy units) to kcal mol$^{-1}$ K$^{-1}$. The concentrations (in mol l$^{-1}$) of the participating strands must be taken into account for dimer simulations.\textsuperscript{138} If $[A_0]$ and $[B_0]$ are the strand concentrations of A and B, respectively, then the total strand concentration, $C_t$, is $[A_0] + [B_0]$.\textsuperscript{123, 139} Usually it is assumed that $[A_0] = [B_0] = C_t/2$. The melting temperature is calculated as shown in Equation 3.2.

$$T_m = \frac{1000 \times \Delta H}{\Delta S + \ln \left( \frac{C_t}{f} \right)}$$

(Eq. 3.2)

where $R$ is the universal gas constant and $f = 4$. When $[A_0] = C_t$ it is referred to as homodimer melting and equation 3.2 holds with $f = 1$.\textsuperscript{80}

The DINAMelt web server\textsuperscript{80} strives to combine up-to-date thermodynamic parameters with suitable algorithms that compute more than just melting temperatures. Ultraviolet (UV) absorbance, heat capacity ($C_p$) and concentrations of various dimer and monomer species as a function of temperature are also simulated for molecules in solution at thermodynamic equilibrium at the designated temperature.
To predict the melting behaviour of surface attached DNA dimers and monomers is beyond the DINAMelt server, as it is complicated by an unclear “solution concentration” and potentially slow kinetics.

Table 3.2 summarizes the computed melting temperatures for 4.65 µM of HPP and the equivalent amount of any target at two different temperatures, 37 and 44 °. A 0.137 M concentration of Na⁺ was used. The computational model does not take end-modification of the sequences into account and thus the same data is assumed valid for all the duplexes formed between the probes (HPP-1 to HPP-4) and the different targets (cDNA, long cDNA, 1MM-std, 1MM-center, 1MM-end, NC and NC-2).

<table>
<thead>
<tr>
<th>Sample (probe and/or target)</th>
<th>Hybridization temperature (°C)</th>
<th>Melting temperature (°C)</th>
<th>Delta G (kcal mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probe</td>
<td>37</td>
<td>44.7</td>
<td>-1.5</td>
</tr>
<tr>
<td>Probe</td>
<td>44</td>
<td>44.7</td>
<td>-0.1</td>
</tr>
<tr>
<td>Probe + cDNA</td>
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<td>64.2</td>
<td>-24.0</td>
</tr>
<tr>
<td>Probe + cDNA</td>
<td>44</td>
<td>64.3</td>
<td>-20.2</td>
</tr>
<tr>
<td>Probe + 1MM (std)</td>
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</tr>
<tr>
<td>Probe + NC</td>
<td>37</td>
<td>-2.6</td>
<td>-15.0</td>
</tr>
<tr>
<td>Probe + NC</td>
<td>44</td>
<td>-2.0</td>
<td>-15.0</td>
</tr>
<tr>
<td>Probe + long cDNA</td>
<td>37</td>
<td>67.0</td>
<td>-27.7</td>
</tr>
<tr>
<td>Probe + long cDNA</td>
<td>44</td>
<td>67.1</td>
<td>-23.4</td>
</tr>
<tr>
<td>Probe + 1MM (center)</td>
<td>37</td>
<td>59.3</td>
<td>-19.4</td>
</tr>
<tr>
<td>Probe + 1MM (center)</td>
<td>44</td>
<td>59.3</td>
<td>-16.2</td>
</tr>
<tr>
<td>Probe + 1MM (end)</td>
<td>37</td>
<td>64.7</td>
<td>-22.6</td>
</tr>
<tr>
<td>Probe + 1MM (end)</td>
<td>44</td>
<td>64.8</td>
<td>-19.2</td>
</tr>
<tr>
<td>Probe + NC-2</td>
<td>37</td>
<td>-4.6</td>
<td>10.1</td>
</tr>
<tr>
<td>Probe + NC-2</td>
<td>44</td>
<td>-3.8</td>
<td>10.1</td>
</tr>
</tbody>
</table>

Table 3.2. Summary of the computed melting temperatures for HPP-4 and its fully complementary (cDNA and long cDNA), one-base mismatch (1MM-std, -center and –end) and non-complementary targets (NC and NC-2), respectively. The annotation “Probe” is used to highlight that the simulation does not take end-modifications into account and thus the predicted Tₘ is the same for all the probes used.
The first calculations were carried out for a hybridization temperature of 37 °C, which is a commonly used temperature as it reflects the regular body temperature. The calculation yielded an estimated melting temperature close to 44 °C for the HPP (in solution). Another set of calculations was then carried out to determine any possible effects on the melting temperatures of the various probe/target duplexes for a hybridization temperature, which matched the melting temperature of the HPP involved.

Generally the melting temperatures computed for hybridization at 37 and 44 °C show little difference, a maximum of 0.1 °C, in all cases. However, greater differences can be seen in values for the free energy, ΔG. For the single-stranded, folded probe a melting temperature of 44.7 °C was predicted. This Tm is relatively low, especially if hybridization is carried out at 44 °C. There exists a potential risk that temperature alone can cause the opening of the folded hairpin, also indicated by the very low ΔG at 44 °C, which would be of great concern in a DNA sensor design, where the origin of the signal must be the hybridization event exclusively. The fully complementary sequences show the highest melting temperatures at 64.2 and 67.0 °C for cDNA and long cDNA, respectively. The long DNA is complementary not only to the loop part of the HPP but also to part of the stem and is thus expected to form a more stable duplex with the probe compared to cDNA, in agreement with the simulated results. A 1-base mismatch is predicted to have a melting temperature of 59.3 °C if it is positioned in the centre of the target sequence, compared to 64.8 °C if it occurs in an end position of the sequence. Interactions with non-complementary target (NC and NC-2) are expected to be very unstable with melting temperature at -2.6 °C and -4.6 °C, respectively, and a duplex is unlikely to form in solution. The sequence of NC-target has 5 (out of 18) randomly positioned bases, which are complementary to the probe and that is seen as a slightly higher melting temperature predicted for NC, compared to NC-2.
3.3.2 Experimentally determined melting profiles for the modified and unmodified HPPs

To validate the predicted melting behaviour of the probes and the targets, the melting profiles of the DNA fragments in solution were experimentally determined. A typical melting curve is presented in Figure 3.9, where the melting of a duplex between HPP-3 and long cDNA is plotted as temperature vs. absorbance at 260 nm. The experimental data is fitted to the sigmoid, Boltzmann equation in the software Origin 7.5 to obtain the melting temperature.

![Figure 3.9](image)

Figure 3.9. A representative melting profile of a dsDNA (HPP-2 and cDNA) obtained experimentally. The circles symbolize the measured data points and the solid blue line represents the sigmoid Boltzmann fit.

Generally, the HPPs exist in their hairpin secondary structure at low temperatures and probe/target duplexes form spontaneously since the hairpin stem is less stable than the formed duplex. When the temperature is raised the duplexes become more destabilized and eventually the hydrogen bonds between the probe and the target are disrupted and the duplex is denatured. Table 3.3 summarizes the obtained, actual melting temperatures for the various
probes alone exhibited an experimentally determined, averaged melting temperature of 48.0 °C, which is slightly higher than the computed melting temperature of 44.7 °C. The melting temperature of the HPPs corresponds well to the results obtained by Bonnet et al, who measured a melting temperature of 54 °C for a hairpin structure with a 5-base stem and a 15-base loop. The higher melting temperature observed by Bonnet et al. for the hairpin structure, can be explained by the shorter loop part of their probe, providing a more stable hairpin structure.

Randolph et al. investigated the fluorescence and hybridization of multiply-labelled DNA probes and the effects of end modification such as labelling density, fluorophore charge and linker length on fluorescence intensity. The change in fluorescence upon duplex formation, the quantum yield of fluorescence, probe–target stability and specificity was also included in the study by Randolph et al. It was found that although the target specificity of the multiply-labelled DNA probes was as high as for the unmodified control probe, a less stable probe–target duplex was formed. Dye–dye and dye–nucleotide interactions appeared to stabilize a single-stranded conformation of the probe, resulting in a lower melting temperature for the duplex. To ascertain that the singly-labelled HPPs investigated here did not experiences similar destabilization due to their end-modifications, the individual melting temperatures of the HPPs (listed in Table 3.3) were carefully considered. Slight differences can be observed for the differently modified probes (HPP-1, HPP-2, HPP-3 and HPP-4) and their targets but the variations are within the experimental error and thus it was concluded that the end-modification of the HPPs did not significantly alter the melting temperature of the HPP/target duplexes. This is in accordance with a study by Riccelli et al., who investigated both the effect of 3’-single-stranded dangling-ends and of loop biotinylation on the thermodynamic stability of hairpin structures. They found that neither the dangling-ends nor the biotinylation affected the stability of the hairpin structure, regardless of the length of the
dangling-end and the position of the biotinylation in the loop. These observations support the use of hairpins as probes for DNA detection as considerable freedom is allowed in the design of the probes.

<table>
<thead>
<tr>
<th></th>
<th>HPP-1 ( T_m ) (°C)</th>
<th>HPP-2 ( T_m ) (°C)</th>
<th>HPP-3 ( T_m ) (°C)</th>
<th>HPP-4 ( T_m ) (°C)</th>
<th>Average ( T_m ) (°C)</th>
<th>Predicted ( T_m ) (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probe</td>
<td>48.5 ± 0.7</td>
<td>49.7 ± 0.3</td>
<td>46.7 ± 0.4</td>
<td>47.1 ± 0.4</td>
<td>48.0</td>
<td>44.7</td>
</tr>
<tr>
<td>cDNA</td>
<td>58.4 ± 0.8</td>
<td>60.2 ± 0.5</td>
<td>58.5 ± 0.5</td>
<td>60.2 ± 0.4</td>
<td>59.3</td>
<td>64.3</td>
</tr>
<tr>
<td>long cDNA</td>
<td>61.6 ± 0.4</td>
<td>62.7 ± 0.2</td>
<td>61.6 ± 0.3</td>
<td>61.9 ± 0.3</td>
<td>61.7</td>
<td>67.1</td>
</tr>
<tr>
<td>1 MM centre</td>
<td>47.6 ± 0.4</td>
<td>47.9 ± 0.2</td>
<td>49.0 ± 0.3</td>
<td>49.1 ± 0.3</td>
<td>48.4</td>
<td>59.3</td>
</tr>
<tr>
<td>1 MM std</td>
<td>51.0 ± 0.3</td>
<td>51.6 ± 0.4</td>
<td>52.5 ± 0.3</td>
<td>52.4 ± 0.3</td>
<td>51.9</td>
<td>60.5</td>
</tr>
<tr>
<td>1 MM end</td>
<td>55.3 ± 0.4</td>
<td>56.9 ± 0.3</td>
<td>56.8 ± 0.4</td>
<td>56.9 ± 0.5</td>
<td>56.5</td>
<td>64.8</td>
</tr>
<tr>
<td>NC-2</td>
<td>51.5 ± 0.2</td>
<td>51.8 ± 0.2</td>
<td>71.4 ±1.2</td>
<td>40.8 ± 0.4</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>NC-2</td>
<td>41.8 ± 1.3</td>
<td>72.2 ± 2.1</td>
<td>48.1 ± 0.7</td>
<td>43.1 ± 0.5</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 3.3. Summary of the experimentally determined melting temperatures for the studied HPPs and their fully complementary (cDNA and long cDNA), one-base mismatch (1MM-std, -centre and –end) and non-complementary targets (NC and NC-2), respectively.

Overall, the HPP/long cDNA duplexes showed a higher melting temperature, an average of 61.7 °C, compared to the HPP/cDNA at an average of 59.3 °C. This is expected and in agreement with the predicted behaviour (Table 3.2). In the long cDNA a part of the stem is complementary to the target, in addition to the loop, which increases the melting temperature of the duplex. Interestingly, Bonnet et al. observed a two-state melting process when the probe was in the presence of its complementary target. Firstly, the formed duplex was disrupted at 42 °C and when the hairpins were released they re-adopted their original hairpin structure. Secondly, the hairpin itself was destabilized at the previously observed 54 °C. This observation has implications for the use of HPPs in different sensing designs. The stability of 1-based mismatched duplexes were also measured and, as listed in Table 3.3, these duplexes showed a decreased melting temperature compared to the fully complementary
ones, averaged 48.4 °C (1MM centre), 51.9 °C (1MM std) and 56.5 °C (1MM end), respectively. The same behaviour was observed by Bonnet et al., who found that the disruption of the duplex occurred already at 28 °C, whereas the released hairpin was disrupted again at 42 °C.139 It has also been shown that the position of the 1-base mismatch alters the thermodynamic behaviour of the formed duplexes, which can be observed as differences in the melting temperatures.140 Throughout Table 3.3 a pattern can be found regarding the effect of the position of the single-base mismatch on the stability of the formed duplexes. The mismatch was either a guanine (G) base replaced with cytosine (C), or a cytosine base replaced with a guanine. The single-base mismatches were positioned as follows: 1) in 1MM (std) at the 6th base, 2) in 1MM centre at the 12th base and 3) in 1MM end at the last base, starting from the 5’-end of the sequence (see Table 2.1 and 2.2). The results, listed in Table 3.3, showed that the mismatch in the centre of the target sequence had the largest and the end-positioned mismatch had the smallest effect on the stability of the double-stranded product. Average melting temperatures for all the probe/1MM duplexes were measured to 48.4 °C for 1MM centre, 51.9 °C for 1MM (std) and 56.5 °C for 1MM end. The least stable 1MM duplexes have an average melting temperature of 48.4 °C, which is approximately 11 °C lower compared to cDNA (59.3 °C), which is slightly lower than the corresponding difference of 14 °C reported by Bonnet et al.139 Only 1MM (std) was used for the evaluation of the performance of a HPP DNA sensor (detailed in Chapter 5 and 6), and it was shown that the 7 °C difference between the melting temperatures of cDNA and 1MM was enough to ensure a very good selectivity of the sensor.48

Duplexes formed with two different non-complementary targets (NC and NC-2) were also studied (sequences are listed in Table 2.1 and 2.2). NC contains 5 bases, which are complementary to the probe, whereas NC-2 is fully non-complementary to the probe. As shown in Table 3.3 the melting temperatures for all the HPPs/NC and NC-2 duplexes
showed values in same range as for 1MM targets, which was surprisingly high. Despite the relatively high stability of the non-complementary duplexes the difference between them and complementary duplexes was still sufficient for distinction of the two.

On the whole, the predicted melting temperatures were higher than the experimentally observed ones, except for the probe itself (see Table 3.3). The difference between the calculated and measured melting temperatures was between 5 and 11 °C for the different duplexes. As reported by Markham et al., predictions can be misleading if kinetics are dominant in the solution. The predictions assume that the molecules in solution are at thermodynamic equilibrium at each temperature but kinetics could be dominant if the equilibrium is reached slower than expected. In this case, however, the simulations adequately predicted the stability of the different duplexes, relative to each other, and the same conclusions could be drawn from both the simulated and the experimentally measured melting temperatures.

3.3.3 Conclusions

Melting temperatures of oligonucleotides provide a measure of the stability of the formed duplex and is dependent on the base composition, sequence length, and the solution conditions such as ionic strength, pH, and buffer composition. Simulation of the thermodynamic behaviour of various single-stranded and double-stranded DNAs can be very useful in the design of suitable probes for various applications. In the case of hairpin structures, the appropriate ratio of bases in the loop versus the stem is of great importance, especially when used as DNA sensors as the sensitivity and selectivity of the HPPs depend primarily on their secondary structure.

The computed melting temperatures corresponded relatively well with the obtained experimental data for the probes in solution and also matched the stability of the various
HPP/target duplexes, relative to each other. However, the experimentally determined melting temperatures were generally 5 - 11 °C lower than the computed values for all HPP/target duplexes investigated. In solutions containing only the probes, the end-modification of the HPPs did not significantly affect their melting behaviour although some interference in the stability of the various HPP/targets seemed to occur.

As predicted by the DINAMelt server, and confirmed by the empirical data, the duplexes formed with complementary targets showed greater stability and thus exhibited higher melting temperatures compared to duplexes with mismatches or non-complementary target. This provides evidence that these HPPs have the potential for discrimination of single-base mismatches if utilized in DNA-sensing.
Chapter 4 - Neutron Reflectometry Study of a Poly(ethylene glycol ) and Hairpin Probe Self-Assembled Monolayer used for DNA-sensing

Characterization of the mSAMs, used as the sensing platform in the DNA-detection design, was attempted with techniques such as ATR-FTIR and high-resolution AFM, as described in section 3.2. Neither of these methods could be used to fully characterize and describe the system. Neutron reflectometry (NR) has been shown to successfully probe soft samples, such as membranes and thin films, on solid surfaces and provide information about the density and the thickness of the studied layer.\textsuperscript{74, 141, 142} NR was thus selected as a complementary technique for the characterization of the mSAM, before and after hybridization with target ODN. Polarized neutron reflectometry (PNR)\textsuperscript{90, 94, 143} was employed to assess the melting behavior of the surface-attached HPPs for a comparison with the melting profiles obtained for HPPs in solution. This chapter presents results for different density m-PEG:HPP-4 mSAMs, before and after hybridization. The HPPs were expected to increase the thickness of the monolayer upon hybridization due to their increased length when unfolded. PNR was utilized for a preliminary in-situ investigation of the melting behavior of surface tethered HPPs. The effect of increased temperature versus hybridization on the conformational changes in the HPPs was also investigated and the results compared to the melting studies described in section 3.3.

4.1 Introduction

NR has emerged over the last twenty years as a sensitive profile analysis method, especially for soft matter and biological samples. Detailed information on the concentration profiles perpendicular to sample surfaces and solid/liquid interfaces can be obtained. NR has gained high resolution with the availability of short wavelength neutrons, which are able to
probe the sample on a molecular level.\textsuperscript{88} The difference in scattering length density (SLD) of hydrogen (H) and deuterium (D) provides the utilization of contrast variation (see section 2.4.7.1 for more details), highlighting different parts of the interface.\textsuperscript{142} An advantage of NR is that NR is non-destructive and very small quantities of sample are required (< 10\textsuperscript{-6} g).\textsuperscript{144} Polarized neutron reflectometry (PNR) measures the neutron spin-dependent reflectivity, based on a magnetic layer, such as Fe, within the substrate. By applying an external magnetic field contrasts are achieved through the difference in reflectivity between up- and downspin neutrons due to changes in the SLD of the magnetic reference layer\textsuperscript{94, 142} (see section 2.4.7.3 for details). Two independent sets of data are thus obtained from a single sample without any additional sample preparation\textsuperscript{145}, which is particularly advantageous for biological samples or when in-situ experiments are conducted.

Gold surfaces have been used as a substrate for surface immobilization of ssDNA probes with various spacer or linker molecules in a range of different DNA detection designs\textsuperscript{49, 51, 146-148} as they offer convenient attachment of thiol-modified molecules to its surface\textsuperscript{51} and can simultaneously act as the working electrode in electrochemical detection methods. Poly(ethylene glycol) (m-PEG) is known for its ability to make surfaces more biocompatible\textsuperscript{102, 103, 149} and used for two main purposes: 1) to reduce non-specific adsorption of DNA probes to the gold surface and 2) to control the probe density on the substrate, which is a crucial parameter in the design of a sensitive and selective sensor.\textsuperscript{14, 15, 49, 147, 148} Various thioalkanes and thioalcohols\textsuperscript{34, 49, 147} have been used for creating a mixed monolayer to control the surface density of the probes, which is a crucial parameter in the design of a sensitive and selective sensor.\textsuperscript{14, 15, 49, 147, 148} m-PEG is an uncharged and very flexible molecule,\textsuperscript{77} able to provide highly inert interfaces\textsuperscript{101}, and is unlikely to interact significantly with the DNA. Therefore, m-PEG provides an alternative to thioalkanes or thioalcohols as interstitial spacers in mixed SAMs.
In 1998 a pioneering neutron reflectivity study on self-assembled DNA monolayers on gold was published by Levicky at el. The conformational changes of the surface-tethered ss-DNA were studied as a result of 6-mercapto-1-hexanol (MCH) treatment, followed by hybridization. NR was determined to be a valuable technique for this study because it enabled depth-profiling with Ångström-level resolution. Concentration profiles for the samples were obtained and the ss-DNAs were observed to pass from a compact to an extended configuration upon MCH treatment. The authors concluded that control of the monolayer formation was achieved and that the ss-DNAs were terminally tethered to the gold surface. Upon hybridization the ds-DNA strands were found to position themselves towards the normal of the substrate, increasing the thickness of the monolayer.

Recently, Steichen et al. reported on the interfacial behavior of a hairpin DNA probe, which was immobilized on gold. Their aim was to show the conformational change of unlabeled hairpin probes, as they unfold from a closed hairpin structure to rigid double strands when hybridized with complementary target. The conformational change was expected to increase the DNA layer thickness on the gold substrate, which was confirmed by the acquired NR data. The transformation was more marked for the HPPs compared to thickness changes in layers based on ss-DNA.

4.2 Neutron Reflectometry analysis

All the experimental reflectivities were corrected for background and normalized to the incident beam intensity before analysis. The structure of the sample was determined by fitting the experimental data to a layer composition model which included the thickness, the scattering length density (SLD) and the roughness of the different layers. Initially, the
Si/SiO₂/Cr/Au or Si/SiO₂Fe/Au wafers were characterized to obtain the thickness and the SLD of the substrate layers and the attained values were subsequently fixed for the fitting of the m-PEG/HPP SAMs. All samples were described by their SLD profiles as a function of the distance, z, from the Au substrate. The ga_refl program,\textsuperscript{92} which was used for fitting of the data (introduced in section 2.7.4.7) is able to fit multiple datasets simultaneously within a range of constraints added to the structure model. It is a C++-based application that utilizes optical matrices to model the reflectivity data and a genetic algorithm for model refinement.\textsuperscript{151} Both non-magnetic and magnetic (polarized) data can be fitted with ga_refl and the number of datasets that can be fitted simultaneously is not restricted. The genetic algorithm used in ga_refl employs an optimization strategy inspired by observations in evolutionary biology. Briefly, it explores sets of possible values of fitting parameters (called population of chromosomes) in a semi-direct manner and thus avoids being trapped in local minima.\textsuperscript{151} The fitness of the population is then evaluated against a test function (a chi-squared error function in this case), which, in the terminology of evolutionary biology, then determines the probability for a given chromosome to “breed” and thus contribute to the next generation. The best fitted pair of chromosomes is more likely to be chosen to breed the next generation and this process is repeated as many times as necessary to populate the new generation. The effects of random mutation, introduced into the new population, is applied before the new population replaces the previous one.\textsuperscript{151} The fitness of a population needs to be evaluated in order to gain insight in to how well the model reflects the actual data and ga_refl uses a chi-squared function for this purpose. The chi-square value obtained from the model reflects how “close” they are to values expected from the fitted model. The $\chi^2$ is a reduced $\chi^2$, weighted by the uncertainty in each point and by the degrees of freedom. If the chi-square value, $\chi^2$, is large the observed and expected values are not close and the model is a poor fit to the data.\textsuperscript{143} Apart from layer thickness and SLD values for the components in the
model, the amount of water present (and consequently the amount of DNA) in the mSAM can be estimated through the following relation:

$$\rho_{\text{HPP-layer}} = v_f\rho_{\text{solv}} + (1 - v_f)\rho_{\text{DNA}}$$  \hspace{1cm} \text{(Eq. 4.1)}$$

where $\rho_{\text{HPP-layer}}$, $\rho_{\text{solv}}$ and $\rho_{\text{DNA}}$ are the SLD values of the HPP-layer, of the solvent and of DNA, respectively. Equation 4.1 thus gives the density of the total layer, under the assumption that it contains only HPP and solvent in it. Calculation of the volume fraction of HPP, $(1 - v_f\text{solv})$, present in the layer above the Au was incorporated in the structure model used to fit the reflectivity data and values were obtained accordingly.

4.3 Characterization of mSAMs before and after hybridization with complementary target

The samples chosen for the NR characterization represented SAMs with different densities of m-PEG:HPPs as well as reference samples with only m-PEG or HPP monolayers. Specifically, four different monolayers, consisting of: 1) only m-PEG molecules, 2) only HPPs, 3) the HPP only sample but backfilled with m-PEG and 4) simultaneously immobilized m-PEG:HPPs with the molar ratio 2:1. m-PEG was used to displace any non-specifically attached HPPs and encourage the HPPs to attach to the surface exclusively through the Au-thiol bond. The general experimental design (NR and PNR) is shown in Scheme 4.1.

The theoretical maximum height of a m-PEG molecule, with six repeated units, is 2.5 nm, and the height of the HPP is estimated to be 4-5 nm. The double stranded DNA, which forms during hybridization, has a maximum height of 13 nm and thus the thickness of the monolayer was expected to alter upon hybridization with complementary target DNA, providing a detectable change in the surface properties.
Scheme 4.1. A schematic (not to scale) illustration of the experiment performed with polarized neutron reflectometry. For NR experiments at ANSTO Cr was used as the adhesive layer between silicon and gold, whereas Fe was used in PNR experiments to achieve magnetic contrast. H represents the external and B the internal magnetic fields applied in PNR.

Figure 4.1 shows the neutron reflectivity profile for a monolayer of m-PEG molecules, after 1 h of immobilization and the SLD profile acquired based on the fitted reflectivity data is shown in Figure 4.2. Three contrasts, D$_2$O, H$_2$O and a mixture of the two, with a SLD = 2.5×10$^{-6}$ Å$^{-2}$ (CM 2.5), were used for the measurements, represented by the blue, red and green lines in Figure 4.1 and 4.2, respectively. Experimental data are displayed as diamond markers in the reflectivity profile, whereas the solid lines correspond to the fitted values. These notations will be used for all the presented results and different contrasts throughout this chapter, unless stated otherwise.

The layer thickness was calculated to 1.6 nm with a volume fraction of 50.1 % for the m-PEG layer. The thickness was lower than the maximum height of 2.5 nm for an extended m-PEG molecule positioned normal to the surface. It has been shown that self-assembled alkanethiols on gold have an average tilt of ~30° from the surface normal. Ehler et al. found tilt angles around 40° for alkanethiols, with a methylene chain length of 11 – 19 units, on gold. The
shortest chains \(n = 11\) exhibited the largest tilt \(45^\circ\), which the authors attributed to polycrystallinity in the surface structure.\textsuperscript{152}

![Figure 4.1](image.png)

Figure 4.1. The reflectivity profiles for a monolayer of m-PEG, immobilized at room temperature for 1 h. The diamonds represent the experimental data and the solid lines are the fitted data for three different contrasts, D\(_2\)O (blue line), CM 2.5 (green line) and H\(_2\)O (red line).

The tilt angle of the m-PEG SAM investigated in this study was calculated to be around \(50^\circ\). (Calculated by comparison of the measured layer thickness with the maximum theoretical length of the longest molecule in the SAM, which is 2.5 nm for m-PEG, 5.5 nm for the HPP and 13 nm for the HPP/target duplex). The calculated tilt angle was higher compared to the alkanethiol layer,\textsuperscript{75,152} which suggests that the short immobilization time resulted in a less dense and not as ordered layer of m-PEG on the Au surface. This is in accordance with a reported tilt-angle of \(50^\circ\) for moderately sparse layers of \(C_{18}H_{37}SH\) alkylthiols on Au (111) surfaces.\textsuperscript{153} Also, a sharp transition from the m-PEG- to the solvent layer was observed in the SLD profile for the m-PEG SAM (Figure 4.2) indicating a well defined m-PEG-layer. As the SLD value obtained for the m-PEG layer was notably different from both the Au substrate and from the solvent layers the structural model appears to fit the data reasonably well.
although the $\chi^2$-value is relatively high, as shown in Table 4.1 at the end of this section. This high $\chi^2$-value is characteristic of all the earliest measurements made on the Platypus reflectometer at ANSTO, and it is primarily due to systematic error near the critical edge. Nevertheless, the fit quality, as can be seen in Figure 4.1 (and subsequent reflectivity figures), is high.

![Diagram](image)

Figure 4.2. The neutron scattering length density (SLD) profile for a monolayer of m-PEG immobilized at room temperature for 1 h. Three different contrasts, D$_2$O (blue line), CM 2.5 (green line) and H$_2$O (red line), were used for the measurements.

The second reference layer investigated contained only HPPs, but was subsequently also backfilled with m-PEG molecules and hybridized with cODN for 1 h at 37 °C. The reflectivity data for these three samples are displayed in Figure 4.3, where the experimental figures are represented by diamond markers and the fitted curves are shown as solid lines. The corresponding SLD profile is presented in Figure 4.4. Firstly, the HPP-only layer gave rise to
a measured a thickness of 2.0 nm and a volume fraction of 7.7 % (Figure 4.3.A and 4.4, dotted lines). The relatively low volume coverage of 7.7 % can be explained by the short immobilization time of the HPPs (30 min), which yielded a relatively low amount of material in the SAM. Based on the obtained layer thickness and volume fraction the coverage of HPPs on the Au surface the average density corresponded to $1.5 \times 10^{12}$ HPP molecules cm$^{-2}$. This is lower than the density of single-stranded probes determined by Levicky et al. (~ 6 chains/cm$^{-2}$ for single-stranded probes),$^{150}$ likely influenced by the short immobilization time and the hairpin structure itself, which is double stranded at the stem where it binds to the gold surface.

The fairly low thickness of the HPP layer, which was of the same magnitude as the diameter of a double stranded DNA helix,$^{11}$ indicated that the HPPs were positioned on the Au with little protrusion into the solvent. This observation is consistent with the idea that, in the absence of spacers (such as MCH or m-PEG), DNA adsorbs non-specifically to gold.$^{51, 150}$ and these interactions are likely to have had a large influence on the HPP, pulling it down towards the gold surface. The tilt angle of the HPPs was calculated to be around 68°, which differs from the tilt of the m-PEG-layer. However, considering the shorter immobilization time (a less dense layer is formed) for the HPPs and their greater length, it stands within reason that the tilt angle is higher for the HPP- than the m-PEG-layer. Also, the determination of the tilt angle is based on a theoretical maximum length of the HPPs and needs to be addressed with some caution. It is also important to bear in mind that all the values obtained are averages over the sample area studied. Horizontal resolution is restricted with NR, which is sensitive to changes normal to the substrate surface. Thus, all the measured data and the calculated tilt angles are averages over a distribution of behaviors, with some molecules at normal angles whereas others are more prone to tilt towards the Au surface.
Figure 4.3. The reflectivity profiles for a monolayer of A) only HPPs, B) after backfilling with m-PEG and C) after hybridization with complementary target ODN at 37 °C for 1 h. The diamonds represent the experimental data and the solid lines are the fitted data. Three different contrasts, D$_2$O (blue), CM 2.5 (green) and H$_2$O (red) were used for the measurements before backfilling or hybridization (A) whereas two contrasts D$_2$O (blue) and H$_2$O (red), were used for studying the backfilled and hybridized layers (B and C).
When the HPP layer was backfilled with m-PEG (Figure 4.3.B and 4.4, dotted-solid lines) the structural model for the fits were changed from a 1- to a 2-layer model to account for a possible separation of the m-PEG and HPPs or dsODN layer. Upon backfilling with m-PEG, there was an increase in both the layer thickness and the volume fraction, from 2 nm to a total of 2.7 nm and from 7.7 % to a total of 24.5 %, respectively, and the surface coverage also increased accordingly. This transformation is also visible when the shape of the SLD profile (Figure 4.4), particularly the D$_2$O contrast, for the backfilled HPP layer (dotted-solid lines) is compared to the HPP layer (dotted lines). These results suggest that the m-PEG molecules are able to reduce non-specific adsorption between DNA and Au and prop up immobilized DNA into a more upright position, consistent with previous findings regarding various thioalkanes$^{14, 15, 49, 51, 147, 148}$ and further supported by the decrease in the attained tilt angle from 68° to 61°. The relatively large tilt angle even after backfilling can partially be explained by the presence of the C$_6$-linker at the 5’-end of the probe sequence. The length of the linker is of the same order as the m-PEGs and the more the HPPs protrude from the m-PEG-layer the more rotational freedom they attain.

After the hybridization with cODN further alterations were distinguishable in the reflectivity- and, more readily, in the SLD profiles (Figure 4.3.C and Figure 4.4, solid lines, respectively). The total thickness of the two layers in the model increased further to 5.1 nm while the total volume fraction decreased to 11.8 % (see Table 4.1). These results are in agreement with the hypothesis that hybridization should induce a change in layer thickness, although the modeled thickness implies that the HPP/target duplexes were not arranged normal to the substrate but at a tilt angle of 56°. Thiolated fifteen base-paired duplexes have been shown to tilt at about 45° when self-assembled on gold.$^{154}$

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Figure 4.4. The neutron SLD profiles for a monolayer of only HPPs (dotted lines), after backfilling with m-PEG (dotted-solid lines) and after hybridization (solid lines) with complementary target ODN at 37 ºC for 1 h. Three different contrasts, D$_2$O (blue lines), CM 2.5 (green line) and H$_2$O (red lines) were used for the measurements of the pure HPP layer, whereas two contrasts, D$_2$O and H$_2$O, were used for the backfilled- and the hybridized layers.

The HPP/target duplexes formed in this study are longer and more flexible than the fifteen-based duplexes investigated by Kelley et al.$^{154}$ and therefore a larger tilt-angle is expected. When the HPPs hybridizes with its complementary target the loop unfolds and a more stable and rigid structure is formed.$^{14, 15, 148}$ The hybridization process thus bares a part of the substrate surface that has previously been covered by the hairpin structure. As the target sequence is located in the loop part of the HPP, the double stranded part of the ODN complex will proposedly be pushed into the solvent or arrange itself on top of the m-PEG-layer. Either of these scenarios would reduce the volume fraction of material close to the substrate as the modeled results indicate. Furthermore, it can be assumed that any addition of material, in terms of calculated volume fractions, is solely due to attachment of the target ODN. The
surface coverage after hybridization of the backfilled layer was estimated to be $5.8 \times 10^{12}$ HPP molecules cm$^{-2}$ and the extracted density of bound cODN in the backfilled layer corresponded to $0.76 \times 10^{12}$ HPP molecules per cm$^{-2}$.

Steichen et al. also investigated the structural change of a DNA hairpin occurring after hybridization but they concentrated solely on a 1/1 hairpin DNA/4-mercaptobutan-1-ol molar ratio (MCB)-layer. The DNA_HPP concentration before hybridization was reported as $7.2 \times 10^{12}$ HPP molecules cm$^{-2}$. This surface coverage is higher than the HPP only-layer studied here, which exhibited $1.5 \times 10^{12}$ HPP molecules cm$^{-2}$. However, the DNA_HPP/MCB assembly was conducted over night (> 16 h) and is not directly comparable to the fast immobilization used for the pure HPP-layer here. Upon hybridization, a 4 nm increase in layer thickness was observed upon hybridization for the DNA_HPP/MCB, whereas the thickness of the backfilled HPP-layer increased with a similar 4.5 nm, confirming the structural change in the HPP.

Figure 4.5 and 4.6 show the reflectivity- and the SLD profile, respectively, for a mSAM with a molar ratio of m-PEG:HPP 2:1 before and after hybridization with cODN (for 1 h at 37 °C). It has been shown that probe density is a critical factor in the construction of efficient DNA sensors and consequently mSAMs with various HPP concentrations were investigated. The fitting procedure yielded a total thickness of 4.0 nm and a 20.1 % volume fraction for the m-PEG:HPP layer above the substrate before hybridization (Figure 4.5.A and 4.6, dotted lines).
Figure 4.5. The reflectivity profiles for the m-PEG:HPP 2:1 mSAM, A) before and B) after hybridization with complementary target ODN at 37 ºC for 1 h. The diamonds represent the experimental data and the solid lines are the fitted data. Three different contrasts, D$_2$O (blue), CM 2.5 (green) and H$_2$O (red) were used for the measurements before hybridization (A) whereas two contrasts D$_2$O (blue) and H$_2$O (red), were used after hybridization (B).
Based on the estimated tilt angle of 48° and the sharp transition seen in the SLD profile (Figure 4.6, dotted line) it could be concluded that the m-PEG:HPP 2:1 SAM forms a relatively dense layer with the components in a more upright position than the backfilled layer. A comparison between the backfilled HPP sample and the m-PEG:HPP 2:1 show similar volume fraction values for the two cases but there was a difference in the layer structure, reflected in the thickness difference between the two cases.

![Figure 4.6](image)

Figure 4.6. The neutron SLD profile for the m-PEG:HPP 2:1 mSAM before (dotted lines) and after (solid lines) hybridization with complementary target ODN at 37 ºC for 1 h. The data were collected at three different contrasts: D₂O (blue lines), CM 2.5 (green line) and H₂O (red lines).

The surface coverage was estimated to 7.9×10^{12} HPP molecules cm⁻², in which the m-PEG molecules are included but not separately accounted for. This generalization is justified by the assumption that the only additional component after hybridization is the complementary ODN, which binds solely to the HPP and not to m-PEG. Thus the difference in surface
coverage before and after hybridization can be assigned to bound target ODN, which in turn reflects the density of HPPs in the mSAM. The surface coverage of $7.9 \times 10^{12}$ HPP molecules cm$^{-2}$ is therefore comparable to the surface coverage of the m-PEG-backfilled HPP layer before hybridization ($6.6 \times 10^{12}$ HPP molecules cm$^{-2}$). The higher surface coverage for the m-PEG:HPP 2:1 compared to the backfilled mSAM is not completely unexpected since the immobilization time was longer. The result, however, is similar to the concentration of hairpin DNA on a gold surface, $1.0 \times 10^{13}$ mol cm$^{-2}$, reported by Steichen et al.$^{144}$ After the cODN was introduced (Figure 4.5.B and 4.6, solid lines) the change in the total thickness of the mSAM was insignificant. However, the volume fraction increased from 20.1 to 34.5 % suggesting that the target did bind to probe sequence in the HPP. The tilt angle was determined to be 72°, which was considerably larger than before the hybridization. The increased tilt angle implies that the molecules in the SAM have moved from their rather upright position to lie partially on top of the m-PEG layer and thus no significant change was observed in the layer thickness after hybridization. When the HPPs opened to form the HPP/target duplex a space was bared at the underlying Au surface, which would give the mSAM the liberty to increase its tilt angle. Although there was no significant increase in thickness due to the hybridization, an increase in surface coverage was observed and estimated to be $1.4 \times 10^{12}$ HPP molecules cm$^{-2}$. The amount of bound ODN was thus found to be $6.1 \times 10^{12}$ HPP molecules cm$^{-2}$, indicating that the m-PEG:HPP mSAM was more efficient as a DNA sensor than the backfilled HPP-layer. This result is in agreement with the electrochemical response found for a HPP-based DNA sensor$^{48}$ although contradictory to the response reported by Steichen et al.$^{144}$ This discrepancy is possibly due to the differences in the HPPs used here and those employed by Steichen et al. The main differences lie within the numbers of base-pairs in the stem of the investigated HPPs. HPP-4, used here, has a 9 base-pair long stem, which is quite stable when the complementary target is absent.
However, as the target is only complementary to the loop part of the HPP and the remaining arm sequences are relatively long, the HPP/target duplex is more flexible than the DNA_{HPP} with 6 base-pairs in stem used by Steichen et al.\textsuperscript{144} The comparatively mobile HPP-4/target duplex is thus more likely to lie on top of the spacer molecules than the more rigid DNA_{HPP}/target duplex.

An attempt was made to characterize a simultaneously immobilized layer of m-PEG:HPP with a molar ratio of m-PEG:HPP 10:1 (immobilization for 1 h at room temperature). The reflectivity- and the SLD profiles are shown in Figure 4.7 and 4.8, respectively.

![Figure 4.7. The reflectivity profiles for the m-PEG:HPP 10:1 mSAM, immobilized for 1 h at room temperature. The diamonds represent the experimental data and the solid lines are the fitted data. Three different contrasts, D$_2$O (blue), CM 2.5 (green) and H$_2$O (red) were used for the measurements.](image)

A total thickness of 5.7 nm was observed, with a volume fraction of 32.9 % and the estimated tilt angle was 19°. Simultaneous immobilization of the two components in the mSAM seem to yield a thicker and denser layer compared to the backfilling strategy presented in Figure 4.3 B and 4.4 (dotted-solid lines). This result is in accordance with AFM-data, presented in Section
3.2.2. It was found that the RMS-value for the m-PEG:HPP 10:1 layer was higher than the m-PEG:HPP 2:1, indicating that the latter ratio produced a smoother and denser layer.

The major features in the reflectivity- and SLD-profiles (Figure 4.8) arose from the m-PEG molecules but the contribution of the HPPs can be seen when the SLD-profile of a pure m-PEG-layer (Figure 4.2) is compared to the m-PEG:HPP 10:1-layer. The SLD of m-PEG is not as close to that of the solvents as the SLD of the m-PEG:HPP 10:1-layer, which is a clear indication of the presence of the HPPs. Also, the thickness of a sole m-PEG-layer was calculated to 1.6 nm, whereas the m-PEG:HPP 10:1 mSAM had a 1st-layer, predominantly representing the m-PEG molecules, of only 1 nm. Although the formation of the m-PEG:HPP 10:1 mSAM was observed, the density of the HPPs proved to be on the detection limit of the instrument and thus hybridization studies were not modeled.

Figure 4.8. The neutron SLD profile for the m-PEG:HPP 10:1 mSAM after immobilization for 1 h at room temperature/ The data were collected at three different contrasts: D₂O (blue lines), CM 2.5 (green line) and H₂O (red lines).
Table 4.1 summarizes the detailed layer thicknesses and volume fractions for all the investigated layers, derived from the 2-layer model fit of the reflectivity data. Even though the uncertainties ($\chi^2$-values) are relatively large across the investigated samples, due primarily to known systematic error most significant at low $Q$, some conclusions can still be drawn based on the observed trends. For the two mSAM samples (backfilled HPP and m-PEG:HPP 2:1), the 2-layer model showed similar thickness and volume fractions for the first layer (closer to the substrate), 2.5 versus 2.1 nm and 23.7 versus 19.9 %, respectively, before hybridization. The thickness of the second layer, considered to contain the fractions of the HPP structures that were longer than the m-PEG-molecule, was very thin (~0.2 nm) with a volume fraction of less than 1 %.

<table>
<thead>
<tr>
<th>2-layer model (except for PEG and HPP only*)</th>
<th>chi_sqr</th>
<th>1st layer thickness (nm)</th>
<th>1st layer volume fraction (%)</th>
<th>2nd layer thickness (nm)</th>
<th>2nd layer volume fraction (%)</th>
<th>Total thickness above Au (nm)</th>
<th>Total volume fraction above Au (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG only</td>
<td>47</td>
<td>1.6</td>
<td>50.1</td>
<td>N/A</td>
<td>N/A</td>
<td>1.6</td>
<td>50.1</td>
</tr>
<tr>
<td>HPP only</td>
<td>64</td>
<td>2.0</td>
<td>7.7</td>
<td>N/A</td>
<td>N/A</td>
<td>2.0</td>
<td>7.7</td>
</tr>
<tr>
<td>HPP + BackFill PEG</td>
<td>36</td>
<td>2.5</td>
<td>23.7</td>
<td>0.2</td>
<td>0.79</td>
<td>2.7</td>
<td>24.5</td>
</tr>
<tr>
<td>HPP + PEG BackFill after hybridization</td>
<td>315</td>
<td>6.7</td>
<td>7.6</td>
<td>0.5</td>
<td>0.49</td>
<td>7.2</td>
<td>8.1</td>
</tr>
<tr>
<td>2:1 PEG:HPP</td>
<td>53</td>
<td>2.1</td>
<td>19.9</td>
<td>1.9</td>
<td>0.17</td>
<td>4.0</td>
<td>20.1</td>
</tr>
<tr>
<td>10:1 PEG:HPP</td>
<td>69</td>
<td>1.0</td>
<td>23.0</td>
<td>4.2</td>
<td>9.85</td>
<td>5.2</td>
<td>32.9</td>
</tr>
</tbody>
</table>

Table 4.1. Summary of layer thickness and volume fraction values for the investigated SAMs, derived from a 2-layer model fit of the reflectivity data (Platypus, ANSTO). *A 1-layer model was applied for the m-PEG- and HPP only SAMs.

After hybridization the thickness increased in both the first layer and the second layer, while the opposite was seen for the volume fraction, which decreased in both layers upon hybridization. This is in accordance with the hypotheses that when the HPPs unfold in the presence of their complementary targets they protrude more, observed as an increase in thickness, but part of the substrate is bared underneath, demonstrated as a decrease in material in the layers. For the mSAM consisting of m-PEG:HPP in a 2:1 ratio, a decrease in thickness
of the first layer is seen as part of the stem was removed but an increase is observed in the
second layer as the target is bound to the probe. Interestingly an increased volume fraction
observed in both the first and the second layer after hybridization of the m-PEG:HPP 2:1
mSAM. The greater volume fraction for the hybridized layer clearly demonstrated that the
target ODN has bound to the probes. The formation of a mSAM with an even lower probe
density was also detected, although it was close to the detection limit of the instrument. In
order to validate the 2-layer model of the mSAM more experiments are needed, preferably
with polarized neutrons, as the inclusion of magnetic contrast is likely to improve the model
fit considerably.

4.4 Polarized neutron reflectometry for investigation of the melting
behavior of the surface-attached probes in the mSAM

Sterical constrains, due to high probe density in the sensing layer have been shown to
affect the hybridization efficiency.\textsuperscript{155, 156} If the hybridization temperature is increased, close to
the melting point of the HPP, these constraints can be overcome.\textsuperscript{48} The mechanism of the
hybridization event, and the dynamics of the HPPs, at the elevated temperature is not fully
understood and PNR was employed to investigate the melting profile of the surface attached
HPPs. Preliminary determination of the melting temperatures of unbound oligonucleotides
(ODNs) (both probes and duplexes) in solution has been carried out by detecting the change
in UV-absorbance with increasing temperature, as reported in section 3.3.3. However, these
results can only be seen as an indication of the melting temperature of the surface attached
HPPs. The temperature dependent behavior of surface attached ODNs is not very well known
and especially the unfolding of the HPPs is difficult to detect with other techniques (such as
Surface Plasmon Resonance) since it does not include any mass changes. Electrochemical
techniques have to some extent been used to measure the melting of single-stranded probes
and their targets,\textsuperscript{126, 157, 158} but to the author’s knowledge NR has not previously been
employed to probe the melting dynamics of DNA, and especially HPPs. Using NR it was possible to simultaneously determine the melting temperature, and the conformational changes of the DNA-probe during the hybridization process. The unfolded and fully extended probe would have an approximate maximum height of 13 nm and thus a significant change in the thickness of the monolayer upon increasing the temperature above the melting point of the hairpin is expected even if the HPP does not adopt a fully extended conformation. The magnetic contrast method, using polarized neutrons, was necessary for these samples to allow contrast change at elevated temperatures without disturbing the delicate hairpin probe system, as would be necessary using solvent contrast. Due to time restrictions and the efforts involved in obtaining beam time at neutron sources, the results presented here are indicative only and further melting studies of the surface-attached probes should be conducted to elucidate the exact melting temperature.

Figure 4.9 shows the SLD profile for the unmodified substrate, Si/SiO$_2$/Fe/Au (blue line), measured in air and for SAMs of HPPs immobilized for one (red line) or two hours (green line) measured in D$_2$O/PBS buffer. Firstly, HPPs were immobilized on the Au for 1 h (Figure 4.9, red line) and then to maximize the amount of HPP present at the surface an additional immobilization step of 1 h was conducted with a ten-fold concentration of HPPs (Figure 4.9, green line). The SLD for the HPP layer was found to be approximately 5.4×10$^{-6}$ Å$^{-2}$, with a thickness of 1.2 nm and a volume fraction of 44.9 % coverage after 1 h. The estimated surface coverage was 5.3×10$^{12}$ HPP molecules cm$^{-2}$. After immobilization for another hour there was no increase in the thickness of the HPP layer but the volume fraction grew to 60.0 %. Thus the surface coverage also improved to 7.1×10$^{12}$ HPP molecules cm$^{-2}$. These results indicate that a SAM of HPP was formed on the Au surface in close vicinity of the Au layer. As mentioned previously in section 4.3 the HPPs can reach a maximum theoretical height of approximately 6 nm if positioned normal to the surface. The HPP layer created after 2 h of
immobilization showed a thickness of only 1.2 nm, which indicates that the majority of the HPPs are lying down on the Au surface. Additionally, as no spacer molecules were utilized, non-specific adsorption is expected to occur between the phosphate backbone of the HPPs and the Au.\textsuperscript{14, 51, 150} The formed HPP layer was slightly thinner than expected, even for flat HPPs as the diameter of a double stranded helix is about 2 nm,\textsuperscript{11} although the layer showed a volume fraction of more than 50 % (Table 4.3). A longer immobilization time would have been likely to yield a thicker layer, but due to time restrictions on the instrument, this could not be explored.

Figure 4.9. The magnetic SLD profile for a bare Au surface (blue line), after immobilization with HPP-4 for 1 h (red line) and 2 h (green line) in 35 °C. The data was acquired in D\textsubscript{2}O/PBS buffer for up-spin neutrons.

To investigate the melting behavior of \textit{surface-attached} HPPs, neutron reflection was measured at five different temperatures: 22, 35, 45, 55 and 65 °C. The temperature intervals were chosen to restrict the experiment time but still cover the major events. 22 °C corresponds to the standard room temperature, 35 °C is in the range of common hybridization
temperatures, 45 °C is close to the calculated (44.7 °C) and experimentally determined (47.1 ± 0.4 °C) melting temperatures for the HPP-4 in solution (as listed in Table 3.3, section 3.3.3), and 55 °C and 65 °C were chosen expecting fully dissociated HPPs.

Figure 4.10. Neutron SLD profile for two magnetic contrasts: A) up spin and B) down spin, of a HPP SAM measured at 5 different temperatures: 22 °C (blue lines), 35 °C (red lines) 45 °C (green lines), 55 °C (orange lines) and 65 °C (light blue lines). The data were acquired in a D₂O environment.
Figure 4.10 illustrates the difference in SLD profiles between A) the up- and B) down spin magnetic contrasts for the temperature related measurements, conducted in D₂O/PBS. The only difference between Figure 4.10.A) and B) is in the magnetic Fe layer, above the SiO₂- and below the Au layer, and thus only the data obtained with the up spin neutrons will be presented hereafter. However, all available contrasts have been included in the fitting procedure and are consequently accounted for and reflected in the \( \chi^2 \) -values, even when not explicitly shown.

The reflectivity profile for the up spin neutron measurements of the HPP layer at different temperatures is displayed in Figure 4.11. The subsequent data sets are offset on the y-axis (reflectivity) with respect to the 22 °C data set, for identification purposes. The reflectivity profile shows that the fits (solid lines) are quite well correlated with the experimental data, although the best fits are achieved for \( Q \)-values lower than 0.16 Å⁻².

![Figure 4.11](image_url)

Figure 4.11. Neutron reflectivity profile for an up-spin magnetic contrasts of a HPP SAM measured at 5 different temperatures: 22 °C (blue), 35 °C (red) 45 °C (green), 55 °C (orange) and 65 °C (light blue). The diamonds represent the experimental data and the solid lines represent the fits. The data were acquired in a D₂O environment and are all offset on the y-axis, with respect to the 22 °C data set, for clarity.
The SLD profiles for the HPP layer at different temperature were calculated based on the fitted reflectivity data and the up-spin profiles are shown in Figure 4.12. The changes in the HPP layer, due to the increase in temperature, are highlighted by the narrowed x-axis range and all the layer thicknesses and volume fractions acquired from the model are summarized in Table 4.3 along with the respective \( \chi^2 \) values. At room temperature (22 °C) the HPPs are expected to be in their stem-loop conformation\textsuperscript{123, 139} and thus the data acquired at that temperature is used as a reference point for the subsequent measurements. Hybridizations are typically carried out at 37 °C, which corresponds to the biological temperature at which DNA naturally undergoes the hybridization process in the human body.\textsuperscript{11} The temperautre regulation for the experimental setup was difficult and a monitoring of the temperature during the measurement showed 35 °C, although the aim was 37 °C.

![Figure 4.12](image.png)

Figure 4.12. Neutron SLD profile for an up-spin magnetic contrasts of a HPP SAM measured at 5 different temperatures: 22 °C (blue line), 35 °C (red line) 45 °C (green line), 55 °C (orange line) and 65 °C (light blue line). The data were acquired in a D\textsubscript{2}O environment.
In Figure 4.12 a shift in the SLD profile is seen at 35 °C (red line), compared to the reference profile at 22 °C (blue line), due to an increased thickness and volume fraction of the HPP layer from 1.2 to 2.0 nm and from 60.0 to 86.5 %, respectively (Table 4.2). The immobilization of the HPPs is a spontaneous process and thus the layer formed is likely to be heterogeneous, especially at lower temperatures and short immobilization times. The increase in layer thickness and volume fraction at 35 °C indicates that the HPPs covered most of the Au substrate, possibly due to an alignment of the HPPs to achieve a more homogenous layer with less electrostatic repulsion between the negatively charged HPPs. That reduction of the non-specific adsorption between the ODN backbone and the substrate would account for the increased thickness was ruled out because non-thiolated DNA reportedly adsorbs so strongly to a Au surface that it cannot be removed by either extensive rinsing with buffer or water, or heating the gold surface to 75 °C.\(^{51}\)

<table>
<thead>
<tr>
<th>1-layer model</th>
<th>(\chi^2)</th>
<th>Thickness (nm)</th>
<th>Volume fraction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPP only, 1 h, 22°C</td>
<td>4.1</td>
<td>1.2</td>
<td>44.9</td>
</tr>
<tr>
<td>HPP only, 2 h, 22°C</td>
<td>5.2</td>
<td>1.2</td>
<td>60.0</td>
</tr>
<tr>
<td>HPP (2 h), 37°C</td>
<td>5.3</td>
<td>2.0</td>
<td>86.5</td>
</tr>
<tr>
<td>HPP (2 h), 45°C</td>
<td>8.1</td>
<td>1.5</td>
<td>94.3</td>
</tr>
<tr>
<td>HPP (2 h), 55°C</td>
<td>10.2</td>
<td>2.7</td>
<td>46.8</td>
</tr>
<tr>
<td>HPP (2 h), 65°C</td>
<td>6.1</td>
<td>1.9</td>
<td>22.7</td>
</tr>
<tr>
<td>HPP (2 h) + cODN, 22°C</td>
<td>4.0</td>
<td>2.6</td>
<td>18.4</td>
</tr>
</tbody>
</table>

Table 4.2. Summary of layer thickness and volume fraction values, at different temperatures or after hybridization, for a HPP SAM, derived from a 1-layer model fit of the polarized neutron reflectivity data (NG-1 reflectometer, NCNR).

When the temperature was elevated further to 45 °C the shape of the SLD profile changed slightly (Figure 4.12, green line) and the slope steepened somewhat. The HPP-layer shrunk slightly in terms of thickness, likely due to a change in configuration from rigid HPPs to random coils.\(^{139}\)
However, the almost complete coverage of the Au surface was achieved as determined by the volume fraction of 94.3% (Table 4.2). As the temperature intervals in this study were quite wide and clear differences can be noted between the SLD profiles at the various temperatures, the exact melting temperature of the surface-attached HPPs is difficult to determine based solely on these results. It is tempting to propose a melting temperature in the vicinity of 45 °C (since the volume fraction is very high at that temperature) which would be surprisingly close to the simulated \( T_m \) of 44.7 and the experimentally determined \( T_m \) of 47.1 for HPP-4 in solution (Section 3.3.3, Table 3.3). Proposedly, as the base pairs in the stem of the HPP become destabilized and the hydrogen bonds start to break the HPPs become more mobile and thus free to interact with neighboring HPPs. Importantly, stems from different destabilized HPPs might even base pair into new “stems” (double-stranded regions), affecting the layer arrangement. Brewood et al. reported a \( T_m \) of 62.0 °C for hairpins attached to gold interdigitated microelectrode (GIME). The authors found that same hairpin in solution had a \( T_m \) of 47.8 °C (predicted \( T_m \) above 40 °C), which is in agreement with previous studies, where it has been showed that attachment to a surface stabilizes DNA strands and thus increases the \( T_m \). The results presented here do not show clear signs of a stabilizing effect due to the surface-attachment of the HPPs. This disagreement could be accounted for by the differences in the hairpin structures themselves. The HPP-4 consists of a 9 base pair long stem with 18 bases in the loop part (see section 2.2.1, Table 2.1) whereas the HPP used by Brewood et al. consisted of a 20-base pair long stem with only 4 bases in the loop. Although both HPPs exhibited similar \( T_m \) in solution, the greater length of the stem in the HPPs attached to the interdigitated microelectrodes would indeed enhance the stability of the HPPs compared to the shorter stem HPP-4s. Additionally, the buffer used by Brewood et al. contained a higher salt concentration, which contributed to the stabilization of the HPP and, consequently, to a higher \( T_m \).
A relatively low $T_m$ of the HPP-4 would be supported by the electrochemical response for a DNA sensor based on mSAM of HPP-4 and m-PEG molecules as a hybridization temperature of 44 °C, was found to be advantageous (compared to hybridization at 37 °C) especially for high probe density mSAMs. These results are discussed in detail in Chapter 6.

However, at 55 °C a more pronounced change in the SLD profile (Figure 4.12, orange line) can be distinguished. This can be explained as the volume fraction of HPPs on the surface decreases significantly from 94.3 to 46.8 % while the thickness of the layer increases from 1.5 nm to 2.7 nm (Table 4.3). At this temperature the HPPs are likely to be released from their original structure and exist in a random coil configuration, i.e. they behave like highly flexible single stranded probes and thus a larger fraction of the solvent was entwined with the probes, similarly to the volume fraction observed at 22 °C. The major differences in the layer at 22 °C, compared to 55 °C, is the structural conformation of the HPPs on the surface, as seen in Figure 4.12, the orange line. As discussed previously, at 22 °C the HPPs are likely to lie flat on the surface, in rigid stem-loop structures, whereas at higher temperatures enhanced conformational freedom allows for a larger variety within the layer and allow part of the random coils to protrude into the solvent layer, observed as an increase in layer thickness.

When the temperature was raised to 65 °C the SLD profile changed further, as shown in Figure 4.12, light blue line. As listed in Table 4.3 there was a notable decrease in the layer thickness and also a loss of material on the surface as the volume fraction of HPP was reduced from 46.8 to 22.7 %. It has been shown that a thiol-Au bond can be broken by the application of a fs-long laser pulse. The laser pulse led to the desorption of thiolated DNA strands from a Au nanoparticle surface, observed through changes in the nanoparticle surface plasmon absorption band. More recently, Herdt et al. investigated the integrity of the thiol-Au bond for Au-DNA conjugates when exposed to high temperatures. Gold particles were
functionalized with ODNs, tagged with fluorophores, and the dissociation of the thiolated DNA-Au bond was characterized over time with fluorescence measurements, gel electrophoresis and ion-exchange chromatography.\textsuperscript{161} It was found that, above 70 °C, there were two reasons for the decomposition of the aqueous Au-DNA conjugates: 1) desorption of thiol-terminated DNA from the gold nanoparticle surface and 2) chemical degradation of DNA in the presence of colloidal gold.\textsuperscript{161} Since the substrate used in this study for immobilization of the HPPs was not of colloidal character, and the temperature was not raised above 65 °C during the measurements (due to current set-up restrictions), a degradation of the ODN strand itself was unlikely. However, an indication of partial thiol-Au bond dissociation could be observed as a clear change in the SLD profile (Figure 4.12, orange line) at 65 °C and as the substantial decrease in volume fraction (from 46.8 to 22.7 %), as listed in Table 4.3. In order to determine the $T_m$ for the surface-attached HPPs, smaller temperature intervals should be used and both the heating and cooling down should be monitored.

After the temperature profile was measured the HPP SAM was cooled down to room temperature and subsequently hybridized with cODN at 35 °C. In Figure 4.13 the SLD profiles for a HPP SAM, measured at 22 °C (blue line), and at 65 °C (red line) are plotted together with the profile for hybridized duplex (green line), measured at 35 °C. The SLD profiles reveal a condition dependent conformational change for the HPPs. A volume fraction of only 18.4 % for the hybridized layer (Table 4.3) provides support for the assumption that the thiol-Au bonds have partially been broken during the previous melting profile measurements. If all HPPs had remained bound to Au substrate through their thiol groups, a volume fraction similar to, or larger than, that of the HPP layer before any treatment (~ 60 %, Table 4.3) would have been expected after hybridization with cODN. The volume fraction was, however, notably reduced suggesting that 65 °C was enough to weaken the thiol-Au bonds sufficiently to detach a number of the HPPs.
The thickness of the hybridized layer, 2.6 nm, was similar to the 2.7 nm measured for the non-hybridized layer of HPPs at 55 ºC, in support of the view that the HPPs protrude into the solvent layer at 55 ºC. The SLD profile (Figure 4.13, green line) for the hybridized duplex exhibit some similar characteristics with the random coil HPP-layer (Figure 4.13, red line) but show a more marked difference between the solvent and the ODN layer, suggesting that the hybridized layer was denser and more rigid than the random coil, as expected. However, a fully extended hybridized HPP/target complex has an approximate height of 10 - 13 nm, which is far from the measured 2.1 nm.

As previously mentioned, the diameter of a double helix is about 2 nm and thus it can tentatively be concluded that the double strands were lying down on the Au surface, instead of positioning themselves in a position normal to the surface. This observation is supported by
consideration of the non-specific adsorption of the pyrimidine and purine bases in the duplex to the Au surface,\textsuperscript{51} the presence of a C\textsubscript{6}-linker between the thiol group and the first base at the 5’-end of the HPP and the low amount of ODNs present on the Au substrate. It is possible that the C\textsubscript{6}-linker increased the flexibility of the HPP/target duplex close to the surface so that the adsorption of the nitrogen rich bases to the Au surface was strong enough to position the hybridized strands horizontally on the substrate.

4.5 Conclusions

NR was used for the characterization of mSAMs of m-PEG and/or HPPs to gain insight of the composition of the formed layers. The experimental data were fitted to a structure model with the program ga_refl. General conclusions regarding the structure of the investigated SAMs could be drawn from thickness- and volume fraction values, determined from the model fit. Conformational changes in the mSAM, induced by hybridization with cODN, were also detected. All the studied SAMs exhibited layer thicknesses that were smaller than expected, based on the maximum theoretical heights of the participating molecules, but at least a partial explanation was offered by consideration of an approximate tilt-angle. The distribution of the HPPs (versus the m-PEG molecules) could not be determined, although important information concerning the densities of the mSAMs was obtained. The surface coverage measured for pure HPP-layers was found to be between \(1.5\times10^{12}\) HPP molecules per cm\(^2\) and \(7.1\times10^{12}\) HPP molecules per cm\(^2\), depending on the immobilization time. Further measurements are needed in order to confirm the results obtained in this study, but important preliminary information about the nature and dynamics of the mSAMs, which had thus far not been achieved (see section 3.2), was gained through neutron reflection measurements.
An early exploration of the melting behavior of the HPP-4 was also conducted by means of polarized neutron reflectometry. Magnetic contrasts are achieved by changing the spin of the neutrons used and this enhances the contrast variation in a simple and straightforward way, without any intrusion on the sample. As the temperature intervals employed were rather wide, there was not a clear sign of melting in the reflectivity and the SLD profiles although differences were clearly visible. These results showed, however, that PNR is a suitable technique for studies of melting temperatures for surface-attached HPPs but further experiments are needed in order to establish a more thorough understanding of the thermodynamic behavior of the immobilized HPPs.
Chapter 5 - Quantum dots as labels in a hairpin DNA sensor

Following characterization of the various components of the sensing platform the performance of the sensor was investigated. Sensitivity and selectivity are important factors for reliable and practical DNA sensors. The sensitivity is often described as a detection limit of a sensor, whereas the selectivity illustrates the tendency of a sensor to respond to other than fully complementary target sequences. The best sensors exhibit great sensitivity as well as selectivity towards its target. The focus of this chapter is to evaluate the performance of two, QD-labeled hairpin sensors: 1) an optical, CdSe/ZnS-modified sensor using fluorescence emission for signal detection and 2) an electrochemical, CdTe-labeled sensor, for which the response was detected with impedance. The CdSe/ZnS-labeled hairpin sensor was based on a mixed self-assembled monolayer (mSAM) of QD-modified hairpin probes and TGA or m-PEG molecules, whereas only m-PEG was used in the mSAMs for the CdTe-modified hairpin sensor. The results obtained with the optical sensor were relatively poor and thus the study focused on the evaluation of the superior electrochemical sensor. The interfacial processes at the modified electrode-solution interface were observed as changes in impedance and charge transfer resistance. The sensitivity and selectivity of the sensor were evaluated and the impact of the CdTe-modification on the sensor performance was considered.

5.1. Introduction

The availability of rapid and specific biosensors is of great importance for many areas of biomedical research and modern biotechnology. Sensors that can detect minimal concentrations of specific gene fragments are needed and a promising alternative approach to traditional DNA assays utilizes nanostructured materials such as quantum dots. Many of the available strategies for gene detection involve fluorescent\textsuperscript{14}, radioactive or chemoluminescent markers\textsuperscript{162}, which often make use of hazardous compounds and are generally time-
consuming. Fluorescence spectroscopy has been successfully used to detect target ODNs, especially in a range of solution assays using molecular beacons, dyes and also quantum dots in the designs. As mentioned in section 1.3 molecular beacons are single stranded nucleic acid probes, which in the absence of their target strands form hairpin-like secondary structures. MBs have been adapted to surface-immobilized systems which rely on optical as well as electrochemical means of detection. Typically, an electrochemical DNA sensor consists of a solid electrode and a surface-immobilized probe which upon hybridization with its complementary strand, the target, generates an electrochemically detectable signal. In general, either the single-stranded (ss) probe or the formed double-stranded (ds) DNA is tagged with a redox label that provides the actual signal. Electrochemical impedance can provide the speed and sensitivity that is needed for the detection of the hybridization event in the development of an inexpensive, simple and portable DNA sensor.

As mentioned in section 1.5, quantum dots (QDs) exhibit unique properties, such as high chemical stability and resistance to photodegradation. Their optical properties are readily tunable and core/shell structures of CdSe/ZnS, CdSe/ZnSe or CdSe/CdS type have traditionally been of interest, since they exhibit efficient photoluminescence in the visible part of the spectrum. The nanocrystals are hydophobic due to the organometallic synthesis routes used in their preparation and surfactant molecules that cap their surface. Although the cap prevents the QDs from aggregating, water-soluble particles are needed in biological systems. Charvet et al. have developed two new surface ligands that replace the initial capping ligands achieving water-soluble, biotinylated CdSe/ZnSe nanocrystals. These are then suitable for fluorescent biological labeling and particle aggregation could be kept at a minimum while the original nanocrystal diameter was essentially maintained. The ligands
consist of a thiolated diethyleneglycol derivative and an alkylthiol substituted biotin molecule (Scheme 5.1, 1.A and 1.B, respectively). The “biotinylated” nanocrystals could then easily be conjugated with biomolecules, using the specific and strong interaction between biotin and streptavidin. An alternative approach to the conventional organometallic preparations routes, where the hydrophobic QDs produced need to be functionalized to obtain water-soluble QDs, is the aqueous synthesis of CdTe QDs. The aqueous route is beneficial as the size of the resulting QDs can be kept to a minimum, which can be crucial for further applications. CdTe-QDs have also been shown to work well in a versatile immuno-sensor, where the CdTe quantum dots were used as electrochemical and fluorescent labels for protein detection, thus also being promising candidates for DNA detection.

Du et al. demonstrated that fluorophore-tagged DNA hairpins attached to gold films could function as sensitive and selective sensors for target ODNs. Two DNA hairpin sequences were evaluated and it was found that hybridization with the complementary sequence caused an increase in signal by over a factor of 20, while nonspecific sequences resulted in a minimal response. The same authors went on to study a similar hairpin DNA sensor, using a ferrocene-tag, in more detail and the thermodynamic and kinetic response of the sensor was investigated. The hybridization efficiency was found to be sensitive to the secondary structure of the hairpin, as well as to the surface distribution of DNA hairpins on the substrate. Exchanging the fluorophores with nanocrystals could potentially improve the sensitivity and selectivity of hairpin DNA sensors further.

Fan and co-workers have developed an electrochemical DNA sensor (E-DNA sensor) with high selectivity and sensitivity based on the conformational change in a surface attached DNA stem-loop structure. The change in electron transfer tunneling distance, induced by the
hybridization event, between the ferrocene-label of the probe and the electrode was monitored. The sensitivity of the sensor was found to be 10 pM.\textsuperscript{34} The same E-DNA sensor has also been used to detect specific sequences of PCR products with the ferrocene-label replaced by a methylene blue indicator. The methylene blue-labelled sensor showed similar sensitivity and selectivity as its predecessor.\textsuperscript{166} Another hairpin DNA-sensor, reliant on methylene blue interacting differently with single stranded and double stranded DNA, was reported by Jin et al. The intercalator was accumulated separately into the hairpin probe the ds-DNA before and after hybridization and the sensor performance was evaluated with cyclic voltammetry (CV). The sensor exhibited a detection limit of 0.2 nM.\textsuperscript{10} An electrogenerated chemi-luminescence (ECL) biosensor, based on hairpin probes, has been developed recently. The hairpin probe was tagged with a ruthenium complex\textsuperscript{167} in a similar fashion to the ferrocene-labelled sensor presented by Fan et al.\textsuperscript{34} The ECL-sensor, however showed a slightly decreased sensitivity of 90 pM compared to 10 pM achieved with the E-DNA sensor.\textsuperscript{34, 167}

Shiddiky and Shim have developed an intricate trace analysis of DNA, where a three-channeled microchip was used for preconcentration, separation and electrochemical detection of target.\textsuperscript{173} The preconcentration and separations steps have been improved by modification of the active matrix with gold nanoparticles and a conducting polymer/gold nanoparticle-modified electrode was used for the detection. The detection limit was as low as 5.7 amol in 50 µl, which corresponds to a concentration of 114 fM.\textsuperscript{173} Rochelet-Dequaire et al. successfully used polystyrene microwells as substrates for the over-all hybridization reaction. Target DNA was passively adsorbed on the bottom of the microwells, followed by hybridization with a gold nanoparticle labelled probe. Before oxidative dissolution of the gold NPs they were autcatalytically grown to enhance the performance of the sensor. The released
gold was then detected with anodic stripping voltammetry, down to 600 aM concentrations.\textsuperscript{174} Liu and Lin have pushed the detection limit even further, to 21.5 aM in a solution assay, based on a DNA polymerase I-induced coupling of nucleotide-modified nanoparticles to the mutant site on the duplex DNA.\textsuperscript{175} Although effective, the assay is multi-stepped including magnetic capture, separation and washing steps.

5.2 The optical CdSe/ZnS-labeled hairpin sensor

5.2.1 Functionalization of the CdSe/ZnS QDs

The functionalization of the CdSe/ZnS QDs was firstly done according to the procedure presented by Charvet et al.\textsuperscript{57} and as described in detail in Chapter 2.1.3. A mixture of two ligands: DSBA (Ligand 2) and 8-thio-3,6-dioxaoctanol (Ligand 1) was used to replace the original ligands on the surface of the nanoparticle, each ligand contributing differently to the properties of the resulting QDs. Ligand 2 was used primarily to achieve water-solubility of the particles whereas Ligand 1 provided the biotin molecule for future creation of a biotin-avidin-biotin bridge between the QD and a probe ODN. Two other ligands, dihydrolipoic acid and thioglycolic acid (Scheme 5.1. – structure 2 and 3, respectively), were also used for functionalization of the CdSe/ZnS QDs. A detailed description of the procedure is found in Chapter 2.1.2.1 and 2.1.2.2.

The ligands could be attached to probe ODN through different mechanisms. In the case of Ligand 1 and 2, a biotin-avidin-biotin bridge was created to link the QDs to the ODN. Both the QDs and the ODN were biotin-modified and by adding avidin to the solution the bridge formed spontaneously. Avidin is a glycoprotein found in avian egg white and is a homotetramer with a molecular mass of 68 000 daltons and it interacts stoichiometrically with biotin, binding one biotin per subunit.\textsuperscript{176, 177} Due to the specific and high affinity interaction
(\(K_a\)) \(10^{15}\) M between avidin and biotin,\(^{177}\) the avidin-biotin system is a well-known and utilized tool. The interaction between biotin and avidin has been widely used in a variety of applications such as immunoassays and purification schemes, especially since biotin-modified antibodies, proteins, and DNAs are commercially available.\(^{169}\)

![Scheme 5.1](image)

Scheme 5.1. Ligands used for functionalization of the CdSe/ZnS QDs. 1.A) Ligand 2, 1.B) Ligand 1 (see chapter 2.1.2.2 and 2.1.2.1, respectively, for details), 2) dihydrolipoic acid (DHLA) and 3) thioglycolic acid (mercaptoacetic acid (MAA))

Functionalization of the QDs with a biotin containing component allows for utilization of the strong affinity between biotin and avidin for the attachment of these QDs to the ODN probe of interest. For the DHLA- and TGA-capped QDs there is an equally established attachment scheme: using carbodiimide chemistry to form a peptide bond between a carboxylic acid and an amide group. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) is used to catalyze the spontaneous formation of the covalent bond.\(^{178, 179}\)

It is of importance that the QDs retain their properties, particularly a high fluorescence intensity, upon functionalization. In Figure 5.1 the fluorescence of the modified CdSe/ZnS QDs is compared to the luminescence of the original QDs in chloroform.
Figure 5.1. CdSe/ZnS QDs in a) chloroform and b) functionalized with: A) Ligands 1 and 2, B) DHLA and C) TGA. The excitation wavelength was 360 nm.

Figure 5.1.A and C shows the Ligand 1 and 2- and the TGA- functionalized QDs, respectively, and although they do exhibit fluorescence after the modification, a clear loss in luminescence can be observed. This might be due to only partial success of replacing the original ligands on the surface of the nanoparticles, resulting in unstable or aggregated particles. However, for the DHLA-functionalized QDs (Figure 5.1.B) the functionalization does not have a significant impact on the luminescence efficiency, indicating a successful functionalization. Over time, some aggregation could be observed for all the differently functionalized particles as the ligands become unstable and are removed from the surface of the nanocrystals.
5.2.2. Quenching of the functionalized CdSe/ZnS by an unmodified and a m-PEG-modified Au surface

To ensure that the fluorescence of DHLA- and TGA-functionalized nanoparticles could be quenched by a gold surface in the same manner as the previously studied Cy3 dye and Ligand 1 and 2-modified CdSe/ZnS QDs (Chapter 3.1.2 and 3.1.3), a set of corresponding experiments were conducted. Figure 5.2 summarizes the quenching ability of a bare (A and C) and a m-PEG-modified gold surface (B and D) to quench the fluorescence of DHLA- (A and B) and TGA-functionalized CdSe/ZnS QDs (C and D). As previously shown, the fluorescence of a Cy3 dye is completely quenched by both an unmodified and a m-PEG-modified gold surface (Figure 3.2). Modified Au surfaces were capable of quenching the fluorescence from the functionalized CdSe/ZnS QDs significantly, whereas the unmodified Au surface was, however, even more efficient in quenching the fluorescence from the QDs. These results are expected since the m-PEG monolayer increases the distance between the QD and the Au, thereby effecting the quenching efficiency of the Au surface. The fluorescence of the DHLA- and TGA-functionalized QDs are quenched much in the similar fashion as the Ligand 1 and 2-functionalized particles. The unmodified Au-surface (Figure 5.2.A and C) was able to quench the luminescence of the TGA-functionalized QDs (Figure 5.2.C) more efficiently than the DHLA-functionalized ones (Figure 5.2.A). After excessive rinsing with PBS buffer the QDs were largely rinsed off the Au surface, an indication that there is very little unspecific adsorption between the QDs and the Au. The m-PEG-modified Au surface showed lower quenching ability of the fluorescence of both the DHLA- and TGA-functionalized QDs (Figure 5.2. B and D) compared to the unmodified Au. After rinsing with PBS buffer the fluorescence was diminished, comparably to the previously obtained results.
Based on the results presented in Section 5.2.1 and 5.2.2, Ligand 1 and 2- and DHLA-functionalized CdSe/ZnS QDs were chosen as labels for the optical hairpin DNA sensor. Although the TGA-modified QDs exhibited similar emission intensity as the Ligand 1 and 2-functionalized QDs, they provided no significant advantage for the sensor design. With DHLA- and Ligand 1 and 2-modified QDs the effect of two different linking mechanisms between QDs and HPPs on the sensor response could be evaluated.
5.2.3. Optical response of a CdSe/ZnS-labeled hairpin sensor

Fluorescence spectroscopy is known to be a sensitive detection method for DNA detection and the optical response of a CdSe/ZnS-labeled hairpin sensor was duly investigated. Scheme 5.2 shows a schematic illustration of the fabrication of the QD-labeled sensor. Firstly, the mSAM was immobilized on the clean gold surface and secondly, the CdSe/ZnS QDs were attached to the HPPs through a biotin-avidin bridge. The complementary target ODN was then introduced, in a third step, and the hairpin was expected to open upon hybridization, removing the QDs from the close proximity of the quenching gold, and thus restore the fluorescence of the QDs.

![Scheme 5.2](image)

Scheme 5.2. Schematic illustration of the fabrication of the optical QD-labeled sensor: 1) Immobilization of HPP and m-PEG molecules to the gold substrate, 2) attachment of CdSe/ZnS QDs through a biotin-avidin or an amide bond and 3) hybridization with complementary target ODN.

Figure 5.3 shows the fluorescence emission spectra of: a) a bare Au surface, b) after immobilization of the hairpin probe (HPP-1), avidin and functionalized CdSe/ZnS QDs and after hybridization with 2.33 µM complementary target ODN at 37 ºC for c) 2.5 h, d) 17.5 h,
e) 41.5 h and f) 65.5 h. There is no evident fluorescence even after very long hybridization time, indicating that the sensor was not responding.

![Fluorescence emission spectra](image)

Figure 5.3. The Fluorescence emission spectra of: a) a bare Au surface, b) after immobilization of the hairpin probe (HPP-1), avidin and functionalized CdSe/ZnS QDs and after hybridization with 2.33 µM complementary target ODN at 37 ºC for c) 2.5 h, d) 17.5 h, e) 41.5 h and f) 65.5 h. The excitation wavelength was 360 nm.

There are two possible main scenarios as to why this sensor design failed: 1) the functionalization and/or attachment of the CdSe/ZnS QDs were unsuccessful, resulting in a too small amount of QDs on the surface to produce detectable fluorescence and 2) although successfully functionalized and attached, the size of the QDs, particularly together with the biotin-avidin bridge, caused too much steric hindrance for the HPPs to open upon hybridization. One QD could also potentially have bound to several HPPs (as there were several biotin-molecules available on the surface of the QD), causing severe restrictions on loop opening. Considering the first scenario in more detail there are several troublesome steps. If the purification of the synthesized Ligands 1 and 2 was ineffective, the actual number
of ligands used for the functionalization was lower than estimated translating into fewer biotin sites for further attachment to the HPPs. Too few QDs were then bound to the HPPs to be able to generate sufficient fluorescence emission for a detectable signal.

If the second scenario was prevalent the issues were quite different. If the functionalization of the QDs and their attachment to the HPPs were successful, steric effects could still be a concern. The size of the QDs was around 6 nm in diameter,\textsuperscript{59} which is close to the height of the HPPs, i.e. the QDs were big enough to potentially cause steric hindrance for the hybridization and also to impair on the ability of the loop to unfold. Additionally, avidin is also a large protein\textsuperscript{176, 177} and thus the biotin-avidin link added to both the bulk that the HPPs had to lift off the surface as well as the steric constrains for the hybridization itself. Additionally, avidin has four available sites for biotin-binding\textsuperscript{177} and there was therefore a possibility that several QDs were bound to the same HPP or that even some HPPs were linked to each other. HPPs, which either had several QDs attached to themselves or which were liked to other HPPs likely were not able to unfold to generate any fluorescence emission. A mix of the two scenarios is the most likely explanation for the lack of response of the CdSe/ZnS-labeled sensor.

To eliminate the potentially detrimental effects of the bulky biotin-avidin link, dihydroleipoic acid (DHLA) was used for functionalization of the CdSe/ZnS QDs\textsuperscript{7, 71, 180, 181} and the sensor response was investigated. As seen in Scheme 5.1. DHLA has two thiols, which will bind to the ZnS-shell of the QDs, increasing the stability of the functionalized QDs. The carboxylic acid group in DHLA also provides convenient attachment to amide-modified HPPs using carbodiimide chemistry (EDC),\textsuperscript{178, 179} reducing the size of the bridge between the QDs and the HPPs significantly compared to the biotin-avidin link.\textsuperscript{7, 71, 180, 181}
Figure 5.4. The fluorescence emission spectra of the DHLA/CdSe/ZnS-labeled hairpin (HPP-3) sensor: a) before and b) after hybridization with 4.65 µM of complementary ODN. The excitation wavelength was 340 nm.

Figure 5.4 shows the fluorescence spectra of the DHLA-functionalized CdSe/ZnS-labeled hairpins (HPP-3) a) before and b) after hybridization with complementary target ODN. The difference in fluorescence emission before and after hybridization was negligible, and although some fluorescence emission at 570 nm was observed in the normalized spectra it was not sufficient for sensing purposes. The alteration of the linking mechanism between the QDs and the HPPs had only a subtle effect on the sensor performance, which was not profound enough to achieve a fully functional sensor. Even though the amide bond was significantly smaller than the biotin-avidin bridge, all the other issues, as discussed above, still remained and the optical detection strategy was abandoned in favor of electrochemical detection methods.
5.3 The electrochemical CdTe-labeled hairpin sensor

5.3.1 Characterization of the synthesized CdTe QDs

In the electrochemical sensor design, the size of the synthesized CdTe QDs is of great importance. The aim was to use very small QDs to impose as small an impact as possible on the hybridization dynamics (unfolding) of the HPPs. CdTe QDs were chosen as they can be easily synthesized through a straightforward, aqueous route\textsuperscript{66, 171} and thus their size can be kept to a minimum. To determine the diameter of the synthesized CdTe QDs, transmission electron micrographs were collected as shown in Figure 5.5. Ten randomly chosen CdTe-particles were analyzed with the software ImageJ and an average diameter of 4 nm was obtained, which corresponds to the previously observed sizes for CdTe QDs synthesized in aqueous medium.\textsuperscript{53, 54, 171}

Figure 5.5. Transmission Electron Microscope (TEM) images of the synthesized CdTe QDs. The diameter of the circled particle was determined to 3.6 nm and the average diameter of 10 particles was 4 nm, calculated with the software ImageJ.\textsuperscript{55}
The nanoparticles exhibited a characteristic UV-vis absorbance with a maximum at 464 nm and fluorescence emission with a maximum at 516 nm (Figure 5.6). In accordance with previous quenching studies (Section 3.1), it was confirmed the CdTe fluorescence was quenched by the gold substrate (results not shown) and thus they were also considered as potential labels in the optical sensor design. However, no optical sensor response was obtained after hybridization of CdTe-modified HPPs with complementary target ODN. Since preservation of the fluorescence after attachment of the QDs to the probe is not essential for electrochemical detection, electrochemical AC impedance was employed to evaluate the sensor response.

Figure 5.6. Absorption spectra (A) and emission spectra (B) of a 3 mM CdTe-solution. The excitation wavelength was 360 nm.
5.3.2 mSAM formation and attachment of CdTe QDs

The stepwise construction of the DNA sensor is shown in Scheme 5.3. The HPPs and m-PEG molecules were self-assembled on the electrode by thiol-adsorption to gold\textsuperscript{51} and the electron transfer processes in the mSAM were investigated through AC impedance measurements. The mSAM, which had been previously characterized (Section 3.2 and 4.3) was chosen as the sensing platform, with predetermined HPP:m-PEG molar ratios (IS2:1, IS10:1 and IS25:1).

Scheme 5.3. Schematic illustration of the fabrication of the electrochemical QD-labeled sensor: 1) Immobilization of HPP and m-PEG molecules to the gold electrode, 2) attachment of CdTe QDs through an amide bond and 3) hybridization with complementary DNA.

Probe density is an important parameter in the design of a sensitive and selective sensor\textsuperscript{155, 156} and steric hindrance and probe-probe interactions can interfere with the hybridization step. As less sample volumes can be used for AC impedance measurements, compared to NR, lower probe densities could be employed. Although the monolayer was assembled through simultaneous (competitive) attachment of m-PEG and HPPs, the formed layer is likely to be
heterogeneous with clusters of HPPs dominating some areas. Efforts to determine the
distribution of the HPPs in the mSAM has been made with high-resolution AFM (section
3.2.1) and ATR-FTIR (section 3.2.2) but it has proven an arduous and complex task, due to
the nanoscale environment. 182

Because HPPs are more rigid than their single-stranded counterparts they are expected to
immobilize in a position closer to the normal of the Au surface than linear single-stranded
probes, but non-specific interactions between the DNA backbone and the Au surface might
still occur. In order to minimize these interactions m-PEG was used to displace any non-
specifically attached HPPs and to ensure that the attachment to the surface was exclusively
through the Au-thiol bond. The m-PEG molecules used are uncharged and very flexible which
minimizes interaction with the HPPs. 110 The approximate height of a m-PEG molecule with n
= 6 is 2.5 nm, 149 which is slightly less than the estimated height of the stem in the HPP, 3
nm. 159 The complementary target is matched with the loop part of the HPP and therefore the
flexible m-PEGs are not expected to interfere with the hybridization process or unfolding of
the hairpins.

The formation of the mSAM at the Au electrode surface resulted in a significant increase in
the ‘semi-circle’ in the Nyquist plot, reflecting an increase in the charge transfer resistance
($R_{ct}$) at the interface as seen in Figure 5.7.A-D.a-b. All the experimental impedance curves
were fitted to an equivalent circuit model that included a solution resistance in series with a
parallel circuit containing a constant phase element, the charge transfer resistance and
Warburg impedance. 183 The increase in $R_{ct}$ seen after immobilization of HPP and m-PEG was
expected since the molecules in the SAM hindered the electron transfer processes at the
electrode surface. The negative charges on the phosphate backbone of the attached DNA
probes repelled the negatively charged redox couple Fe(CN)$_6^{3-/4-}$, thus further adding to the large $R_\text{ct}$. Electron transfer processes through the m-PEG molecules in the mixed monolayer were ruled out, since a SAM based exclusively on m-PEG molecules has been shown to block the electron transfer processes at the electrode surface.$^{48}$

![Nyquist plots](image)

Figure 5.7. Nyquist plots, $-Z''$ vs. $Z'$, for A and C: m-PEG:HPP 10:1 and B and D: m-PEG:HPP 25:1 for an Au electrode in SCC buffer containing 5.0 mM Fe(CN)$_6^{3-/4-}$ a) bare Au surface and b) after immobilization of the mSAM, c) after covalent attachment of CdTe QDs, d) after hybridization at 37 °C (A-B) or hybridization at 44 °C (C-D) for 1 h with 4.65 $\times 10^{-6}$ M of complementary target ODN. For clarity the insets in Figure B and D zoom in on the responses after the attachment of CdTe QDs and hybridization.

Fe(CN)$_6^{3-/4-}$ is a commonly used redox couple$^{47,163,184,185}$ but initially hexaamineruthenium(III)chloride$^{163,185}$ was also utilized as a redox marker to detect the hybridization event. However, as the hexaamineruthenium(III)chloride did not yield as well a defined and –
separated response as the Fe(CN)$_6^{3-/4-}$, it was discarded and the Fe(CN)$_6^{3-/4-}$ was employed for all electrochemical measurements included in this thesis.

Figure 5.7.A-D.b-c, show that the covalent attachment of the CdTe QDs to the NH$_2$-modified, 3’-end of the HPPs unexpectedly resulted in a significant decrease in impedance. To confirm this efficient electron transfer through the QD layer to the electrode surface, impedance spectra were measured after adsorption of only CdTe QDs on a bare gold electrode. No rinsing was done before the measurement to ensure the presence of the QDs on the electrode. A decrease in impedance was observed for the CdTe-coated gold electrode, as shown in Figure 5.8.A, confirming the previous response.

Du et al. has very recently found the same apparent decrease in interfacial resistance when assembling thiol-capped CdTe on a gold nanoparticle/chitosan microsphere-modified glassy carbon electrode. The authors suggested a synergistic effect between the gold nanoparticles and the CdTe QDs, which facilitated the electron transfer in the system. Fan et al. found that the stem-loop structure of their probe was imperative for efficient electron transfer from the ferrocence-tagged probe to the gold electrode. This was achieved only at high ionic strength, when the ferrocence unit was sufficiently stabilized. The CdTe-modified HPPs in this study exhibited an excellent electron transfer indicating that their secondary structure was retained even at modest ionic strength (0.137 M) and indicated that the CdTe QDs themselves had a stabilizing effect on the HPP structure. The CdTe QDs proposedly act as redox mediators, likely through a fast direct electron tunneling process. Direct electron transfers are unusual due to the fact that a very short distance is needed between the redox site and the electrode.
However, before hybridization the CdTe QDs are indeed in close proximity to the gold electrode, providing suitable conditions for a fast direct electron tunneling mechanism, supported by the substantial decrease in the measured charge transfer resistance upon attachment of the CdTe QDs to the mSAM.

It has been reported that thiol-capped CdTe QDs exhibit strong adsorption to gold. Poznyak et al. found that just dipping a gold plated electrode in a deaerated colloidal solution of the QDs for 5 min resulted in adsorption of QDs to the surface. To investigate the extent of non-specific adsorption of CdTe QDs to the gold electrode used for the HPP sensor, the impedance was measured after extensive rinsing of an adsorbed CdTe-layer on a bare gold electrode.

Figure 5.8. Nyquist plots, $-Z''_m$ vs. $Z'_m$ and for an Au electrode in SCC solution containing 5.0 mM Fe(CN)$_6^{3-}$/Fe(CN)$_6^{4-}$: A.a) bare Au surface and A.b) after drying CdTe QDs on Au, without any rinsing and B.a) bare Au surface and B.b) after rinsing off dried CdTeQDs with excess of buffer.
The post rinsing impedance was very similar to the one measured for the bare gold electrode (Figure 5.8.B). As the mSAM was formed before the QDs were attached, the m-PEG molecules in the mSAM were deemed sufficient to minimize any non-specific adsorption between the QDs and the gold electrode. To ensure that the QDs were added solely to the NH$_2$-modified, 3’-end of the HPP, attachment was carried out post mSAM-formation at 44 °C. At this temperature the hydrogen bonds in the stem of the HPP are slightly destabilized, providing advantageous conditions for QD-attachment.

5.3.3 Response of the electrochemical CdTe-labeled sensor

Hybridization between the hairpin and the target involves both the opening of the stem-loop structure of the probe as well as the formation of the probe-target duplex, where the probe-target binding is the driving force for the opening of the loop. The hybridization process can be described as a step-wise event: 1) the target interacts with the probe sequence in the loop and attaches itself to the hairpin, 2) when a sufficient number of bases pairs have been formed the hairpin opens and 3) the rest of the complementary bases pair up to form the target-probe duplex. The responses of the sensors based on mSAMs with molar ratios of m-PEG:HPP of 2:1, 10:1 and 25:1 (IS2:1, IS10:1 and IS25:1, respectively) were investigated. As the IS2:1 and IS10:10 showed similar trends, the following, detailed discussion will focus on the results obtained with the IS10:1 and IS25:1 sensors.

The Nyquist plots in Figure 5.7 show the response of the IS10:1 sensor (A, C) and of the IS25:1 sensor (B, D) to a fully complementary target ODN, when hybridized at 37 °C and 44 °C, respectively. The sensor based on the mSAM with the higher density of HPPs (IS10:1) shows an increase in $R_{ct}$ upon hybridization, regardless of the hybridization temperature (Figure 5.7.A and C), whereas the lower probe density sensor (IS25:1) shows a decrease in $R_{ct}$.
for both hybridization temperatures. The dynamics of the hairpins is dependent on the stability of the hydrogen bonds between the base pairs, which keep the stem together. It has previously been shown that the hybridization temperature plays an important role in the hybridization event; but for the CdTe QD-modified sensor the probe density alone was the restrictive factor, supported by the previous hypothesis that the CdTe QDs stabilizes the stem-loop structure of the probes. For the higher probe density (Figure 5.7.A, C) the increase in $R_{ct}$ reflects the hybridization and the formation of a stable complex between the probe and the complementary target ODN, but not necessarily the unfolding of the hairpins. If the hybridization could be considered a step-wise process, as mentioned above, the results suggest that the hybridization can halt at step 1, especially since the target sequences is situated only in the loop part of the probe and the attached CdTe QDs add to the steric constraints in the layer. An accumulation of negative charges in the monolayer, generated by the partially attached targets to the probe, resulted in accumulation of material on the electrode surface, contributing to the inhibited electron transfer. The redox probes in the solution thus experience a stronger repulsion from the mSAM after hybridization than before it, increasing the $R_{ct}$. 

Hairpin probes occupy a larger area on the electrode surface, compared to single stranded probes, particularly with the CdTe QDs attached. Once the HPPs unfold completely easy access to the gold is provided for the Fe(CN)$_6^{3-/4-}$, observed as a decrease in the impedance, as seen in Figure 5.7.B and D for the lower probe density sensor. An important consideration is the possibility that the HPPs will not be able to fully unfold due to the added weight and steric constraints imposed on the HPPs by the attachment of the QDs. The results, however, indicate that the HPPs are indeed able to at least lift the QDs high enough from the surface to expose an area of bare gold on the electrode. Non-specific interactions between the QDs and the gold
are minimized by the m-PEG layer and no additional force is needed to detach the QDs from the electrode surface.

The sensitivity of this electrochemical sensor can be determined from the dependence of the change in the charge transfer resistance upon hybridization of the hairpin probe with a range of concentrations of complementary target ODN. Although the relationship can be anything but linear, a linear approximation can be used around a given concentration and then sensitivity can be determined by the slope of the log-linear relationship of response vs. concentration. Based on the results shown in Figure 5.7, the observation was made that the hybridization temperature did not alter the sensing mechanism of the sensor and thus the sensitivity of the sensor was investigated at the higher hybridization temperature (44 °C). For the IS10:1 sensor, the response is approximately linear with the logarithm of the target concentration with a slope of $0.691 \frac{\Delta R_{ct}}{R_{ct}(ML)}/(\log \text{concentration, M})$ (Figure 5.9.A) whereas a slope of $-0.143 \frac{\Delta R_{ct}}{R_{ct}(ML)}/(\log \text{concentration / M})$ (Figure 5.9.B), was obtained for the IS25:1 sensor. The sensor responses were approximately linear with the logarithm of the target concentration in the whole range applied (fM - μM). The sensor response measured for the mSAM after QD attachment was used as a ‘zero’ response and all the results obtained after hybridization with target ODN was compared to that ‘zero’ response. Thus it might seem confusing that the sensor response can still pass through zero, as seen e.g. in Figure 5.9.B. Although the QD-modified mSAM has been defined as a “ground-level” for the sensor response, it is not impossible to detect impedance that is higher than this artifical ground level, for hybridization with the lowest (or other) target concentration. This was observed, e.g., for the IS10:1 sensor in Figure 5.9.A. From Figure 5.9.B it can thus be concluded that the sensor responded with an increase in impedance (and subsequently in $\Delta R_{ct}$) when hybridized with the lowest target concentration, whereas a decrease in impedance was observed for the
succeeding target concentrations. Proposely the lowest target concentration is not sufficient to unfold the hybridized HPP, similarly to the sensor response in Figure 5.9.A. It is important to consider the response trends for these sensors, rather than the absolute response values, as the behaviour of the individual HPPs in the mSAM does not seem to be completely linear. Further investigations of the dynamics of the mSAMs at various probe densities and at different temperature are still needed in order to fully elucidate the hybridization mechanisms in the layers.

Figure 5.9. $\Delta R_{ct}/R_{ct}(\text{ML})$, taken as the sensor response before and after hybridization with different concentrations of complementary ODN (4.66×10$^{-15}$ M to 4.66×10$^{-6}$ M) in SCC solution containing 5.0 mM Fe(CN)$_6^{3-}$/Fe(CN)$_6^{4-}$. The sensing monolayer was immobilized with predetermined, molar ratio of HPP:m-PEG molecules, A) IS10:1 and B) IS25:1. Hybridizations were carried out at 44 ºC. The standard error from the fitting is set to 15 %.

However, when the difference in response between the CdTe-modified mSAM and the first hybridized layer was $\geq 3 \, k\Omega$ the sensor response was considered reliable (the same condition was applied for all the electrochemical sensors presented in this thesis) and thus, for
the CdTe-labelled sensor, a reliable response was observed at 4.7 fM target concentration (taken from the experimental data), which corresponds to 188 zmol \((10^{-21})\) in a 40 µl-sample. The detection limit for a similar type of hairpin sensor, where amplification of the sensor response was achieved by intercalation of a cationic dye into the dsDNA, was found to be 50 pM \(^{148}\), and when methylene blue was used as an hybridization indicator 0.2 nM concentrations were detected.\(^{10}\) 10 pM concentrations were detected in a DNA sensing protocol where single stranded probes were attached to a mixed SAM, composed of 2-mercaptoethanol and 11-mercaptoundecanoic acid\(^ {147}\) and electrochemical sensors based on ferrocene- and methylene blue-tagged hairpins show the same sensitivity.\(^ {34, 166}\)

An ECL-sensor, based on a ruthenium-labeled HPP showed a detection limit of 90 pM.\(^ {167}\) The sensor investigated here exhibits an even higher sensitivity (4.7 fM), very similar to a “sandwich-type”, flow injection chemiluminescence assay, based on signal amplification with gold QDs (4.8 fM), reported by Zhang et al.\(^ {189}\) The advantage of the CdTe QD-modified sensor is the simple and direct detection design resulting in a robust and fast sensor. Oxygen does not have to be removed from the sample solution during detection and the response is obtained in approximately 1.5 h (including the hybridization time). There are reports on even more sensitive DNA sensors with detection limits in the attomolar range\(^ {173-175}\) but they are based on conceptually different designs and not directly comparable to the CdTe-modified sensor presented here. Examples of repeated experiments of the responses for the IS10:1 and the IS25:1 -sensors, over the whole concentration range, are shown in Figures 5.10.A, B and 5.11.A, B, respectively. The corresponding Nyquist plots (impedance data) are presented in Figure 5.12 and 5.13. From these figures it can be concluded that the trend of the sensor response was reproducible for each of the individual sensors, although absolute values differed from sensor to sensor.
Figure 5.10. $\Delta R_{ct}/R_{ct}$(ML), taken as the sensor response before and after hybridization with different concentrations of complementary ODN ($4.66 \times 10^{-15}$ M to $4.66 \times 10^{-6}$ M) in SCC solution containing 5.0 mM Fe(CN)$_6^{3-}$/Fe(CN)$_6^{4-}$. The sensing monolayer was immobilized with predetermined, molar ratio of HPP:m-PEG molecules, A) IS10:1 and B) IS10:1 repeat. Hybridizations were carried out at 44 ºC. The standard error from the fitting is set to 15 %.

Figure 5.11. $\Delta R_{ct}/R_{ct}$(ML), taken as the sensor response before and after hybridization with different concentrations of complementary ODN ($4.66 \times 10^{-15}$ M to $4.66 \times 10^{-6}$ M) in SCC solution containing 5.0 mM Fe(CN)$_6^{3-}$/Fe(CN)$_6^{4-}$. The sensing monolayer was immobilized with predetermined, molar ratio of HPP:m-PEG molecules, A) IS25:1 and B) IS25:1 repeat. Hybridizations were carried out at 44 ºC. The standard error from the fitting is set to 15 %.
Figure 5.12. Nyquist plots, $-Z_\text{im}$ vs. $Z_\text{re}$, for A) HPP:m-PEG 10:1 and B) HPP:m-PEG 10:1 repeat for an Au electrode in SCC buffer containing 5.0 mM $\text{Fe(CN)}_6^{3-/4-}$: a) for bare electrode, b) after mSAM formation, c) after covalent attachment of CdTe, after hybridization at 44 °C for 1 h with d) $4.66 \times 10^{-15}$ M, e) $4.66 \times 10^{-14}$ M, f) $4.66 \times 10^{-13}$ M, g) $4.66 \times 10^{-12}$ M, h) $4.66 \times 10^{-11}$ M, i) $4.66 \times 10^{-10}$ M, j) $4.66 \times 10^{-9}$ M, k) $4.66 \times 10^{-8}$ M, l) $4.66 \times 10^{-7}$ M and m) $4.66 \times 10^{-6}$ M of fully complementary target ODN.

Figure 5.13. Nyquist plots, $-Z_\text{im}$ vs. $Z_\text{re}$, for A) HPP:m-PEG 25:1 and B) HPP:m-PEG 25:1 repeat for an Au electrode in SCC buffer containing 5.0 mM $\text{Fe(CN)}_6^{3-/4-}$: a) for bare electrode, b) after mSAM formation (not included as the impedance was so high that it obscured the rest of the data), c) after covalent attachment of CdTe, after hybridization at 44 °C for 1 h with d) $4.66 \times 10^{-15}$ M, e) $4.66 \times 10^{-14}$ M, f) $4.66 \times 10^{-13}$ M, g) $4.66 \times 10^{-12}$ M, h) $4.66 \times 10^{-11}$ M, i) $4.66 \times 10^{-10}$ M, j) $4.66 \times 10^{-9}$ M, k) $4.66 \times 10^{-8}$ M, l) $4.66 \times 10^{-7}$ M and m) $4.66 \times 10^{-6}$ M of fully complementary target ODN.
It is known that HPPs go through a conformational change upon heating\textsuperscript{13, 140} and thus the melting profile for the NH\textsubscript{2}-modified HPP (HPP-3) was measured in PBS-buffer as shown in Figure 5.14. A separate profile was measured for the buffer alone, and subtracted from the probe curve in order to correct for any contribution from the buffer to the sample profile. The melting temperature of a DNA duplex is generally referred to as the midpoint transition where half of the base pairs are broken,\textsuperscript{190} as previously described in Chapter 3.3. The data was fitted to a sigmoidal, Weibull 5-parameter equation to obtain a melting temperature of 46.7 ± 0.4 °C, as listed in Table 3.3, Chapter 3.3.

![Figure 5.14. The melting profile for 0.5 μM of HPP-3 (the HPPs used in the electrochemical CdTe-labelled sensor). The squares symbolized the measured data points and the solid blue line represents the sigmoid, Weibull-5 fit.](image)

Cations have a stabilizing effect on DNA duplexes and the relatively low ionic strength of the PBS buffer used is a likely reason to the low melting temperature of the HPP.\textsuperscript{189} Based on the
A low melting temperature measured for the HPP (46.7 °C) it is arguable that the whole response (Figure 5.7.B, D and Figure 5.9.B) is due to the opening of the loops by temperature alone and not by target hybridization. This is theoretically possible but ruled out since the electrochemical measurements were conducted at room temperature, not at 44 °C, with a minimum of 5 min in SSC-buffer at room temperature prior to impedance measurements. The HPPs, which might have opened because of the increased hybridization temperature, would have closed again before the impedance was measured. Also, the solution based melting profile cannot fully account for melting behavior of the surface-attached HPPs as they tend to exhibit higher melting temperatures then their solution counterparts. In the PNR study (Section 4.4) we were not yet able to determine an exact melting temperature for the surface-attached HPP-4, although there were some indications that it might be lower than previously reported for surface-attached probes, possibly even in vicinity of the $T_m$ measured for the probe in solution. However, the added weight of the CdTe QDs to the probes was expected to have a stabilizing effect on the surface-attached HPPs.

The selectivity of the hairpin probes is generally attributed to the conformation of the stem-loop structure. To investigate the selectivity of the CdTe-modified sensor the response was measured after hybridization with 4.66 µM of non-complementary (NC) and then fully complementary (FC) target ODN at the two different hybridization temperatures. The Nyquist plots in Figure 5.15.A and B show the response of the IS10:1 and IS25:1 sensors, respectively, for the NC- and the FC-target ODN when hybridized at 37 °C. The IS10:1 (Figure 5.15.A) show a clear response for both the NC- and the FC-target, indicating low selectivity of the sensor. When the probe density was decreased the selectivity of the sensor improved. The IS25:1 sensor (Figure 5.15.B) responded to the NC-target, but surprisingly the response was an increase in impedance, contrary to the observed response for complementary
target. This is an indication of non-specific adsorption between the NC-target and the HPPs, potentially due to 5 bases (of a total 18) that were compatible with the probe sequence. The sensor was, however, able to discriminate between NC- and FC-target and the opposite response for NC-targets could be utilized to enhance the selectivity of the sensor.

Figure 5.15. Nyquist plots, $-Z_{\text{im}}$ vs. $Z_{\text{re}}$, for A, C) HPP:m-PEG 10:1 and B, D) HPP:m-PEG 25:1 for an Au electrode in SCC buffer containing 5.0 mM Fe(CN)$_6^{3-}$/Fe(CN)$_6^{4-}$: a) after covalent attachment of CdTe, after hybridization at 37 °C (A and B) or at 44 °C (C and D) for 1 h with 4.66×10$^{-6}$ M of b) non-complementary and c) fully complementary target ODN.

Figure 5.15.C and D show the selectivity response of the IS10:1 and IS25:1 sensors, respectively, when hybridized at 44 °C. No significant response was observed for the NC-target in both cases as the signal was only 12% (IS10:1) and 8% (IS25:1) of the response obtained for FC-target. The large concentration of the NC-target used (4.7 µM) indicates
selectivity in excess of $10^6$, in agreement with results obtained with ferrocene- and methylene blue-tagged hairpin sensors.\textsuperscript{34,166}

5.4 Conclusions

CdSe/ZnS nanoparticles (QDs) were functionalized with three different ligand combinations: 1) Ligand 1 and 2, 2) dihydrolipoic acid (DHLA) and 3) thioglycolic acid (TGA). Of the three combinations the DHLA-modified QDs exhibited the greatest fluorescence in an aqueous environment whereas Ligand 1 and 2- and TGA-functionalized QDs showed similar emission intensities after the functionalization. The quenching ability of an unmodified and a poly(ethylene glycol) (m-PEG)-modified gold surface of the fluorescence of the QDs was also studied. It was found that both surfaces were able to considerably quench the fluorescence from the variously modified QDs, although some emission was still distinguishable for the m-PEG-modified gold surface. This is expected as the m-PEG-layer keeps the QDs somewhat away from the quenching gold. Since the TGA-modified QDs did not exhibit any significant advantages over the other functionalized QDs in any regard, DHLA- and Ligand 1 and 2-functionalized QDs were chosen as labels for the optical sensor design and the effect of the linker system on the sensor response was examined. Two QD-labeled hairpin-DNA sensors have been investigated, both based on mixed self-assembled monolayers of poly(ethylene glycol) and hairpin structured probes immobilized on a gold electrode. The optical sensor, based on fluorescence emission, did not show any reliable response with either ligand 1 and 2- or DHLA-functionalized QDs as labels. Steric constrains and possible cross linking between several QDs and one HPP, or even between individual HPPs, caused difficulties and no sensor response was obtained.
An electrochemical, CdTe-labeled sensor has also been presented. Mixed monolayers with two different probe densities, molar ratios of HPP:m-PEG of 10:1 and 25:1 (IS10:1 and IS25:1), were studied in detail and used for the evaluation of the sensor performance. Both the sensitivity and selectivity of the sensor were investigated and depending on the probe density the sensor showed completely opposite response trends with regard to the change in charge transfer resistance and in the impedance at the electrode interface. The IS10:1 and IS25:1 sensors both showed a high sensitivity towards target ODN and were able to detect concentrations as low as 4.7 fM. Non-complementary targets could be discriminated from fully complementary targets with the IS10:1 and IS25:1 at the higher hybridization temperature.

The use of CdTe QDs as labels potentially provides an additional, optical readout for this sensor, although with the previously encountered complications with the CdSe/ZnS-labeled HPPs and the relatively poor initial optical response in mind, considerable efforts would be needed to develop that system.
Chapter 6 - Effect of Probe Density and Hybridization Temperature on the Response of an Electrochemical Hairpin-DNA Sensor

To further improve the performance of the CdTe-QD labeled sensor, and also to simplify the design, a label-free sensor was developed and is discussed in this chapter. Without the QDs attached to the HPPs, optical detection methods were not feasible and thus the focus was on electrochemical methods to investigate the sensor performance. The interfacial processes at the modified electrode-solution interface are observed as changes in impedance, charge transfer resistance and current. The sensitivity and selectivity of the sensor is evaluated without any amplification of the response signal.

6.1 Immobilization of HPP and m-PEG molecules onto a gold electrode

The DNA sensor was constructed in accordance with the previously described CdTe-modified electrochemical sensor, shown in Scheme 5.3, except for the CdTe-attachment step. NR-measurements, as presented in Section 4.3, were conducted for both backfilled and simultaneously assembled layers and thus two different immobilization strategies\(^{51}\) (IS), were used to create the mixed SAM. Firstly, HPPs were allowed to immobilize on the Au surface with the m-PEGs subsequently backfilled to passivate the surface. Secondly, simultaneous attachment of both components was performed using a mixture of m-PEG and HPPs, at a predetermined molar ratio (IS2:1, IS10:1 and IS25:1). Mixed monolayers are preferred as sensing platform, compared to layers with only ss-probes, as possible steric hindrance and probe-probe interactions during the hybridization step can be minimized, as explained in Section 4.1. A relatively high concentration of probes, which is achieved with immobilization strategy (IS1), resulted in a relatively ordered monolayer considering the short immobilization
This was confirmed by the volume fraction of material above the gold substrate obtained in the NR-study (Section 4.3, table 4.1). A relatively dense monolayer would force the hairpin probes to an upright position, which would be beneficial for hybridization, but may also create steric constraints for the hybridization and subsequent unfolding of the hairpins. Thus sparsely populated layers are usually preferred.

Figure 6.1. A) Nyquist plots, $-Z_{im}$ vs. $Z_{re}$ and B) Electrochemical AC voltammograms for an Au electrode in SCC buffer containing 5.0 mM Fe(CN)$_6^{3-}$/Fe(CN)$_6^{4-}$: a) bare Au surface and b) after immobilization of the hairpin probe (HPP) and m-PEG molecules (IS1).

In Figure 6.1.A it is shown that the formation of the mSAM (IS1) at the Au electrode surface resulted in a significant increase in the ‘semi-circle’ in the Nyquist plot, reflecting an increase in the charge transfer resistance ($R_{ct}$) at the interface, in accordance with the data presented in Section 5.3.2 (Figure 5.7). All the experimental impedance curves were fitted to an equivalent circuit model (Scheme 2.9, section 2.4.9.1) that included a solution resistance ($R_s$) in series...
with a parallel circuit containing a constant phase element (CPE), the charge transfer resistance ($R_{ct}$) and Warburg impedance ($W_0$).

Figure 6.2. A) Nyquist plots, $-Z_{im}$ vs. $Z_{re}$ and B) Electrochemical AC cyclic voltammograms for an Au electrode in SCC solution containing 5.0 mM Fe(CN)$_6^{3-}$/Fe(CN)$_6^{4-}$: a) bare Au surface, after b) 30 min and c) after 2 h of m-PEG self-assembly.

The modified electrodes were further investigated by AC voltammetry and the increase in impedance for a pure m-PEG-layer is mirrored as a decrease in the detected current in the AC voltammogram (Figure 6.2.B). The AC voltammogram for the mSAM-layer showed a similar decrease in current (Figure 6.1.B), supporting the impedance data in Figure 6.1.A.

6.3 Hybridization dynamics and sensitivity of the label-free DNA sensor

Hybridization between the hairpin and the target involves both the opening of the stem-loop structure of the probe as well as the formation of the probe-target duplex, where the probe-target binding is the driving force for the opening of the loop. In Section 5.3.3 the
hybridization process is described as a step-wise event, according to Tsorukas et al.\cite{140} Briefly, the first step involves the attachment of the target to the probe sequence in the loop and the second step is the opening of the HPP, when a sufficient number of bases pairs have been formed. The final formation of the target-probe duplex is then considered to be the third step.\cite{140} The Nyquist plots in Figure 6.3.A and B describe the sensor response for various concentrations of complementary target ODN at a hybridization temperature of 37 °C and 44 °C, respectively. The sensors were prepared by the first immobilization strategy (IS1), resulting in a relatively high probe density layer. The dynamics of the hairpins is dependent on the stability of the hydrogen bonds between the base pairs, which keep the stem together\cite{139} and consequently the hybridization temperature can play an important role in the hybridization event; this is reflected in the distinctively different trends observed in Figure 6.3.A and B.

Based on the results for the lower hybridization temperature a general increase in $R_{ct}$ (Figure 6.3.A) can be seen, which reflects the hybridization, but not necessarily the unfolding of the hairpin, and the formation of a stable duplex between the probe and the complementary target ODN. If the hybridization would be considered a step-wise process, as mentioned above, the results suggest that the hybridization can halt at step 1, especially in a sterically crowded environment. However, when complementary target of a low concentration (pmol l\textsuperscript{-1} range) was introduced a decrease in impedance and $R_{ct}$ was observed. A decrease in $R_{ct}$ is an indication of an enhanced electron transfer from the solution to the electrode surface. The hairpin probes occupy a larger area on the electrode surface, compared to single stranded probes, and a decrease in the impedance and in the $R_{ct}$ would be expected if the hairpin probes fully unfold upon hybridization, thus exposing a bare area of gold underneath, which becomes easily accessible for the Fe(CN)$_6^{3-/4-}$.\cite{191}
Figure 6.3. Nyquist plots, $-Z_m$ vs. $Z_re$ for an Au electrode in SCC buffer containing 5.0 mM Fe(CN)$_6^{3-}$/Fe(CN)$_6^{4-}$: a) bare Au surface, b) after immobilization of the hairpin probe and m-PEG, after hybridization at A) 37 °C and B) 44 °C with c) 4.66×10$^{-15}$ M, d) 4.66×10$^{-13}$ M, e) 4.66×10$^{-11}$ M, f) 4.66×10$^{-9}$ M, g) 4.66×10$^{-7}$ M of complementary ODN.

Upon further hybridization the SAM becomes more dense and crowded and the overall increase in $R_{ct}$ can be explained by an accumulation of negative charges in the monolayer, generated by both the formation of the fully hybridized probe-target duplexes as well by the
partially attached targets to the probe sequence in the hairpin loop. The redox probes in the solution thus experience a stronger repulsion from the SAM after hybridization than before it, increasing the impedance.\textsuperscript{148}

An additional concern regarding the effect of steric constraints during hybridization also needs to be considered: even in a case of simultaneous (competitive) immobilization of HPPs and the m-PEGs a highly heterogeneous distribution of the probes would be expected in the SAM. Some areas may suffer from probe aggregation and thus also from additional steric constraints for the conformational change during hybridization, as observed by H. Du et al.\textsuperscript{15} The authors concluded that enough target had to be present to open whole “clusters” of molecular beacons before a signal could be detected. A certain extent of bonding between probes could also be possible in locally dense, clustered, areas.\textsuperscript{15} This, along with the rigidity of the hairpins at the lower hybridization temperature could offer an additional explanation to the varying response observed in Figure 6.3.A. In order to decrease the impedance the hairpins need to fully unfold when the complementary target is introduced. If there are clusters of probes in some areas a sufficient amount of target is needed to unfold all the probes at the same time, as they will be entangled in each other and not very prone to hybridize one by one.\textsuperscript{15} The target will still bind with the probe but is unable to open up the hairpin, which results not only in accumulation of negative charges but also of material on the electrode surface, both contributing to the inhibited electron transfer at the electrode surface. On the other hand, once such a cluster of probes simultaneously opens up bare gold is exposed and a decrease in impedance is observed. It is reasonable to believe that at very low target concentrations the sterically favored hairpins will open up first and that the crowded probes will, at least initially, cause the accumulation of target ODN on the surface.
The problem with unfolding of the probes upon hybridization for high probe density sensors can be overcome if additional energy (increased hybridization temperature) is introduced during the hybridization event. When a higher hybridization temperature was used an opposite trend could be seen as the impedance decreased continuously upon hybridization with complementary ODN (Figure 6.3.B). As mentioned in section 3.3, it is known that HPPs go through a conformational change upon heating.\textsuperscript{13, 140} Figure 6.4 shows the melting profile for 2 µM of HPP-4, which is the probe employed in the label-free electrochemical sensor presented in this chapter. The data was fitted to a sigmoid, Boltzmann equation to obtain a melting temperature of 47.1 ± 0.4 °C as listed in Table 3.3 (Section 3.3.3).

![Melting profile for 2 µM of HPP-4](image)

Figure 6.4. The melting profile for 2 µM of HPP-4. The circles symbolized the measured data points and the solid blue line represents the sigmoid, Boltzmann fit.

As the melting temperature measured for the HPP (47.1 ° C) is quite close to the higher hybridization temperature it has to be considered that the response seen, in Figure 6.3.B and Figure 6.5.B, can be partly due to dissociation of the loops by temperature and not by target.
hybridization alone. Although the 3 °C difference between the melting temperature of the HPP and the hybridization temperature should be sufficient to rule out any false responses, additionally the electrochemical measurements were conducted at room temperature, not at 44 °C, with a minimum of 5 min in SSC-buffer at room temperature prior to impedance measurements. Any base pairs, which might have dissociated because of the increased temperature, would have had re-associated before the impedance was measured. As seen in Figure 6.6.G and H there is no sensor response at very low target concentrations, which provides further evidence that the sensor response is indeed due to duplex formation. At 47.1 °C the stems of the HPPs could have been destabilized to some extent making it easier for the target DNA to break the bonds between the base pairs and hybridize efficiently, i.e. unfold the hairpin, with the complementary target. Anne et al has shown that even rigid double stranded DNA helices can move in an elastic bending motion (at 37 °C) when attached to a surface. Thus a destabilization of the stem at the higher hybridization temperature could be expected to enhance the flexibility of the probe within the monolayer and the target would find the actual probe sequence in the loop part of the probe more easily. The improved flexibility of the probe would thus facilitate the base-pairing with the complementary ODN during the hybridization process. The progressive decrease in the normalized charge transfer resistance, \( \Delta R_{ct}/R_{ct}(ML) \), within the applied concentration range of target ODN also suggested that saturation of the probes had not been reached within this concentration range. \( \Delta R_{ct} \) represents the difference in charge transfer resistance before and after hybridization with target ODN and \( R_{ct}(ML) \) is the charge transfer resistance for the mixed monolayer before addition of any target ODN. The sensitivity of this electrochemical sensor can be determined from the dependence of the change in the charge transfer resistance upon hybridization of the hairpin probe on the concentrations of complementary target ODN. Sensitivity is mainly determined by the slope of the linear relationship of response vs. concentration. For the
probe hybridized at 37 °C the sensor response an attempt was made to fit the response linearly with the logarithm of the target concentration. The linear fit results in a slope of 0.143 \([\Delta R_{ct}/R_{ct}(\text{ML})]/[\log \text{ concentration, (M)}]\) (Figure 6.5.A) and a \(|R|\) of 0.83, which is too low for the response to be considered linear in the investigated concentration range. However, a higher sensitivity, with a slope of -0.169 \([\Delta R_{ct}/R_{ct}(\text{ML})]/[\log \text{ concentration, (M)}]\) (Figure 6.5.B), is found when hybridizing at 44 °C. For the sensor represented in Figure 6.5.B the sensor response is approximately linear with the logarithm of target concentration in the n-μM range.

Figure 6.5. Normalized charge transfer resistance change, \(\Delta R_{ct}/R_{ct}(\text{ML})\), taken as the sensor response, before and after hybridization at A) 37 °C and B) 44 °C with different concentrations of complementary ODN \((4.65\times10^{-10} \text{ M to } 4.65\times10^{-6} \text{ M})\) in SCC buffer containing 5.0 mM Fe(CN)$_6^{3-}$/Fe(CN)$_6^{4-}$. The IS1 strategy was used to form the sensing monolayer. The standard error from the fitting is set to 10 %.

The results show that the detection limit could be several orders of magnitude smaller under fully optimized conditions as a reliable response was observed at 4.7 nM target concentration.
(taken from the experimental data). The detection limit for a similar type of hairpin sensor, where amplification of the sensor response was achieved by intercalation of an cationic dye into the dsDNA, was found to be 50 pM,\textsuperscript{148} whereas 10 pM concentrations have been detected in a DNA sensing protocol where single stranded probes have been attached on top a mixed SAM, composed of 2-mercaptoethanol and 11-mercaptopoundecanoic acid.\textsuperscript{147} The sensor investigated here exhibit similar sensitivity, even with an unamplified response.

To gain further insight in the mechanism of the hybridization for the hairpin sensor and to improve the sensitivity of the sensor, the same molar ratios of m-PEG:HPPs were used as for the CdTe-labelled sensor presented in Section 5.3. The molar ratios used were 2:1, 10:1 and 25:1 and the prepared sensors (IS2:1, IS10:1, IS25:1) were hybridized with a range of complementary target concentrations, from $4.65 \times 10^{-15}$ M to $4.65 \times 10^{-6}$ M at both 37 °C and 44 °C. Figure 6.6 summarizes the hybridization response from the IS1 sensor at both 37 °C (A) and 44 °C (E) and from the IS2:1, IS10:1, IS25:1 sensors at 37 °C (B, C, D) and at 44 °C (F, G, H). Figure 6.6 summarizes the Nyquist plots for the investigated sensors with varying probe densities (A,E: IS1, B, F: IS2:1, C, G: IS10:1 and D, H: IS 25:1) at two different hybridization temperatures (A-D: 37 °C and E-H: 44 °). In order to compare the the sensor responses in a more accessible way, the results were plotted as $\Delta R_{ct}/ R_{ct}$(ML) versus concentration. As $\Delta R_{ct}$ is derived from the fitted impedance data, as mentioned previously (Section 6.1), it directly reflects the impedance data in Figure 6.6.
Thus, visualized in Figure 6.7.A-D is the $\Delta R_{ct}/R_{ct}(\text{ML})$ response trend when the probe density was gradually decreased but the hybridization temperature was kept at 37 °C. The dynamic range for the sensor response was defined as the concentration range where the linear fit gives $|R| > 0.900$. In Figure 6.7.A, B and F the $|R|$ value was below 0.900 in the whole concentrations range and thus the response of these sensors were not fitted. Also, for the sensor presented in figure 6.7.A the response was not considered reliable for the lowest cODN concentration as the difference in response between the mSAM and that hybridized layer was not $\geq |3| \text{k}\Omega$. The response trends can, however, still be observed for all the sensors.
A progressive shift and stabilization in the response trend of the sensor seemed to be occurring under the investigated conditions (Figure 6.7.A-C), indicating that there still was not enough space for the hairpins to fully open during the hybridization when low concentrations of targets was used, seen as an increase in charge transfer resistance. At higher concentrations of target the impedance starts to decrease, indicating that the probes are able to unfold, resembling the response for lower probe densities at 37 °C (Figure 6.7.C, D) and for most probe densities at 44 °C (Figure 6.7.E, G and H).

Figure 6.7. Comparison of $\Delta R_c/R_c$(ML), taken as the sensor response before and after hybridization with different concentrations of complementary ODN (4.65×10$^{-15}$ M to 4.65×10$^{-6}$ M) in SCC solution containing 5.0 mM Fe(CN)$_6$$^{3-}$/Fe(CN)$_6$$^{4-}$. The sensing monolayer was immobilized using either the IS1 strategy (A and E) or IS2:1, IS10:1 and IS25:1 (B, C, D, F, G and H) with predetermined ratios of m-PEG:HPP molecules. In Figure A-D hybridizations were carried out at 37 ºC whereas 44 ºC was used in Figure 5.E-H. The standard error from the fitting is set to 5 %.
For some of the sensors, Figure 6.7.B, C, F and H, the response trend is going through 0, similarly the CdTe-labelled sensor presented in Figure 5.9. As mentioned earlier, in Section 5.3.3, the reason for this is that the measured impedance (and thus the $\Delta R_{ct}$) is higher for the first hybridized layer compared to mSAM, which had been set as an artificial ‘zero’, although a decrease in impedance was observed for the succeeding target concentrations. If the lowest target concentrations are not sufficient to unfold the hairpins upon hybridization, although the target ODNs do bind specifically to the probes (as seen in Figure 6.7.A) the $\Delta R_{ct}$ response will pass through the artificial zero.

Table 6.1 summarizes the experimentally determined detection limits for the various sensors and their sensitivity to complementary target, determined as the slope of the linear relationship range of the response vs. the log(concentration).

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>IS1</td>
<td>0.47</td>
<td>-0.169</td>
<td>37</td>
</tr>
<tr>
<td>IS2:1</td>
<td>0.0047</td>
<td>-0.068</td>
<td>37</td>
</tr>
<tr>
<td>IS10:1</td>
<td>470</td>
<td>-0.092</td>
<td>37</td>
</tr>
<tr>
<td>IS25:1</td>
<td>0.047</td>
<td>-0.123</td>
<td>44</td>
</tr>
<tr>
<td>IS10:1</td>
<td>0.47</td>
<td>-0.035</td>
<td>44</td>
</tr>
<tr>
<td>IS25:1</td>
<td>0.47</td>
<td>-0.035</td>
<td>44</td>
</tr>
</tbody>
</table>

Table 6.1. Summary of the experimentally determined detection limits for the various sensors. The detection limit was extracted from the experimental data, and defined as the lowest concentration, within the dynamic range of the sensor ($R > 0.900$), where a response could be observed. The sensitivity was determined as the slope of the linear relationship range of response vs. concentration.

The most reliable and sensitive response was observed with the IS25:1 sensor at 37 °C (Figure 6.7.D) and the sensor responded to targets concentrations as low as $4.66 \times 10^{-15}$ M.
Generally a good sensor response was achieved at low probe density for hybridizations performed at 37 °C or high probe density for high hybridization performed at 44 °C, for target concentrations between 4.66×10⁻¹⁴ and 4.66×10⁻⁶ M, (Figure 6.7.C, D and E respectively).

![Figure 6.8. Comparison of ΔR_0/R_0(ML), taken as the sensor response before and after hybridization with different concentrations of complementary ODN (4.65×10⁻¹⁵ M to 4.65×10⁻⁶ M) in SCC solution containing 5.0 mM Fe(CN)_6³⁻/Fe(CN)_6⁴⁻ for repeated measurements. The sensing monolayer was immobilized using either the IS1 strategy (A and E) or IS2:1, IS10:1 and IS25:1 (B, C, D, F, G and H) with predetermined ratios of m-PEG:HPP molecules. In Figure A-D hybridizations were carried out at 37 °C whereas 44 °C was used in Figure 5.E-H The standard error from the fitting is set to 5 %.

The response was log-linear with |R| values of 0.917 and 0.977 for Figure 6.7.C and 6.7.D, respectively, which is an indication of an efficient and reliable sensor. In Figure 6.7.E a good response is visible for tested target concentrations between 4.66×10⁻¹⁰ and 4.66×10⁻⁶ M with the |R| value of 0.973). When the hybridization temperature was 44 °C the response showed good linearity at target concentrations higher than 4.66×10⁻¹² M and an inconsistency in the
response at probe densities below $4.66 \times 10^{-12}$ M (Figure 6.7.G-H). The higher probe density sensor, IS2:1 (hybridization at 44°C, Figure 6.7.F) showed a behavior similar to the IS2:1 at 37 °C (Figure 6.7.B), indicating that these immobilization strategies do not produce a reliable sensor response. The results obtained from repeated measurements, corresponding to the data presented in Figure 6.7 on all accounts, are presented in Figure 6.8. Although there are variations in the data, the response trends for all sensors were confirmed. The low probe density sensor, IS25:1 (Figure 6.8.D and H), showed the best reproducibility of the label-free sensor investigated here.

A supplementary approach to evaluate the sensor response is to calculate the heterogeneous standard charge transfer rate constant, $K^0_a$, as a function of complementary target concentration. $K^0_a$ represents the kinetic facility of a redox couple and can be obtained from equation 6.1, which is derived from the Butler–Volmer model of electrode kinetics.

$$K^0_a = \frac{RT}{n^2 F^2 A R_{ct}} \times \frac{1}{C_0^{*1-\alpha} C_R^{*\alpha}}$$

(Eq. 6.1)

where $R$ is the gas constant, $T$ is the temperature (298 K), $n$ is the number of electrons, $F$ is Faraday’s constant, $A$ is the electrode area, $R_{ct}$ is the charge transfer resistance, and $C_0^{*1-\alpha} = C_R^{*\alpha} = C^0$. $C^0$ is the bulk concentration of Fe(CN)$_6^{3-}$/Fe(CN)$_6^{4-}$ (5 mM) and $\alpha$ is the charge transfer coefficient, which is assumed to be 0.5. As shown in Figure 6.7.C-H, the charge transfer resistance, $R_{ct}$, present a decreasing trend with increased target concentration. Only two sensors exhibit an opposite (Figure 6.7.A) or less pronounced (Figure 6.7.B) response trend. Previously, a decrease in $K^0_a$ values with the increase of complementary ODN concentration, has been reported for a DNA sensor based on a functionalized conducting copolymer, poly[pyrrole-co-4-(3-pyrrolyl) butanoic acid]. Decreasing $K^0_a$ values suggest that duplexes, formed after hybridization, create a barrier against movement of both ions and of
the redox couple \( \text{Fe(CN)}_6^{3-}/\text{Fe(CN)}_6^{4-} \) to the electrode surface. In Figure 6.7 the \( K_0^0 \) values for the studied HPP sensors are presented in a corresponding manner to the \( \Delta R_{ct}/R_{ct}(\text{ML}) \) response, displayed in Figure 6.7.

Figure 6.9. Comparison of the heterogeneous standard charge transfer rate constant, \( K_0^0 \), before and after hybridization with different concentrations of complementary ODN (4.65×10^{-15} M to 4.65×10^{-6} M) in SCC solution containing 5.0 mM \( \text{Fe(CN)}_6^{3-}/\text{Fe(CN)}_6^{4-} \). The sensing monolayer was immobilized using either the IS1 strategy (A and E) or IS2:1, IS10:1 and IS25:1 (B, C, D, F, G and H) with predetermined ratios of m-PEG:HPP molecules. In A-D hybridizations were carried out at 37 °C whereas 44 °C was used in E-H.

The trend of a decrease in \( K_0^0 \) values observed in Figure 6.9.A indicates that a barrier has been formed after hybridization, leaving the electrode less accessible to movements of the redox couple\(^5\), supporting the view that the HPPs are not able to unfold upon hybridization due to the high probe density at the moderate hybridization temperature, 37 °C. A generalized, opposite trend can be observed for the other sensors (Figure 6.9.B-H), with \( K_0^0 \) values rising...
as a function of increased target concentrations. The increase in $K_a^0$ values implies an improved accessibility of the redox couple to the electrode as a result of the hybridization of the HPPs with complementary target. This is an indication that the barrier formed by the initial mSAM has been compromised due to the unfolding of the HPP and that a larger part of the Au electrode has become available to the redox couple.

6.4. Selectivity of the label-free DNA sensor

The selectivity of the hairpin probes is generally attributed to the conformation of stem-loop structure$^{13-15, 17, 148}$ and this correlation is also observed for the sensors reported here. The selectivity of a sensor was investigated by measuring the response of the sensor after hybridization with $4.66 \times 10^6$ M single-base mismatched and $4.66 \times 10^6$ M non-complementary target ODN at the two different hybridization temperatures. The results are summarized in Figures 6.10 and 6.11. The Nyquist plots in Figure 6.10.A-D show the sensor response for a single-base mismatch target ODN when hybridized at 37 °C. For the high probe density at low hybridization temperature we observe excellent selectivity (Figure 6.10.A) with no obvious change in the sensor response when the sensor is incubated in a solution of a single-base mismatch target or non-complementary ODN even if their concentration was in the µM-range. This indicates a very high selectivity for the target sequence due to the intact conformation of the hairpins in the sterically crowded environment at high probe densities. The response to a non-complementary target almost identically resembles the one-point mismatch response (Figure 6.9.A) demonstrating that this sensor exhibits an excellent selectivity towards its target sequence, when hybridized at 37 °C.

When the probe density was decreased the selectivity of the sensor, at low hybridization temperature, also started to decrease. For the m-PEG:HPP 2:1 sensor, an opposite response was observed for the one-base mismatch compared to the fully complementary target (Figure
6.8.B). The underlying reason for this response is not fully understood. However, in Figure 6.2.B, where the signal for complementary target was investigated, no clear response trend could be observed indicating irregular behavior of the m-PEG:HPP 2:1 sensor at 37 °C. When the molar ratio of m-PEG:HPP was 10:1 (Figure 6.10.C) the sensor responded to the single-base mismatch, but the response was significantly smaller than for the fully complementary target.

Figure 6.10. Nyquist plots, $-Z''$ vs. $Z''$, and for an Au electrode in SCC solution containing 5.0 mM Fe(CN)$_6^{3-}$/Fe(CN)$_6^{4-}$: A, E) IS1, B, F) IS2:1, C, G) IS10:1, D, H) IS25:1, a) bare Au surface, b) after immobilization of probe and m-PEG and c) after hybridization at either 37 ºC (A-D) or at 44 ºC (E-H) with 4.66×10$^{-6}$ M of one-point mismatch ODN.
Furthermore, the selectivity was not significantly compromised even by the lowest probe density, IS25:1 (Figure 6.10.D), indicating that the stem-loop structure was intact during the hybridization. However, the high selectivity for one-base mismatch targets was lost when hybridization is carried out at 44 °C, as shown in Figure 6.10.E-H. All the sensors showed a response towards the one-base mismatched target, although again an opposite response was observed for IS1 at 44 °C (Figure 6.10.E). The general lack of selectivity at the higher hybridization temperature was not surprising since the selectivity of the hairpins is known to stem from their secondary structure, which is compromised when the hybridization temperature is close to the melting temperature of the HPP.

Figure 6.11. Nyquist plots, –Z_m vs. Z_re and for an Au electrode in SCC solution containing 5.0 mM Fe(CN)_6^{3-/4-}: A, D) IS1, B, E) IS2:1, C, F) IS10:1, D, H) IS25:1, a) bare Au surface, b) after immobilization of probe and m-PEG and c) after hybridization at either 37 °C (A-D) or at 44 °C (D-H) with 4.66×10^{-6} M of non-complementary ODN (NC)
The sensor response towards non-complementary target (NC) is shown in Figure 6.11.A-H. The results corresponded completely to the ones obtained with one-base mismatched targets, for the sensors investigated. Discrimination against non-complementary targets was achieved at the lower hybridization temperature (Figure 6.11.A-D) but all selectivity was lost when hybridization was carried out at 44 °C. The IS1, IS10:1 and IS25:1 showed the greatest selectivity of all the investigated sensors.

6.5. Conclusions

A high-quality DNA sensor can discriminate between its target and mismatched sequences in a reliable and sensitive manner. To realize such a sensor a range of parameters had to be considered and optimized based on the fundamental behavior of the sensing layer. We have presented an electrochemical, label-free sensor based on a mixed self-assembled monolayer (SAM) of poly(ethylene glycol) (m-PEG) and hairpin structured probes immobilized on a gold electrode. Depending on the hybridization temperature the sensor showed completely opposite trends with regard to the change in charge transfer resistance at the electrode interface. We propose that this difference is due to conformational changes in the transition state of the hairpin at the elevated hybridization temperature. The transition state (partially melted state) allows the hairpin a higher structural flexibility, which facilitates the base-pairing with the complementary ODN and thus assists with the unfolding of the probe.

The best prepared sensor showed sensitivity down to 4.7 fM target ODN and was capable of detecting single-base mismatched ODN. The solid-state approach to DNA sensors is advantageous for practical applications and can provide the bases for miniaturization and manufacturing purposes.
Chapter 7 – Summary and future work

7.1 Summary

Sensitive and selective electrochemical DNA sensors, based on a self-assembled monolayer of HPPs and m-PEG molecules, were developed to achieve direct, sensitive and selective detection of DNA. Both optical and electrochemical methods of detection were evaluated. To assess one of the fundamental characteristics of the optical signal-on approach, where fluorescence from the QD-labeled HPPs was to be quenched by the gold substrate when no target was available, and restored upon hybridization with complementary target ODN, a quenching study was conducted. It was found that both an unmodified and a m-PEG-modified Au surface effectively quenched the fluorescence of a Cy3-dye as well as the fluorescence of functionalized CdSe/ZnS quantum dots. However, adhesion between the QDs and the Au-surface was reduced when the Au-substrate was modified with m-PEG and thus a mixed SAM, with both HPPs and m-PEG molecules, were used for the sensor applications.

The sensing platform, composed of the mSAM, was then characterized using ATR-FTIR, TM-AFM and NR. The ATR-FTIR-technique was found to be only moderately useful for characterization of the mSAMs. The recorded spectra were complex due to the large amount of vibrations from the various molecules present in the mSAM and quantitative characterization of the mSAM with ATR-FTR was not possible for this system. The TM-AFM imaging resulted in intial information for the determination of the composition and morphology of the mSAM. Variations in surface roughness between the different mSAM-layers were observed. A SAM, which consisted of only m-PEG molecules, was relatively dense and smooth, whereas the mSAMs (m-PEG:HPP 2:1, 10:1 and 25:1) showed a higher surface roughness. Additionally, NR was used for the characterization of the mSAMs and information regarding the layer structure was gained.
Thickness- and volume fraction values, determined from the model fit, showed hybridization induced conformational changes in the mSAM. The surface coverage of HPPs was dependent on the immobilization time, with $1.5 \times 10^{12}$ HPP molecules cm$^{-2}$ attached in 30 min and $7.1 \times 10^{12}$ HPP molecules cm$^{-2}$ in 2 h, and at a larger initial concentration. Further studies, especially with PNR, would be interesting and could offer more thorough insight in the morphology of the mSAMs.

The stability of a dsDNA sequence is greatly influenced by temperature and thus the performance of a DNA sensor can be dependent on the melting behavior of the participating ODN-strands. Simulation provides a fast and useful way to assess the thermodynamic behaviour of different single-stranded and double-stranded DNAs, and the DINAMelt server was used to predict the melting temperatures of the HPPs and HPP/target duplexes used in the DNA-sensor design. The empirically determined melting temperatures (in solution) were found to correlate relatively well with the predicted values, although they generally were 5 - 11 °C lower than the computed values for all HPP/target duplexes investigated. Surface-attached ODNs are expected to show higher melting temperatures, compared to the same strands in solution and thus the melting behavior of the HPP-4 was also studied by PNR. Clear signs of melting were observed in the reflectivity- and the SLD profiles although a distinct melting temperature could not yet be confirmed. However, PNR, as a technique, has the potential to detect the melting temperature of surface-attached probes.

Two different kinds of QDs were synthesized, CdSe/ZnS and CdTe, and utilized as labels in a DNA sensor. The CdSe/ZnS QDs required functionalization to become water-soluble and for attachment to the HPPs. The ligands used for functionalization increased the size of the QDs, which increased the sterical constraints during hybridization with complementary target.
Additionally, crosslinking was likely to occur between one QD and several HPPs, and no optical signal was detected. Although functionalization was not required for the CdTe QDs, an optical response was not observed with the CdTe-labeled sensor either. However, an electrochemical response could be detected with the CdTe-labeled sensor for target concentrations in the fM-range.

An electrochemical, label-free sensor based on the mSAM was also developed. The effect of hybridization temperature and the probe density were investigated and it was found that the sensor response was greatly influenced by these parameters. HPPs undergo conformational changes at elevated hybridization temperature and if hybridization is carried out close to the melting temperature of the HPP, the HPP is allowed a higher structural flexibility, which is advantageous in the detection of fully complementary target sequences. The best DNA sensor showed sensitivity down to 4.7 fM of target ODN and was capable of distinguishing single-base mismatched ODN from fully complementary ODN.

In conclusion, the objective to develop an efficient, label-free DNA sensor for direct and fast detection of target ODNs has been achieved. Although these sensors presented here have not yet been tested on real samples, they show high sensitivity and selectivity for their complementary target sequences, which is paramount for DNA sensors striving to rival the PCR-amplification-based procedures currently employed commercially.

7.1 Future work

Commercial DNA sensors should either be reusable or sufficiently cheap and easy to manufacture to justify single time usage. Thus, the reusability of the HPP based DNA sensors presented here should be investigated. Alkali solutions have commonly been used for
regeneration of DNA sensors but heating the HPP sensor above the melting temperature of the HPP/target duplex could also be employed. The HPP sensor would also need to be tested for more “real-life”-type samples, where proteins and other possible interfering molecules are present. It would also be important to develop an array of HPPs sensors, selective for different target sequences, which then could be built into a single chip. The different areas on the chip should be individually controlled to enable activation of a particular HPP sensor at any time.

To further pursue the investigation of the melting behavior of surface-attached HPPs would also be of great interest. Particularly PNR demonstrated the ability to sense variations in the SAM of HPP as a function on temperature and if coupled with impedance measurements, a more thorough understanding of the melting could be gained.

New techniques for characterization and quantification of possible sensor platforms are always in demand, and in recent years Surface enhanced Raman spectroscopy (SERS) has evolved into a more analytical technique than before. A specially designed marker sequence could be incorporated into the arms of the HPP and conformational changes in the HPP could be detected based on the SERS signal arising from the marker sequence.

Conversion of existing strategies, which are sensitive to DNA, into protein responsive sensors, could emerge as a new generation of electrochemical biosensors. There are naturally occurring DNA binding proteins, which regulate DNA transcription, coordinate stacking and carry out replication and repair of DNA sequences. Targeting these proteins would open up new possibilities, not only for medical diagnostics but also for forensic analysis and within the dairy industry.
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