Surface Templating Using a Photolabile Terpolymer to Construct Mixed Flims of Oligomers and Oligonucleotides for DNA Biosensor Development

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Chemistry
University of Toronto

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Doctor of Philosophy
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Abstract

A photolabile terpolymer containing 6-nitroveratyloxycarbonyl (NVOC) protected amine, epoxy and trimethoxysilyl functionality in 1:3:2 monomer ratio was synthesized to template glass surfaces for specific site directed coupling of non-probe oligomers and probe oligonucleotides. Non-probe oligomers were introduced to the surface to control the environment of the probes by reducing probe-to-probe and probe-to-surface interactions. The trimethoxysilyl group served as the anchoring site for the terpolymer to be covalently bound to glass and silicon wafers. Amine terminated non-probe oligomers were coupled to the epoxy sites and thiolated 19-mer SMN1 probes were directed to the deprotected amine sites via the heterobifunctional linker, sulfosuccinimidyl-4-[maleimidomethyl]cyclohexane-1-carboxylate (sulfo-SMCC). Characterization of the terpolymer was done using $^1$H NMR, $^{13}$C NMR, MALDI-ToF and elemental analysis. NVOC deprotection was monitored by UV absorption, and surface characterization of the bound terpolymer on silicon wafers was investigated with XPS, ToF-SIMS, ellipsometry and static contact angle.
Neutral polyethylene glycol (PEG), negatively charged methacrylic acid (MAA) oligomer and dC$_{20}$ oligonucleotides were used as non-probe oligomers. The probe density on the surface was estimated to be $2.2 \pm 0.3 \times 10^{12}$ molecules/cm$^2$ and the presence of the oligomers on the surface did not significantly affect probe immobilization efficiency. The mixed films were functional for target hybridization and its selectivity towards partially-mismatched targets was investigated at different solution pH, ionic strength and temperature. It was demonstrated that pH can be tuned to ameliorate non-specific adsorption and ionic strength governed the selectivity of the surfaces. Improved selectivity was achieved at high salt concentration (1 M NaCl) on PEG and dC$_{20}$ mixed films at room temperature. The MAA surface did not show significant improvements in selectivity. This indicated that charge of the oligomers does not dominate control of selectivity. The results suggested that the terpolymer construct played a role in depression of the melting temperature of the hybridized duplex to within 5 to 10 °C of room temperature. With the melting temperature shifted closer to room temperature, it is possible to improve selectivity for room temperature detections of single nucleotide polymorphism.
Acknowledgments

I would like to thank Dr. Ulrich Krull for his guidance, inspiration, support and patience throughout the work of this thesis. I am very grateful for his encouragement, and for his belief in me during my times of distress. The time and effort spent in completing this thesis is very much appreciated.

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### Abbreviations and Symbols

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<th>Definition</th>
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<tr>
<td>α-CHCA</td>
<td>Alpha-cyano-4-hydroxycinnamic Acid</td>
</tr>
<tr>
<td>δ</td>
<td>Chemical Shift (NMR)</td>
</tr>
<tr>
<td>1bp</td>
<td>Single Base Pair Mismatch Target</td>
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<tr>
<td>2bp</td>
<td>Two Base Pair Mismatch Target</td>
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<tr>
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<td>CHN</td>
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<td>MAA</td>
<td>Methacrylic Acid Oligomer</td>
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<tr>
<td>MALDI-ToF</td>
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<td>NVOC</td>
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</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<td>PEG</td>
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<td>TLC</td>
<td>Thin Layer Chromatography</td>
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<tr>
<td>T&lt;sub&gt;m&lt;/sub&gt;</td>
<td>Melting Temperature</td>
</tr>
<tr>
<td>ToF-SIMS</td>
<td>Time-of-Flight Secondary Ion Mass Spectrometry</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume by Volume</td>
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<tr>
<td>v/v/v</td>
<td>Volume by Volume by Volume</td>
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<tr>
<td>Vazo 88</td>
<td>1,1’-Azobis(cyclohexanecarbonitrile)</td>
</tr>
<tr>
<td>w/w</td>
<td>Weight by Weight</td>
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<tr>
<td>XPS</td>
<td>X-Ray Photoelectron Spectroscopy</td>
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</tbody>
</table>
Chapter 1
Introduction

1 Introduction to Biosensors, Nucleic Acids and Nucleic Acid Biosensors

A biosensor is a device that contains a biological recognition interface that translates the interaction between the analyte and the biocomponent into a signal that can be detected by a transducer [1]. Biosensors have been developed to serve as diagnostic tools in the detection and analysis of biomolecules such as nucleic acids and proteins. They are designed to detect specific targets with high sensitivity and selectivity. The technology is being driven towards efficiency and reliability in environmental [1], clinical [2] and forensic [3] analyses. Recent advances have been achieved in multiplexed detection [4]. Miniature portable analytical devices that are simple and user friendly are beneficial for on-site investigations.

Nucleic acids are of interest because the genomic sequence is unique for each organism and it carries information for the basis of life and cellular processes. There are two forms of nucleic acids, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). DNA stores genetic information for heredity from one generation to the other and codes for peptides that are essential to regulate cellular activities, metabolisms and growth. DNA is transcribed into messenger RNA (mRNA), which is translated by means of transfer RNA (tRNA) into proteins with every 3 bases representing a codon for a specific amino acid. The importance of DNA and its implication to life, development and evolution, have resulted in great interest in DNA sequencing, mapping and the study of gene expression. With the human genome and genomes of a number of other organisms fully sequenced, mutated sequences and the significance of single nucleotide polymorphism (SNP) sites was identified. Extensive studies have been focused on determining
the functions of the genes, the relationships and interactions between the genes, and the understanding of the reaction of cells towards physical and chemical stimulants. The outcomes of these studies have provided a huge leap in the understanding of cellular functions and pathways, the identification and classification of diseases, and subsequently, drug discovery and the effects of drugs on individuals.

Nucleic acids are linear polymers that are built from monomers of a five carbon sugar, nitrogenous base and phosphate. DNA has 2-deoxy-D-ribofuranose as the sugar, whereas RNA has D-ribofuranose. Since RNA occurs as single strands, the presence of the hydroxyl group at the 2’ position of the sugar serves to stabilize the folded structure through interactions with the phosphates or bases [5]. In comparison with DNA, RNA is less stable chemically, and is prone to hydrolysis. The nitrogenous bases are either a pyrimidine, a six-membered heterocyclic aromatic ring containing two nitrogens, or a purine, which is a pyrimidine fused with a five-membered imidazole ring system containing additional two nitrogens [6]. The pyrimidines are thymine (T), cytosine (C) and uracil (U), whereas the purines are adenine (A) and guanine (G). In DNA, the four bases are A, T, G, and C, whereas in RNA, T is replaced by U. The sugar and base are linked in a N-glycosidic bond at the 1’ position of the sugar, where the hydroxyl group is replaced by the N1 of the pyrimidine or N9 of the purine. A phosphoric acid connects two sugar units via a phosphodiester bond between the 5’ end of a sugar unit and the 3’ end of the other. DNA occurs naturally as a double helix structure with one strand going from 3’ to 5’ and the other from 5’ to 3’. The two DNA strands are held together via hydrogen bonding between specific bases, A with T, and G with C, along with hydrophobic interactions and stacking forces to stabilize the structure [6].
Nucleic acid biosensors are typically based on a solid substrate with a recognition layer of single stranded probe oligonucleotides on the surface. The probes capture target sequences of nucleic acids from the analyte, and provide a physical change at an interface that is transduced as a signal for detection. The solid substrates that have been investigated include glass, fused silica, silicon wafers, gold, nanoparticles, quantum dots, carbon nanotubes, wires and various conductive materials.

1.1 DNA Structures and Properties

Double stranded DNA (dsDNA) is helical with major and minor grooves where the insoluble nitrogenous bases are paired in the core, and the sugar phosphate backbones are on the exterior. This conformation minimizes the repulsion between the two negatively charged phosphate backbones. The stability of the helix is contributed by the summation of the hydrogen bonds between the bases, the stacking forces and the hydration of the molecule. The hydrogen bonds are short, non-covalent and directional [7]. Hydrogen bonds typically have 3 to 7 kcal/mol, however, due to the geometric constraints within the double helix, they only have 2 to 3 kcal/mol for a distance between 2.6 and 3.1 Å [7]. There are two hydrogen bonds between A and T, and three hydrogen bonds between G and C. This determines the specificity of the dsDNA. The stacking energy originates from the hydrophobic and Van der Waals interactions between the base pairs stacked in the helix core. It has been estimated that the stacking energy ranges from 4 to 15 kcal/mol per dinucleotide depending on the sequence of the base pairs involved [7]. Since the bases are planar and aromatic, the \( \pi \) electrons of the aromatic rings overlap to form a protected hydrophobic core within the two hydrophilic sugar phosphate backbones. In hydrated environments, the water molecules orient around the two sugar
phosphate backbones of the dsDNA, and this supports the helix structure. Cations such as sodium and magnesium counteract the negative charges on the phosphate backbones situated on the exterior of the helix. While each of the individual interactions is relatively weak, the cumulative effect allows the helix to be stable and energetically favoured.

There are several types of hydrogen bonding configurations, such as Watson-Crick, Hoogsteen, wobble and syn-anti base pairing, as shown in Figure 1.1 [7]. In the Watson-Crick model, the hydrogen bonding sites on the purines are solely on the pyrimidine ring, whereas the Hoogsteen base pairing involves the nitrogen on the imidazole ring of the purines. A Hoogsteen GC$^+$ base pair will only form under acidic conditions because the nitrogen on cytosine has to be protonated in order for hydrogen bonding to occur, and only two hydrogen bonds are formed between the bases as opposed to three in the Watson-Crick base pairing [8]. Therefore, at neutral pH, the Hoogsteen GC$^+$ base pair is not stable and it is unlikely to have both Watson-Crick and Hoogsteen base pairing in the same duplex because they have different sugar to sugar distances [8]. Reverse Watson-Crick and reverse Hoogsteen base pairing is possible when one base rotates 180° with respect to the complementary base. A wobble base pair occurs when the position of one base is shifted within the flat plane relative to the complementary base. The syn-anti base pair involves two purines where one is in the favourable anti form and the other in the less favourable syn conformation. The anti and syn conformation is due to the rotation of the base around the glycosidic bond.
Figure 1.1. Different types of hydrogen bonding between the bases are shown, Watson-Crick, Reverse Watson-Crick, Hoogsteen, Reverse Hoogsteen, Wobble and syn-anti base pairing, where the R groups representing the sugar phosphate backbone.
There are several types of dsDNA helical structures. They differ in the right or left handedness of the helix, twisting of the C2’ and C3’ carbons in the sugar and the rotation of the base around the glycosidic bond. These differences result in different height, width, base pair per turn and geometry of the helix. The sugar moiety is flexible, allowing the C2’ or C3’ carbon to twist out of plane [7]. In cases where C2’ twists up towards C5’, it gives a C2’ endo configuration, whereas when C3’ twist down towards C5’, it gives a C3’ endo configuration, as shown in Figure 1.2. This affects the distance between the sugar units with the latter giving a shorter distance. The nitrogenous bases are free to rotate around the glycosidic bond, giving a syn or anti conformation. The anti conformation is more favourable due to less steric hindrance and availability for hydrogen bonding.

![Diagram of sugar conformations](image)

Figure 1.2. (a) Illustration of C2’ endo and C3’ endo conformation of the sugar in the envelop form. (b) Illustration of the syn and anti conformation of the base using A as an example, where R and R’ representing the adjacent nucleotides.
The major types of dsDNA helices are A, B and Z-DNA. In salt solution, which is the condition used in the work of this thesis, B-DNA dominates. It is a right-handed helix with a height of 34 Å and containing an average of 10.5 base pairs per turn [6,7]. It has prominent major and minor grooves for binding of chemical reagents where the functional groups on the bases can be accessed [6]. Hoogsteen hydrogen bonding can be achieved through purines in the major groove. A-DNA, also a right-handed helix, is more compact than B-DNA and has a large base pair tilt. It occurs under low humidity conditions, 75% relative humidity, and in homopurine and homopyrimidine DNA, for example a poly(dG) with poly(dC) helix [7]. The Z-DNA is a left-handed helix and is the most elongated form among the three. It forms in sequences of alternating pyrimidine-purine, and the guanines adopt the syn conformation while the cytosines remain in the anti conformation [7]. The characteristic parameters of the three DNA forms are summarized in Table 1.1.

Table 1.1. List of A, B and Z-DNA structural properties taken from X-ray diffraction data.
Adopted from references [6] and [9].

<table>
<thead>
<tr>
<th>Parameter</th>
<th>A-DNA</th>
<th>B-DNA</th>
<th>Z-DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Helix Sense</td>
<td>Right</td>
<td>Right</td>
<td>Left</td>
</tr>
<tr>
<td>Residue per turn</td>
<td>11</td>
<td>10 (10.5)</td>
<td>12</td>
</tr>
<tr>
<td>Axial Rise (Å)</td>
<td>2.55</td>
<td>3.4</td>
<td>3.7</td>
</tr>
<tr>
<td>Base Pair Tilt (°)</td>
<td>20</td>
<td>-6</td>
<td>7</td>
</tr>
<tr>
<td>Rotation per Residue (°)</td>
<td>33</td>
<td>36 (34.3)</td>
<td>-30</td>
</tr>
<tr>
<td>Diameter of Helix (Å)</td>
<td>23</td>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td>Glycosidic Bond Conformation</td>
<td>Anti</td>
<td>Anti</td>
<td>Anti, syn at G</td>
</tr>
<tr>
<td>Sugar Conformation dA, dT, dC</td>
<td>C3’ endo</td>
<td>C2’ endo</td>
<td>C2’ endo</td>
</tr>
<tr>
<td>dG</td>
<td>C3’ endo</td>
<td>C2’ endo</td>
<td>C3’ endo</td>
</tr>
<tr>
<td>Intrastand Phosphate-Phosphate Distance (Å)</td>
<td>5.9</td>
<td>7.0</td>
<td>7.0</td>
</tr>
<tr>
<td>dA, dT, dC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dG</td>
<td>5.9</td>
<td>7.0</td>
<td>5.9</td>
</tr>
<tr>
<td>Major Groove</td>
<td>Narrow and deep</td>
<td>Wide and deep</td>
<td>Flat</td>
</tr>
<tr>
<td>Minor Groove</td>
<td>Wide and shallow</td>
<td>Narrow and deep</td>
<td>Narrow and deep</td>
</tr>
</tbody>
</table>
DNA can form triplexes and quadruplexes. Triplexes are formed in polypurine and polypyrimidine regions where a Watson-Crick dsDNA associates with another single stranded DNA through Hoogsteen base pairing. If the third strand is pyrimidine rich, forming T•AT or C•GC, it will bind in a parallel manner with the purine of the duplex through normal Hoogsteen base pairing [7]. If it is purine rich, forming G•GC or A•AT, it will bind in an antiparallel orientation to the pyrimidine of the duplex through reverse Hoogsteen base pairing [7]. Due to the requirement of the GC Hoogsteen base pairing, parallel triplexes generally require acidic conditions whereas the antiparallel triplexes are pH independent [9]. Both intermolecular and intramolecular triplexes can be formed. The latter is known as the H-DNA, which consists of a triplex and single stranded region [9]. DNA quadruplexes are formed between four guanine bases that are on the same plane being held together by a total of eight Hoogsteen hydrogen bonds with two hydrogen bonds each between two guanine residues. This occurs in a parallel or antiparallel fashion between two strands of DNA [9].

1.2 DNA Biosensor Fabrication and Challenges

The fabrication of a DNA biosensor begins with the choice of substrate depending on the method of detection, followed by surface functionalization using a chemistry that is compatible with the probes. For optical detection, the substrate used is usually glass due to low cost, optical transparency and durability. Fused silica is more expensive but provides a more uniform and flatter surface compared to glass. Gold coated surfaces are ideal for detection using electrochemical and reflectance methods. In order to immobilize the probe sequences onto the substrate, the surface of the substrate must be modified in order to yield active functional groups such as amine, epoxy or thiol. Probes are typically bound by either adsorption or covalent
bonding. Surface templating can be achieved by incorporating photolabile protecting groups onto the surface. The probe sequences are often only 15 to 21 bases in length because this is already sufficient to hybridize uniquely with complementary targets from the human genome, and is sufficiently short to prohibit self hybridization [7]. Short sequences also limit folding of the probes and maximizes the energy differences for SNP detection [10].

The factors that are taken into consideration in terms of the probability of capturing the targets include the configuration of the probe on the surface, probe density, the use of linker molecules, the probe sequence, temperature and ionic strength of the sample solution. The ideal immobilization technique is through a single end group attachment of the probes onto the surface, while keeping the probes in an upright configuration perpendicular to the surface in order to maximize the probability of hydrogen bonding of all nucleobases with the target nucleobases. This configuration can be induced by repulsion between the negatively charged phosphate backbones through nearest neighbour effects, which is dependent on the probe density. When the probe density is too high, there will be insufficient space between the probes for the incoming targets to hybridize and steric hindrance dominates. When the probe density is low, the probes will adsorb onto the surface resulting in blocking the active sites for hybridization. In order to mimic the environment of solution, immobilized probes are projected from the surface into the solution via flexible linkers, such as linear alkyl and ethylene glycol linkers.

Some major challenges confronted by DNA biosensors are the stability and reproducibility of the recognition surface, the reduction of non-specific interactions and the time frame needed for detection. The stability of the biosensor is affected when there is dissociation of the probes from the surface caused by washing and heating cycles. The lack of reproducibility
typically restricts the sensors to single use only. Non-specific binding can occur, and adsorption of nucleic acid target is deemed to be the first step towards hybridization in solid phase assays. This affects hybridization and more seriously, can result in a false positive signal. Solutions to reduce non-specific interactions include use of capping reagents to block active sites at the surface, and adding molecules such as formamide and ethylenediamine tetraacetic acid (EDTA) to the sample solution to preferentially interact with the interfering substances. The speed of detection is preferred to be on the order of minutes, or even better in seconds, but when diffusion is limited it can take many hours.

1.3 DNA Biosensor Operation for Signal Acquisition

The analysis is typically done in one of two ways; through monitoring of hybridization, or monitoring of denaturation of the duplex with respect to increasing temperature or stringency. The hybridization event indicates the presence or absence of complementary targets, whereas the denaturation process suggests the quality of the hybridization event, indicating whether the hybridization is between fully complementary sequences or with mismatched targets. The hybridization event is dependent on experimental factors such as pH, ionic strength, temperature and hybridization time. Its dependence on probe sequence, length and density has been addressed previously [7,10,11]. The pH of the solution affects the degree of protonation of the functional groups on the DNA. At pH above 11, the duplex denatures readily. When pH is lower than 3, depurination of the bases occurs. This sets the pH working range for DNA hybridization. The ionic strength of the solution controls the counter ion effects, which serve to neutralize the highly negatively charged phosphate backbones. Solutions with high ionic strength stabilize the duplex. Raising temperature overcomes the forces holding the duplex together. However, the
temperature of denaturation is highly dependent on the base composition, which correlates with the number of hydrogen bonds and the stacking forces that participate in the duplex formation. One approach to ameliorate non-specific and mismatched sequences is to perform a hybridization experiment at an elevated temperature somewhere below the melting temperature of the fully complementary duplex. The melting temperature ($T_m$) is defined as the temperature at which 50% of the duplex has denatured. Hybridization time controls the detection limit because it determines the amount of targets in solution that are able to come into contact with the recognition layer. All of these experimental factors have to be monitored carefully to achieve full hybridization and to minimize non-specific binding.

The denaturation process is an equilibrium process often presented in the form of a melt curve as shown in Figure 1.3. Duplexes with high GC content will have higher $T_m$ since there is one additional hydrogen bond for every GC pair compared to AT. Under different ionic strength conditions, the $T_m$ for the same duplex will increase with increasing ionic strength [6]. The presence of a mismatch weakens the duplex. Therefore, the $T_m$ of a duplex with mismatch will be lower than that of the fully complementary duplex. Also, mismatches in the middle of the duplex destabilize the structure more than those at the termini. The general order of stability for mismatch base pairs from the strongest to the weakest is $GT > GG > GA > CT > AA$, $TT > AC$, $CC$ when the mismatch is located in the middle of a poly(dT) and the complementary poly(dA) duplex [12].
Figure 1.3. A melt curve illustration for a mismatch (MM) and fully complementary (FC) target. The former will have a lower $T_m$ than the latter due to the destabilization of the duplex caused by the mismatch.

1.4 Methods of Detection for DNA Biosensors

Detection of target hybridization in DNA biosensors is commonly achieved by piezoelectric, electrochemical and optical transducers. These are surface sensitive techniques, where the signal detected results from a change in surface property of the sensor such as mass, charge or refractive index. Fluorescence detection techniques are associated with tethered dyes [13], tagged targets [14], or cationic polymers [15]. A change in fluorescence emission intensity specific to the presence or environmental change of a dye indicates target hybridization. For simultaneous detection of multiple targets, labeling techniques using a variety of fluorescent dyes, nanoparticles and quantum dots are utilized.
1.4.1 Piezoelectric and Electrochemical Based Detection

Piezoelectric based detection is a mass sensitive technique that relies on the shift in resonant frequency of the acoustic wave due to changes in mass on the piezoelectric surface. An acoustic wave is generated when an alternating electrical field is applied to a piezoelectric material and is detected by electrodes. The resonant frequency of the piezoelectric material is dependent on the piezoelectric material itself and the strength of the electrical field. The probe is immobilized directly onto the surface of the piezoelectric material. When target hybridization occurs, any perturbation on the surface will cause a shift in the resonant frequency. There are two types of piezoelectric based detectors for DNA biosensors, surface acoustic wave (SAW) devices and quartz crystal microbalance (QCM).

Electrochemical detectors are one of the most versatile transducers due to high sensitivity, low cost and compatibility with microfabrication techniques. This technology offers a wide selection of interfacing routes and signal amplification techniques. Voltammetry and electrochemical impedance spectroscopy are commonly used for DNA biosensors. In voltammetry, the redox currents are measured in terms of applied potential. In electrochemical impedance spectroscopy, the impedance, which represents the resistance in current flow, is measured.

Additional details and references are summarized in Table 1.2.
Table 1.2. Summary of piezoelectric and electrochemical based detection technologies used in DNA biosensors.

<table>
<thead>
<tr>
<th>Technology</th>
<th>Detection Method</th>
<th>Estimated LOD $^a$</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Piezoelectric</td>
<td>Shift in resonant frequency of propagating acoustic wave</td>
<td>$10^{-8} - 10^{-9}$ M</td>
<td>SAW [16,17]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$10^{-8} - 10^{-10}$ M</td>
<td>QCM [18,19,20]</td>
</tr>
<tr>
<td>Electrochemical</td>
<td>Changes in redox currents due to interfacial properties of electrodes</td>
<td>$10^{-10} - 10^{-12}$ M</td>
<td>Conductive polymers [21,22,23,24]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$10^{-12} - 10^{-15}$ M</td>
<td>Electroactive intercalators [25,26]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$10^{-13} - 10^{-16}$ M</td>
<td>Enzyme labels [27,28]</td>
</tr>
</tbody>
</table>

$^a$ Limit of detection

1.4.2 Optical Based Detection

The two main branches of optical detection include surface plasmon resonance (SPR) and fluorescence spectroscopy. SPR operates under the condition of total internal reflection of the incident light through a prism, where an evanescent wave is generated and allowed to interact with an underlying metal interface [29]. SPR is a surface sensitive technique and allows real time label free detection. In the Kretschmann configuration, one face of the prism is coated with a thin metal film, typically around 50 nm thick, and is kept in contact with a lower refractive index medium, such as air or other dielectric sample [29]. For the detection of DNA, probe oligonucleotides are immobilized directly onto the surface of the gold metal film. Interactions of the probes with the analyte are detected by the changes in refractive index at the surface of the sensor.
Fluorescence detection requires the use of intercalators, groove binders, fluorescent dye labels or quantum dots. The hybridization event is usually detected as a change in fluorescence intensity or shift in fluorescence wavelengths. Table 1.3 summarizes some of the figures of merit of optical methods.

Table 1.3. Summary of major optical based detection technologies used in DNA biosensors.

<table>
<thead>
<tr>
<th>Technology</th>
<th>Detection Method</th>
<th>Estimated LODa</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPR</td>
<td>Shift in plasmon resonance frequency due to changes in surface refractive index</td>
<td>10^{-7} - 10^{-9} M</td>
<td>Label-free [30,31,32]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10^{-11} - 10^{-15} M</td>
<td>Nanoparticle label [33,34,35]</td>
</tr>
<tr>
<td>Fluorescence</td>
<td>Changes in fluorescence intensities or wavelengths</td>
<td>10^{-8} - 10^{-9} M</td>
<td>Intercalators [36,37]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10^{-9} M</td>
<td>Molecular beacons [38,39]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10^{-9} - 10^{-12} M</td>
<td>Quantum dots [4,40]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10^{-10} - 10^{-16} M</td>
<td>Fluorescence tags [14,41]</td>
</tr>
</tbody>
</table>

a Limit of detection

Fluorescent intercalators and groove binders provide enhanced fluorescence intensity due to confinement and loss of collisional deactivation in hybridized duplexes. Examples of intercalators include ethidium bromide [42] and thiazole orange [13]. Among the two, thiazole orange has been reported to be superior in terms of enhancement of fluorescence intensity upon DNA binding [43]. Efforts have been made to tether thiazole orange onto the probe to develop a
self-contained signaling mechanism so that when hybridization occurs, the dye is in proximity to intercalate [13].

Fluorescent dyes and quantum dots are most often used as labels at the terminal ends of target sequences, or can serve as a donor and acceptor in fluorescence resonance energy transfer (FRET) systems. Fluorescent dyes are substituted polyaromatic rings with delocalized π electrons that allow electronic transitions. The shift in absorption and emission wavelengths in different dyes is due to the nature of the substituent and molecular rigidity of the dyes. Cyanine dyes, such as Cy 3 and Cy 5, as shown in Figure 1.4, are commonly used as detection elements of labelled targets. A variety of dyes are commercially available over a wide range of excitation and emission wavelengths, but they vary in stability and coupling efficiency to the target. In comparison to fluorescent dyes, quantum dots are more stable to photobleaching, have higher quantum yield and provide narrower emission wavelength bands. However, solubility and chemical stability of quantum dots in aqueous solutions can be problematic depending on the surface chemistry.

![Figure 1.4. Structures of Cy 3 and Cy 5 fluorescent dyes.](image-url)
Molecular beacon technology uses a fluorescent dye and a quencher molecule that are bound to each terminal of a single stranded probe oligonucleotide. The probe oligonucleotide forms a ‘stem-loop’ structure through hydrogen bonding between complementary bases on the 3’ and 5’ terminal of the probe sequence. A stem is usually comprised of 5 to 8 bases whereas a loop contains 15 to 30 nucleotides [44]. In a ‘stem-loop’ structure, the fluorescent dye and quencher molecules are in close proximity to each other, resulting in efficient quenching of the emission from the fluorescent dye. In the presence of complementary targets, hybridization with the probe causes spatial separation of the fluorescent dye and the quencher molecule, resulting in an increase in fluorescence emission [44]. Molecular beacons have shown sufficient selectivity that the approach can be used in SNP detection, both in solution [39] and immobilized on solid surfaces [38].

In FRET systems, a donor is excited and the energy is transferred to the acceptor and the emission intensity of the acceptor is detected. In order for FRET to occur, the donor and acceptor pair has to be in close proximity, and there must be a spectral overlap between the donor emission and acceptor absorption spectra. A simultaneous two colour diagnostic system based on FRET using a green and red CdSe/ZnS quantum dots conjugated with two different probe oligonucleotides was introduced by Algar and Krull [4]. The targets complementary to the probes on the green and red quantum dots were labelled with Cy 3 and Alexa Fluor 647 respectively. Upon hybridization with the targets, the quantum dots were excited by a single excitation wavelength near the ultraviolet region, and the emission intensities of the dyes were concurrently detected [4]. It was found that the colours responded in proportion to the target concentration and were relatively independent from each other [4].
Fluorescent cationic polymers interact with the hybridized duplex by electrostatic interactions for detection. Liu and Bazan introduced a tricolour mode of detection for the Cy 5 labelled peptide nucleic acid (PNA) probe, non-complementary and complementary targets [15]. The cationic poly(fluorene-co-phenylene) polymer chain that emits blue light was functionalized with the 2,1,3-benzothiadiazole chromophore that emits green light [15]. Additional N(CH₃)₃ groups were introduced into the polymer in order to increase its water solubility [15]. In the presence of the probe, the blue colour of polymer was detected. In the presence of the non-complementary targets, green was detected due to the contraction and aggregation of the polymer that resulted in FRET from the polymer to the chromophore [15]. Further FRET occurred in the presence of complementary targets where red emission was detected. Polythiophene and related derivatives have also been used for detection of hybridization with PNA probes. Such systems change colour due to conformational change upon hybridization [45,46]. Study has shown that the polythiophene can discriminate between fully complementary, terminal and centered single base pair mismatches, two base pair mismatches and non-complementary targets [46]. These polymers were investigated in solution and it is questionable whether the same level of analytical performance can be maintained in solid-phase assays.

2 Surface Functionalization for DNA Immobilization

Substrates that are commonly used for DNA immobilization include glass, fused silica, silicon wafers, gold and nanoparticles. Quantum dots, carbon nanotubes and wires have also been used due to their unique optical and electrical properties. The choice of substrate governs the conformation of the probe DNA and the maximum density of the probe on the surface. On a
planar surface, a closed pack density can be achieved, but with an increase in steric hindrance for the incoming targets upon hybridization.

Silicon substrate are cleaned and etched to remove contaminants and oxide layers on the surface in order to expose a uniform surface for functionalization. This is often accomplished by acid and base cleaning, which is hydrochloric acid in hydrogen peroxide, followed by ammonium hydroxide in hydrogen peroxide.

Three major routes for DNA immobilization onto a substrate are by adsorption, affinity chemistry and covalent bonding. Adsorption is more suitable for immobilization of long unmodified DNA probes, where the probes are attracted to the surface through electrostatic interactions and are laid down on their sides on the surface. Affinity chemistry with thiol-gold and biotin-avidin interactions has shown success in building self-assembled monolayers (SAM). Short end-modified DNA probes can be covalently tethered to a surface. For covalent coupling, a wide selection of chemistries is available ranging from silane chemistry, silicon-carbon chemistry, to attachment through functional groups of conductive polymers and dendrimers.

The immobilization of DNA probes onto functionalized surfaces is usually performed in high salt concentration (1 M) buffer solutions. This is to maximize the number of probe molecules that are immobilized by reducing the repulsion between individual probes and with the surface through the screening of charges. The optimum salt concentration and type of buffer solution is surface dependent.
2.1 Adsorption

Adsorption is the most straight-forward and low cost method to immobilize DNA probes onto a surface. Due to the negatively charged phosphate backbone on DNA probes, they adsorbed readily onto cationic surfaces, provided by aminosilanes [47,48] or polymers such as polylysine (PLL) [49], polypyrrole (Ppy) [50], polyaniline (PANI), polyethylenimine (PEI) [51] and polyallylamine. The more contact points there are between the DNA probes and the surface, the stronger the adsorption. Therefore, it is well suited for long unmodified DNA probes.

However, there are several drawbacks to this technique. The main issue is that the orientation of the adsorbed material on the surface is random and is not structurally ordered. This results in hindered nucleobases for hybridization and lowered hybridization efficiency. There is minimal to no probe mobility, and non-specific adsorption of other charged molecules can occur during the course of experiments through competitive binding. Moreover, the probes are prone to desorb over time through repeated washings and heatings.

2.2 Thiol Gold Chemistry

Thiolated probes are immobilized directly onto gold substrates by immersing in respective probe solutions. Thiols have high affinity towards gold surfaces and are able to form densely packed SAMs. This is advantageous as it forms a stable and structurally ordered monolayer. However, the hybridization efficiency is compromised due to the high probe density that causes steric and charge hindrance for target hybridization. Thus, spacers such as mercaptohexanol (MCH) or other thiolated derivatives are often introduced to remove some of the thiolated probes on the surface by competitive displacement. The degree of displacement is controlled by the concentration of the thiolated molecule and its reaction time with the surface.
Complete displacement of thiolated probes was observed under high concentration and long exposure time of MCH [52]. Herne and Tarlov suggested that non-specific adsorption of purine and pyrimidine bases on gold is strong because extensive rinsing and heating to 75 °C was not able to remove the non-derivatized DNA from the surface [53]. Further investigation of homooligomer adsorption by Kimura-Suda and coworkers found that preferential adsorption of nucleobases on gold is in the order of A > C = G > T [54]. In a competitive adsorption study with thiolated poly-thymine and unmodified poly-adenine on gold, the resulting surface contained mainly of adenine as opposed to thymine [54]. This indicated that adenine adsorbed more readily than thiolated probes on gold. Therefore, adenine rich probes or targets can be problematic, which affect hybridization efficiency and results. Non-specific adsorption on gold can be minimized by capping the surface with MCH or hydrophobic polymers [55].

2.3 Affinity Biotin-Avidin Chemistry

Biotin-avidin chemistry has grown in popularity for probe oligonucleotide immobilization onto surfaces. Biotin is a small molecule that has strong affinity towards avidin, neutravidin and streptavidin with association constants of $10^{15}$ M$^{-1}$ [56]. Avidin is a protein found in egg-white and its deglycosylated form yields neutravidin. Streptavidin is a bacterial protein from *streptomyces avidinii* that lacks the carbohydrate component in avidin [57]. These proteins are tetrameric and have four identical subunits for biotin binding. The strong binding is held together by combined forces of hydrophobic interactions and an extensive hydrogen bonding network [58]. These interactions support wide pH and temperature ranges, and are not affected by denaturing agents and common detergents used in nucleic acid biosensor fabrication and analysis [59]. However, due to the composition of proteins, avidin is positively charged at
physiological pH and is susceptible to non-specific adsorption of negatively charged species. This is less prominent with neutravidin and streptavidin. There are two ways to utilize the biotin-avidin chemistry for probe immobilization. The surface can first be modified with the protein of choice [60] or have the proteins coupled to the biotin modified surface [61], followed by the introduction of biotinylated probes. The attachment of the proteins or biotin onto amine modified surfaces can be done through activation of carboxylic acid groups on the proteins or biotin with 1-ethyl-3(3-dimethylaminopropyl)-carboimide (EDC) and N-hydroxysuccinimide (NHS).


2.4 Covalent Bonding

Covalent attachment of DNA probes onto solid supports involves single end attachment of the probes to the surface. The probes are functionalized at the 3’ or 5’ end, where the active group is compatible with the surface in order to give monolayer coverage on the surface. This allows the probe to adopt a configuration that extends into solution from a surface, resulting in a more ordered structure compared to adsorption processes. Some examples of covalent binding include the use of two thiol groups to form a disulfide bond [62,63], an amine with a carboxylic acid to form an amide bond [64,65], an amine with aldehyde to form an imine bond [64], and epoxide ring opening by an amine [66,67]. More specialized chemistries can be used, such as
NHS activated surfaces with amine functionalized oligonucleotides [68], or cross-linkers such as p-maleimidophenyl isocyanate (PMPI) [69] and sulfosuccinimidyl-4-(N-maleimidoethyl)-cyclohexane-1-carboxylate (sulfo-SMCC) [70,71,72] on amine or hydroxyl surfaces for the immobilization of thiol modified oligonucleotides.

Covalent immobilization offers many advantages over adsorption processes in terms of orientation of the probes on the surface, and stability and reproducibility of the surface towards washing steps and changes in environment such as salt concentration and temperature. This allows a wide range of experimental conditions for analysis [64]. Moreover, the terminal attachment of probes in covalent immobilization provides the opportunity to achieve higher probe densities and allows the probes to experience greater mobility, thus improving the hybridization kinetics. Different lengths and types of linkers can be incorporated on the DNA probes and the surface to project the probes into solution. This increases the flexibility and mobility of the probes on the surface for higher hybridization efficiency and rates of target capture.

Glass and fused silica substrates are the most commonly used platforms for DNA biosensors due to their low cost, availability and optical transparency. The abundant silanol groups (-Si-OH) on these substrates can be easily functionalized with silane chemistry to provide a more reactive terminal group, such as thiol, amine or epoxide for covalent bonding.

Silicon substrates, such as silicon wafers and crystalline silicon, are also used as platforms for development of DNA biosensors. Porous silicon can be formed by electrochemical etching of silicon wafers to give a pore size between 0.5 and 1.0 μm, which can then be chemically derivatized for the immobilization of DNA probes [73]. Porous silicon is of interest due to its higher probe immobilization capacity compared to flat substrates [73,74]. Moreover,
porous silicon is luminescent and can provide signal enhancement for fluorescent and infrared detection of reactions on a surface [73]. Planar silicon wafers offer a relatively uniform surface in comparison to glass and fused silica substrates, which provides an opportunity to produce SAMs on such surfaces with a direct silicon carbon bond via silicon hydride (-Si-H). However, the silicon substrates are easily oxidized under ambient conditions. Thus, the substrates have to be cleaned and kept under nitrogen in order to maintain a silicon hydride surface for further surface functionalization.

2.4.1 Silicon-Carbon Chemistry

Crystalline silicon offers a well defined structure and a highly organized planar surface. It is possible to achieve formation of a high density and well ordered SAM on a silicon surface via covalent bonding. Silicon substrates are of great interest due to their robustness, electronic properties and applications in microelectronics. In general, silicon hydride surfaces can be modified by substituting the hydride with chloride, which can be subsequently coupled to hydroxyl terminated molecules [69], or by reacting with end group functionalized alkenes [68,71,72,75,76]. The alkenes will bind to the hydride surface through UV irradiation [68,71,72] or in the presence of a 2,2,6,6-tetramethyl-1-piperdinyloxy (TEMPO) derivative [76]. The functionalized silicon surfaces showed superior homogeneity in surface coverage in comparison to glass surfaces.

Since polyethylene glycol (PEG) is known to reduce non-specific adsorption of biomolecules, there has been significant interest in the incorporation of this material onto surfaces. Cha and coworkers grafted the Si(111) surface with high density PEG in a brush-like configuration, and demonstrated that low background signal and high sensitivity for
hybridization detection was achieved [69]. The hydride surface was first chlorinated for the attachment of one hydroxyl end of the PEG onto the surface. A heterobifunctional linker, PMPI, was then introduced. The isocyanate moiety reacted with the free hydroxyl group on the PEG whereas the maleimide group formed a covalent bond with the incoming thiol terminated oligonucleotide. It was found that the PEG film was homogenous and flat at the Ångstrom level, and the film thickness was consistent with the molecular length of PEG used in the experiment [69].

Strother and coworkers modified a Si(001) silicon hydride surface with t-butyloxycarbonyl (t-BOC) protected 10-aminodec-1-ene by using 2 hours of UV irradiation at 254 nm [71]. The t-BOC protecting group was crucial as surface corrosion was observed when the unprotected aminoalkene was introduced directly to the surface [71]. The t-BOC group was removed and a sulfo-SMCC cross-linker was attached to the free amine, with the maleimide end reserved for reaction with the incoming thiol terminated oligonucleotide. Two methods were proposed to control the amine surface density for probe oligonucleotide immobilization. One was to use a mixture of t-BOC protected aminodecene and dodecene in the UV coupling step, while the other was to selectively remove the t-BOC groups by controlling the time of the deprotection reaction [71]. A linear relationship between the density of DNA hybridization sites and mole fraction of aminodecene was observed for the former method. For the latter method, it was found that the deprotection of t-BOC occurred rapidly for up to 15 minutes in 25 % trifluoroacetic acid. The oligonucleotides that were immobilized onto the silicon surfaces exhibited high stability; after 15 cycles of use the fluorescence intensity for hybridization was ~85 % of the initial intensity value, corresponding to only ~1 % loss per cycle [71].
Submicron patterning on Si(100) was achieved by Yin and coworkers using UV light over a phase mask to produce an interference pattern of 500 nm bands with 1 μm periodicity of alkene functionalized NHS esters [68]. Amine terminated oligonucleotides were then immobilized onto the surface. The thickness of the alkene modified layer was determined to be between 8.6 to 11.2 Å, and the patterned surface density was found to be 6.7 × 10^{12} oligonucleotides/cm² [68]. Strong discrimination for SNPs was observed. This patterning technique showed potential for application to the design of bioelectronic devices.

2.4.2 Silane Chemistry

Substrates containing terminal hydroxyl groups can react with silane molecules to form a monolayer. Silanes that are commonly used consist of an active group, such as trimethoxysilane or trichlorosilane, a carbon backbone, and a functional group at the end of the carbon chain. The number of carbons on the backbone determines the distance between the DNA probe and the surface, which can serve as a linker to the surface. The methoxy and chloro groups serve as good leaving groups that facilitate the formation of covalent bonds between the silane and the surface. Polymerization between the silane molecules also occurs, forming a polymerized network layer across the surface. A wide variety of functionalized silane groups are available for surface modifications. Silanes with only one or two leaving groups bonded to the silicon atom are also available. However, the reactivity of such silanes decreases with decreasing number of leaving groups. Silane modification can be done either by coating the substrate with the desired silane and heating in an oven, or by refluxing the substrate in a solution of the desired silane in appropriate solvents.
Thiol modified surfaces can be prepared by using 3-mercaptopropyltrimethoxysilane, where the thiolated DNA probes forms a disulfide bond with the surface. One of the complications with thiol groups is that self-dimerization can occur both on the surface and between the DNA probes, which leads to deactivation. The disulfide bond is heat stable, but is susceptible to reducing agents such as dithiothreitol (DTT). Rogers and coworkers employed the sulfide exchange technique between the disulfide protected DNA probes and the thiol surface for probe immobilization at pH 9.0 in carbonated buffer, and a probe density of 3.0 x 10^{13} molecules/cm^2 was obtained with no significant non-specific adsorption [62]. Studies using ^32P-labeled disulfide DNA probes indicated that 60% and 80% of maximum attachment was achieved within 10 and 25 minutes, respectively, and no significant increase of density of probes was observed after 2 hours of immobilization [62]. Further studies revealed that only 16% of the immobilized DNA probes participated in hybridization with complementary targets, and less than 5% of the DNA probes were detached from the surface after the hybridization step [62].

Carboxylic acid modified surfaces are made from ester functionalized silanes, which can be readily hydrolyzed. The ester serves as a protecting group that is required due to the incompatibility between the carboxylic acid and the silane [65]. Other protecting groups that can be used include tert-butyl and trifluoroethyl ester groups, which can be deprotected with hydrochloric acid and iodos(trimethyl)silane, respectively [64,65,77]. Amine terminated probes can be immobilized onto the carboxylic acid surface in the presence of carbodiimide and succinimide compounds, such as di(isopropyl)carbodiimide (DICI) and NHS [77]. A carboxylic acid surface is negatively charged, which solves the problem of non-specific adsorption of negatively charged oligonucleotides. However, this can also be disadvantageous because repulsion will prohibit high density immobilization of probe oligonucleotides and will hinder hybridization. Therefore, these types of surfaces are generally not suitable for direct probe immobilization.
Amine modified surfaces are made from 3-aminopropyltrimethoxysilane (APTMS) or 3-aminopropyltriethoxysilane (APTES). Their structures are shown in Figure 1.5. These surfaces are popular because of their compatibility with various functional groups, including aldehyde and carboxylic acid groups. However, they have a great tendency towards non-specific adsorption due to strong electrostatic adsorption and hydrogen bonding that occurs between the DNA and the amine surface. Several routes for DNA immobilization onto amine modified surfaces are possible. Direct coupling with aldehyde or carboxylic acid terminated probes will result in an imine or an ester bond, respectively [64]. Imine bonds are believed to be unstable, therefore a reduction step usually follows. For the immobilization of carboxylic acid terminated probes, EDC followed by NHS or N-hydroxysulfosuccinimide (NHSS) are required to activate the carboxylic acid groups for the formation of an amide bond [64]. These reagents are usually added to the reaction mixture in catalytic amounts. The use of heterobifunctional crosslinkers, such as succimidyl 4-[maleimido-phenyl]butyrate (SMPB) and sulfo-SMCC, allows coupling of amine modified surfaces to thiol terminated probes [70]. The succinimidyl group reacts with the
surface amine whereas the maleimide moiety is selective towards thiolated probes. The maleimide groups on these cross-linkers are bulky, which is beneficial for achieving spacing effect between sites of DNA immobilization.

Aldehyde modified surfaces can be obtained by reacting amine modified surfaces with glutaraldehyde, or by reducing carboxylic acid modified surfaces to hydroxylated surfaces followed by oxidization with pyridinium chlorochromate (PCC) [64]. The aldehyde modified surface is one of the most popular platforms for direct probe oligonucleotide immobilization because it offers an electrically neutral surface, in contrast to amine and carboxylic acid modified surfaces. Aldehyde surfaces react with amine terminated probe oligonucleotides without any additional coupling agents. However, a subsequent reduction step with sodium borohydride is required in order to reduce the imine bond and deactivate the remaining aldehyde sites on the surface.

![Figure 1.6. Structure of 3-glycidoxypropyltrimetoxysilane (GOPS).](image)

Epoxide modified surfaces are formed from 3-glycidoxypropyltrimethoxysilane (GOPS), in the presence of catalytic amounts of Hünig’s base (N,N-diisopropylethylamine). The structure of GOPS is shown in Figure 1.6. Similar to aldehyde modified surfaces, epoxide modified
surfaces are electrically neutral. The epoxide ring can be opened under acidic or basic conditions. Nucleophilic amine or thiol terminated probe oligonucleotides can be coupled directly onto the surface via an epoxide ring opening reaction. No coupling reagents or subsequent reduction or oxidation steps are required. Therefore, epoxide modified surfaces are excellent for direct immobilization of probes. GOPS modified silicon and glass surfaces have been characterized by X-ray photoelectron spectroscopy (XPS), time-of-flight secondary ion mass spectroscopy (ToF-SIMS), atomic force microscopy (AFM), scanning election microscopy (SEM), and ellipsometry [66].

In comparison, amine, aldehyde and epoxide are the most popular functionalized surfaces on silicon dioxide based substrates for DNA biosensor platforms. Amine modified surfaces show high reactivity, and are advantageous for both direct coupling of DNA probes and for further functionalization. However, amine modified surfaces are sensitive to oxygen and humidity, which can lead to a more rapid loss of activity compared to other platforms [78]. Amine modified surfaces also experience high amounts of non-specific adsorption due to the positively charged surface. Therefore, aldehyde and epoxide modified surfaces are favored for direct immobilization of DNA probes due to their neutrality, which also effectively reduce non-specific adsorption.

2.4.3 Dendrimers

Dendrimers are organized three dimensional structures grown from a central molecule that yield numerous functionalized arms, such as amines or aldehydes, on the outer surface. These groups are usually fully accessible for covalent surface coupling and DNA probe immobilization. With every generation of growth, the number of functionalized arms on the
dendrimer doubles, adopting a nearly spherical shape. In comparison with planar surfaces, dendrimers offer higher probe binding capacity, greater surface stability and probe spot homogeneity. Hybridization efficiency is also higher due to the larger surface area, which provides spacing between the DNA probes for easier target access. However, these advantages are overshadowed by the synthetic challenges and high fabrication costs in comparison to the simplicity of other probe immobilization techniques.

The first dendrimer linker system for DNA biosensor was introduced by Beier and Hoheisel [79], by growing five amine arms from each amine on an APTMS coated glass surface. This surface was shown to be suitable for both in situ oligonucleotide synthesis and coupling of amine terminated DNA probes via phenyleneisothiocyanate (PDTIC) bifunctional linkers.

Figure 1.7. (a) Illustration of a 4th generation starburst polyamidoamine (PAMAM) dendrimer with 64 amine terminus. The dendrimer was coupled onto APTES modified surface through bifunctional linkers. (b) Structure of a 2nd generation PAMAM dendrimer.
Benters, Niemeyer and Wohrle explored the 4th generation polyamidoamine (PAMAM) starburst dendrimer, containing 64 amines on the outer sphere, for DNA immobilization, as illustrated in Figure 1.7 [80]. The PAMAM dendrimer was first coupled to a disuccinimidyl glutarate (DSG) or PDITC activated APTES surface, follow by activation of the dendrimer surface with DSG or PDITC for amine terminated DNA probe immobilization. The homobifunctional linkers also served to crosslink adjacent dendrimers to improve surface stability towards harsh washing conditions. The stability resulted in a hybridization signal of 80 % of the initial hybridization signal after 110 regeneration cycles [80]. Moreover, compared to GOPS and nitrocellulose-coated slides, the dendrimer surface showed the best spatial homogeneity of signal distribution [80]. A 2-fold and 8-fold increase in probe immobilization and hybridization signals was observed, respectively, compared to DSG and PDITC activated APTES surfaces [80]. Further study was done using G4-PAMAM on GOPS and PDTIC or glutaric anhydride (GA)/dicyclohexylcarbodiimide (DCC)/NHS activated APTES surfaces [81]. To reduce non-specific adsorption, the amines on the PAMAM surface were reacted with GA to give carboxylic groups before further activation with DCC/NHS for immobilization of amine terminated DNA probes. Among the dendrimer surfaces, PAMAM on GOPS gave the highest hybridization signal with a 10-fold increase compared to conventional planar and PLL surfaces [81]. In the detection of SNPs with a 212-mer PCR product, a one base pair mismatch resulted in a decrease of 60 % to 92 % in hybridization signal depending on the position of the mismatch, whereas a two base pair mismatch only showed 3 % of the perfect match signal [81].

Dendrimers with hexachlorocyclo-triphosphazene (N₃P₃Cl₆) as the core molecule have been synthesized and investigated by Le Berre and coworkers [82]. The 4th generation dendrimers have a diameter of 75 Å with 96 aldehydes on the periphery that can react with amines on the APTES surface and amine modified DNA probes to form imine bonds [82].
Although no additional linkers are required for the coupling, as with the PAMAM dendrimers, reduction of the imine bonds with sodium borohydride is needed to give a more stable bond. A detection limit of 0.1 pM was achieved with high specificity towards perfectly matched sequences and the surface was shown to be reusable at least ten times without significant loss in hybridization signal \[83\].

The immobilization of dendrimers on gold and glassy carbon electrodes have been explored using electrochemical experiments. For gold surfaces, 2-aminoethanethiol was first introduced to form a self assembled monolayer, followed by glutaraldehyde as the linker, then G4-PAMAM and reduction of the imine bonds with sodium borohydride \[84\]. The immobilization of amine terminated DNA probes was done through glutaraldehyde with subsequent reduction. Another method is based on use of mercaptoacetic acid as a monolayer on gold, followed by covalent binding of G4-PAMAM in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC) \[85\]. Unmodified DNA probes were then coupled onto the dendrimer surface with EDAC to form phosphoramidate bonds between the amine on the dendrimer and phosphates on the terminal of DNA probes.

2.5 Linkers

There are two kinds of linkers discussed, the linear linkers that are attached to the modified terminus of DNA probes, and the bifunctional linkers that activate the functionalized surface for specific binding of end-modified probes. The primary goal of using a linker is to project the DNA probe from the surface into solution to increase probe mobility and to reduce steric problems associated with target capture. Thus, the nature and length of the linker can affect the local environment on the surface and influence the hybridization rate and efficiency.
Linear alkyl chains (-CH$_2$)$_n$ and polyethers (-OCH$_2$CH$_2$)$_n$ are the most popular linkers for end modification of DNA probes. These represent the hydrophobic and hydrophilic version of a linker, respectively. The main difference between the two is the degree of hydration in aqueous environments at the location where hybridization occurs. The alkyl chains are rarely hydrated and tend to aggregate [86], which limits the freedom for probe mobility. Polyethers hydrate well by forming hydrogen bonds in coordination with the water molecules forming a continuous solvated phase from the surface to the DNA probes, which offers great flexibility to the probes [87]. Moreover, polyethers are superior in reducing non-specific adsorption of DNA [87] and protein [86] that often interfere with experiments. Commercially available customized end-modified oligonucleotides come with an option of a C$_6$ or C$_{12}$ alkyl linker. Polyethylene glycol linkers are incorporated online with the DNA synthesizer through phosphoramidite chemistry, or offline by modifying one terminus to amine or other functional group that is compatible for coupling.

Zhang and coworkers introduced the importance of a linker by investigating the hybridization efficiency of covalently immobilized amine terminated oligonucleotides via carbodiimide mediated condensation with carboxylic groups on nylon membranes [88]. Zero to three repeating units of hexaethylene glycol phosphates with a C$_6$ amine linker were examined and a 4 fold increase in hybridization efficiency was observed in the presence of hexaethylene glycol linker [88]. This was supported by Shchepinov and coworkers, where they studied the linker lengths of different repeating units of propanediol, diethylene glycol and triethylene glycol linkers that were covalently coupled onto amine functionalized polypropylene using phosphoramidite chemistry [89]. It was found that the optimal length for all three linkers was 8 to 10 repeating units, giving 45-60 atoms, 60-75 atoms and 70-90 atoms in length respectively for each linker [89]. Hybridization efficiency of 50 fold increase was observed for 8 repeating
units of propanediols and 150 fold increase was achieved with 8 repeating units of triethylene glycols [89]. Extensive increase in linker length beyond 10-12 repeating units was found to reduce hybridization efficiency, where with 25-30 repeating units, it was equivalent to no linker being present [89]. Peeters and coworkers studied 25-mer DNA probes on gold using six thiolated linkers, alkyl C_6, C_{11} and their derivatives with additional triethylene glycol and hexaethylene glycol units connected through phosphoramidite chemistry [90]. It was found that the C_{11} derivatives yielded higher probe density on the surface as compared to their C_6 counterparts and achieved better sensitivity and lower detection limit [90]. However, lower hybridization efficiency was observed due to increasing steric hindrance with higher probe density [90]. Among the C_{11} derivatives, the pure alkyl C_{11} gave the highest sensitivity and lowest detection limit at 0.3 ± 0.2 nM [90].

Non-probe oligonucleotides have also been used as linkers. Guo and coworkers employed thymines as linkers for amine C_6 terminated DNA probes on phenylisothiocyanate activated glass surfaces and found that improved hybridization signal was achieved with six or more thymines [91]. Signal was enhanced by increasing the length of thymines to dT_{15}. In recent studies, Nonglaton and coworkers showed that among the four homopolymers, dA_{11}, dC_{11}, dT_{11} and dG_{11}, studied as a linker on zirconium phosphonate modified surfaces, only the dG_{11} linker surface gave enhanced hybridization signal compared to no linkers [92]. Further investigation by Lane and coworkers with XPS indicated that the surface coverage for the dG linker surface was 5.0 × 10^{11} ± 0.9 × 10^{11} ssDNA molecules/cm^2, which was twice as large as that for the dA linker surface after stringent washing and heating [93]. This is due to the added stability of the guanines being able to form quadruplex, and the phosphate groups attracting the DNA probes towards the zirconium surface [93].
Homo- and hetero-bifunctional cross linkers serve to covalently couple the end modified DNA to the surface when the functional groups on the DNA and surface are not compatible directly. The active groups on these linkers are highly reactive and are specific towards certain functional groups within a certain pH range. These linkers are stored at low temperatures and preferably in an inert environment. Common amine specific reactive groups are isothiocynates, succimidyls and imidates. For thiols, the groups are maleidos and pyridyldithiols, and for carbonyls, the groups are amines in the presence of EDC. Nitrophenyl azide, hydroxyphenyl azide and phenyl azide groups are also amine specific but require UV photolysis. Each of these groups is removed at different UV wavelengths. The chain between the two reactive groups varies from straight alkanes to cyclohexane and benzene ring. The selection of chemistry determines the solubility of the linker in organic or aqueous solvents, whether the linker is cleavable, and the flexibility of the linker.

Homobifunctional cross linkers are easier to synthesize as compared to heterobifunctional cross linkers in terms of process and purification. Some examples of homobifunctional linkers are 1,4-phenylenediisothiocyanate (PDTIC) [67,80,91,94], and disuccinimidyl glutarate (DSG) [80]. Examples of heterobifunctional linkers include succimidyl-4-[maleimido-phenyl]butyrate (SMPB) [70,95], sulfo-SMCC [71,96,97], p-maleimidophenyl isocyanate (PMPI) [69,98] and sulfosuccinimidyl 6-maleimidylhexanoate (sulfo-EMCS) [99]. The structures of these heterobifunctional linkers are shown in Figure 1.8. Most of the bifunctional linkers are soluble in organic solvents, and the sulfonated derivatives are soluble in water. The procedure for bifunctional linker coupling is to first introduce the linker at high concentration to the functionalized surface followed by addition of the end-modified probes to the surface. In this case, homobifunctional linkers have a higher probability to react both active ends with the surface, resulting in capping of the surface. This can be prevented by choosing a rigid short chain
homobifunctional linker, so that the linker will be forced away from the surface due to steric effects. To eliminate the problem completely, heterobifunctional linkers are the better choice. Due to the high reactivity of the bifunctional linkers, DNA immobilization should follow immediately.

![Figure 1.8: Structures of heterobifunctional linkers, sulfo-SMCC, PMPI, sulfo-EMCS and SMPB.](image)

2.6 Spacers

Spacer molecules serve to control probe orientation and density on the surface by increasing the distance between adjacent probes, and to prevent non-specific interactions between the probe or target molecules with the surface. Short mercapto-alkanes, -alcohols and -ethylene glycols are commonly used as spacers on gold surfaces. These are introduced to the surface either by co-immobilization from solution along with thiolated probes or by displacement of the self assembled thiolated probe surface with mercapto spacers. The degree of attachment of the spacer molecules to the surface is governed by the reaction time and concentration of the
spacers introduced to the surface. Large molecules such as avidin and dendrimers can also be employed as spacer molecules for control of distances and coverage over the surface. The distance between the functional arms on the dendrimers is controlled by the generations of growth.

Small conical molecules with three functional base arms for surface attachments have been introduced as dendron spacers on gold [100,101] and glass surfaces [102]. The structure of these spacers provides uniform and controlled spacing in contrast to single contact point spacers. Moreover, the multiple attachments onto the surface result in greater stability. A rigid thiolated tripodal derivative with an alkyne between two benzene rings and a carboxylic acid terminal for probe oligonucleotide immobilization was used on gold surfaces [101]. It provided a more hydrophobic surface in comparison to its single contact derivative due to the additional benzene ring on each arm, which is beneficial for reducing non-specific adsorption. A more flexible second generation three armed dendron with six carboxylic acid groups at the terminal of each arm for silicon based substrates was introduced by Hong and coworkers [102]. The dendron surface was fabricated from an epoxide modified surface, followed by treatment with ethylene glycol, which resulted in a hydroxyl surface for the coupling of the carboxylic acids on the dendrons. A deprotection step of the anthracene moiety on the dendrons with 1.0 M trifluoroacetic acid yielded an amine terminated surface for probe immobilization [102]. An average separation of 3.2 nm between the amine groups was reported [102]. This separation allowed target molecules to form natural helices [100]. It was found that this method was superior to direct coupling of the dendron onto amine modified surfaces [102]. Ethylene glycol served as a linker and reduced non-specific adsorptions [102].
2.7 Surface Capping

Surface capping is used to reduce background noise and non-specific adsorption by blocking unreacted sites and changing the surface reactivity. Common capping agents used after probe immobilization include bovine serum albumin (BSA) and Denhart’s solution. BSA was found to be effective on amine modified surfaces in reducing non-specific adsorption [103]. Polymers with hydrophobic backbones can be added to prevent adsorption of hydrophilic DNA probes or targets to the surface. Polyacrylic acid was added to cap free amines after the coupling of NHS terminated DNA probes to gold that was coated with thiocytic acid modified polyallylamine [104]. The negative charge on polyacrylic acid formed ionic complexes with the amines on the surface and also served to repel probe and target non-specific adsorption [104]. The effect of electrostatic charges decreased with increasing ionic strength [104]. Opdahl and coworkers introduced dA blocks in the capping of gold surfaces due to the high intrinsic affinity of adenine for gold surfaces [105]. It was found that the surface density of dA was close to saturation and was nearly independent of the length of the dA [105]. Capping of organosilane modified surfaces before probe immobilizations can be achieved by introducing additional alkylsilanes as they can incorporate into the siloxane network. About 30% of the silanol groups on the surface remain unreacted after organosilane functionalization, which can cause non-specific interactions with amino groups on amine modified surfaces [106]. This reduces the number of reactive amines for probe immobilization. Capping with butyltrimethoxysilane following aminosilanization was found to be effective in liberating the amino groups from the surface and it was suggested that the hydrophobic aliphatic chain provided steric hindrance, blocking access of the amino groups to the silanols [106,107].
3 Matrix Isolation DNA Biosensor Platform

A “matrix isolation” DNA biosensor platform has been engineered so that the probe oligonucleotides are on average surrounded by inert oligomers on a surface, as depicted in Figure 1.9. The oligomers contained repeating units of non-probe molecules, such as hydrocarbons or phosphates. This is intended to control the environment of the probes by reducing interactions between the probes and between the probes and the surface.

Figure 1.9. Illustration of a ‘matrix isolation’ DNA biosensor platform, where probe oligonucleotides are on average surrounded by non-probe oligomers.

In conventional DNA biosensors, terminal modified DNA probes modified at one end are typically immobilized to achieve high density on a surface. The probes are expected to be in an upright configuration perpendicular to the surface based on repulsion of the negatively charged phosphate backbones. However, the hybridization efficiency is compromised due to steric hindrance and charge effects. At high probe density, there is not enough space between the probes to accommodate the targets for hybridization and the local charge density increases with
subsequent hybridizations [11]. Moreover, since the probes are in close proximity to one another, the hybridization of one probe decreases the chances of hybridization for the adjacent probe through nearest neighbour effects. The consequence is that most of the probes on the surface do not participate in target hybridization. Oligomers can be introduced to dilute the density of probes on the surface for control of steric and charge effects, while maintaining the upright configuration of probes in an orientation and with a mobility that is suitable for hybridization.

The environment of the probes can be controlled by the selection of the oligomer, being neutral or negatively charged, straight chain or branched, rigid or flexible and long or short. Neutral oligomers such as polyethylene glycols and their derivatives are favourable due to flexibility in aqueous solutions and amelioration of non-specific adsorption. Negatively charged oligomers provide an electrostatic environment that mimics conventional DNA biosensor surfaces, and maintains the repulsion that can orient probes in an upright configuration. Negatively charged oligomers can be designed to have permanent or partial charges. The permanently charged oligomers are made up of phosphate or sulfuric groups, whereas the partially charged oligomers are composed of carboxylic acid groups [108]. The degree of deprotonation on the partially charged oligomers is dependent on the $K_a$ of the acidic groups. Hence, the charges on the partially charged oligomers can be tuned by the pH of solution [108]. The degree of intrachain interactions depends on the structure and groups present on the oligomer. Branched oligomers have greater potential for intrachain interactions in comparison to straight chain oligomers. The stiffness of the oligomer is determined by its backbone [108]. Oligomers with alkyl backbones are more flexible than those with benzenes, alkenes or alkynes. The lengths of the oligomers are important because if the length is too long, the probes will be overshadowed on the surface and target hybridization will be hindered. If the length is too short, then the oligomer becomes equivalent to a spacer.
Probe-to-probe interactions affect the selectivity of the surface in terms of discrimination between fully complementary and mismatch targets. This effect was observed in broad denaturation transitions of hybridized duplexes on surfaces [109]. Having oligomers placed around the probes can reduce or eliminate probe-to-probe interactions [110], thus the length of the oligomers should be close to the length of the probes. This would ensure hybridization of each probe occurs independently of others, and should result in sharper denaturation transitions [109]. The melting temperatures of hybridized duplexes can be lowered and the difference in melting temperature between fully complementary and mismatched targets can widen in comparison to conventional DNA biosensors [109,110]. Thus, the surface selectivity is improved.

3.1 Methods of Building Mixed Film Platforms

In order to build a ‘matrix isolation’ platform, the surface has to be functionalized to contain at least two different active functional groups, one specifically for probe immobilization and the other for oligomer attachment. To achieve probe isolation, the functional group density for oligomer immobilization must be significantly greater than that for the probe. Protecting groups are required on one or both of the functional groups to prevent reaction during the multistep fabrication process on the surface, and to provide for surface templating. The surface can be built up from a mixture of desired compounds that compete for sites or from polymers with multiple functional groups. Factors that need to be considered in surface fabrication include control of the functional group ratios on the surface, the choice of functional groups for subsequent attachments, the protecting groups, and the reaction conditions. The following is a survey of methods that could potentially be modified to make a ‘matrix isolation’ platform.
3.1.1 Protecting Groups and Photochemistry

Protecting groups have been used in multistep synthesis and in building of surfaces that contained multiple functional groups such as amine, thiol, hydroxyl and carboxylic acids. For example, wide selection of protecting groups have been introduced and used on amino acids in peptide chemistry. A recent review about such protecting groups and the deprotection conditions are summarized by Isidro-Llobet and coworkers [111]. The choice of protecting groups depends on the coupling efficiency, purification process, stability to heat and light, and reaction conditions. Deprotection steps should be taken into account to ensure that such processes do not affect or remove other groups that are present on the surface. Acid sensitive groups such as trityl, tert-butyloxycarbonyl (Boc) and benzyloxycarbonyl (Z), base sensitive 9-fluorenylmethoxycarbonyl (Fmoc), and photolabile 6-nitroveratyloxycarbonyl (NVOC) and 2-(2-nitrophenyl)propyloxycarbonyl (NPPOC) groups are often used in protecting amino and hydroxyl groups. Fmoc is stable to acidic conditions and photolysis, thus it can be used in conjunction with the protecting groups stated above. Trityl groups are removed under mild acidic conditions with trichloroacetic acid (TCA) or trifluoroacetic acid (TFA), whereas Boc and Z groups require harsher conditions. Both NVOC and NPPOC [112] groups are stable in basic and acidic conditions. Photolabile protecting groups offer additional advantage for surface patterning and selective deprotection for site specific coupling. The structures of some of the protecting groups used in DNA synthesis for biosensors development are shown in Figure 1.10.
NVOC is a popular nitrobenzyl photolabile protecting group due to its ease in coupling and stability at typical room lighting conditions, showing only a slow deprotection rate \cite{113}. A first order deprotection reaction with rate constant of 0.11 min$^{-1}$ was reported from the study of photodeprotection of NVOC protected hydroquinone using a 365 nm UV lamp \cite{114}.

Deprotection of NVOC can be monitored by the decreasing absorption maxima at 350 nm with time \cite{113}. NVOC have been used in surface patterning for protein immobilization \cite{115, 116} and solid phase synthesis of PNA chips \cite{117}.

Figure 1.10. Structures of photolabile protecting groups (a) NVOC, (b) NPPOC, (c) DMB, and acid sensitive (d) DMT for amino and hydroxyl groups.
NPPOC [112] and 3’,5’-dimethoxybenzoin (DMB) [110] have been used with acid sensitive 4,4-dimethoxytrityl (DMT) groups in solid phase synthesis of oligonucleotides. The removal of DMT under acidic conditions resulted in an orange coloured trityl cation, which allowed confirmation and monitoring of the deprotection reaction [118]. NPPOC and DMB were removed by irradiation with 365 nm mercury lamp. Chen and coworkers showed that NPPOC deprotection on surfaces could be controlled by irradiation dosage and time to regulate oligonucleotide surface density [112]. The investigated surface was first capped with NPPOC protected phosphoramidite nucleobases, followed by partial deprotection with irradiation where the hydroxyl sites were subsequently capped with DMT-dT phosphoramidite [112]. The density of surface hydroxyl groups was found to be controlled by varying exposure time up to 40 seconds (4.0 J/cm\(^2\)) with a 350 W mercury arc lamp since the NPPOC deprotection reaction was a first order reaction with a half-life of 7.6 seconds and rate constant of 0.091 s\(^{-1}\) [112]. Oligonucleotide sequences were synthesized using NPPOC protected phosphoramidite nucleobases following complete deprotection of the remaining NPPOC sites on the surface with 50 seconds exposure time [112]. The DMT sites remained protected since they were not affected by photolysis. Deprotection of DMT after the completion of oligonucleotide synthesis with 3 % dichloroacetic acid provided free hydroxyl groups on the surface [112] that could be further derivatized for mixed film fabrication.

DMB is more photosensitive than NPPOC and undergoes a photoinitiated cyclization reaction with a cleavage rate constant of greater than 10\(^{10}\) s\(^{-1}\) [119]. Due to its photosensitivity, the synthesis of DMB protected molecules must be done in darkness. Piunno and coworkers introduced a mixed film surface of charged ethylene glycol oligomers synthesized by phosphoramidite chemistry using DMB in a solid phase DNA synthesizer [110]. The surface was built by protecting one end of hexaethylene glycol with DMB, and coupling the free end onto a
GOPS modified surface. DMB was selectively deprotected with irradiation, followed by coupling of DMT protected ethylene glycol phosphoramidite. The stepwise synthesis of the ethylene glycol phosphate oligomer was achieved by removal of DMT with 2% dichloroacetic acid. Oligonucleotides were then synthesized using DMT protected phosphoramidite nucleobases by coupling onto the remaining DMB sites on the surface after irradiation.

3.1.2 Mixture of Compounds

The most direct method of fabricating a mixed film on a surface is by competitive immobilization of two compounds from a solution. The immobilization efficiency of each compound is dependent on its size, length and the functional groups it contains. Co-immobilization has been used in thiol-gold and silane chemistry to orient the probes, dilute the probe density and reduce non-specific adsorption on the surface. The ratio of the immobilized probes and oligomers is controlled by the ratios of the molecules in the immobilization solution.

Mixed films on gold have been well studied and characterized. There are three major ways to make a mixed film on gold: competitive desorption of probes with thiolated oligomers [120]; co-immobilization of probes and oligomers from a mixture solution [121,122,123]; and selective desorption [124] or exchange [125] of oligomers on the surface with probes. Competitive desorption involves having a high probe density SAM on the surface followed by immersion in thiolated oligomer solution for probe desorption. The degree of desorption is controlled by the concentration and reaction time of the thiolated oligomers. However, this only works well with oligomers that are much shorter in comparison with the probes because there is less steric hindrance in accessing the surface. For co-immobilization, the immobilized probes and oligomers on the surface are controlled by the ratio between the two in the immobilization
solution. Protecting groups can be used on either one or both of the co-immobilized materials to give added advantages for coupling of different oligomers and varying the probe density. Lee and coworkers introduced a maleimide-ethylene glycol disulfide compound, 

\[ \text{HO(CH}_2\text{CH}_2\text{O)}_4\text{(CH}_2)_{11}\text{SS-(CH)}_{11}\text{(OCH}_2\text{CH}_2)_6\text{OCH}_2\text{CONH(CH}_2)_2\text{C}_4\text{H}_2\text{NO}_2] \]

for building a mixed film on gold [126]. The disulfide bond was broken upon immobilization onto gold, giving equal amounts of the two components on the surface. The 19-mer thiolated probe oligonucleotides were directed to the maleimide sites, thus the probe density could be controlled by the concentration of the probes and the ionic strength of the buffer solution [126]. Unreacted maleimide sites were capped with MCH.

Selective desorption or exchange of oligomers from the surface with probe oligonucleotides is more specific and provides more control over the surface structure in comparison to competitive desorption and co-immobilization methods. However, side reactions and cross linking on the surface can occur. Satjapipat and coworkers investigated the selective desorption of mercaptopropionic acid (MPA) in MPA/hexanethiol and MPA/MCH SAM on gold with electrochemistry [124]. A stripping potential between the reduction potential of the shorter and the longer alkanethiol was used to selectively desorb the shorter alkanethiol [124]. In this study, MPA was selectively desorbed from the surface in an alkaline solution to yield bare gold sites for the immobilization of 17-mer thiolated probe oligonucleotides. The size of the sites was controlled by the ratio of MPA and respective alkanethiols. It was found that higher probe density was achieved with smaller sites because the alkanethiols oriented the probes to extend into solution, whereas in large domains, non-specific interactions between the probes and surface increased and resulted in lower probe density [124]. This technique was used because the diffusion of alkanethiols from a densely pack region to a bare gold region was reported to be 96 hours or longer and the attachment of thiolated probes to densely cover the bare gold sites only
required 4 hours [124]. Therefore, the desorbed site remained available for probe immobilization as long as it was done immediately following the desorption step.

An irradiation-promoted exchange reaction (IPER) was used to fabricate a surface composed of oligo(ethylene glycol) substituted alkanethiols and biotinylated alkanethiols on gold [125]. The surface was first immobilized with oligo(ethylene glycol) substituted alkanethiols, followed by partial removal by IPER for the immobilization of biotinylated alkanethiols. The degree of removal depended on the irradiation dosage and the length of the oligomers. It was reported that irradiation strength higher than 1 mC/cm$^2$ caused extensive cross-linking and hindered the exchange [127]. Two different constructs using 3 and 7 repeating units of ethylene glycol oligomer were investigated. It was found that the longer molecules had stronger intermolecular interactions in the SAM and were more difficult to exchange, whereas the shorter molecules showed higher exchange efficiency with IPER [125]. Without IPER treatment, a 6 % exchange for the biotinylated alkanethiol was observed on the shorter oligomer surface as opposed to no exchange on the longer oligomer surface. With 0.5 mC/cm$^2$ and 0.75 mC/cm$^2$ irradiation dosage, 21 % and 31 % exchange for the biotinylated alkanethiol on the shorter oligomer surfaces was achieved respectively, but only 3.5 % and 10.5 % exchange was observed for the longer oligomer surfaces that were subjected to the same irradiation dosages [125].

In silane chemistry, mixed films have been achieved by competition of two different silanes on a surface. The compositions of the silanes on the surface were controlled by the reactivity, chain length, size, and concentration of the silanes. Deposition could be done simultaneously in a one step method, or by sequential two step deposition method. In the one step method, a mixture of the desired silanes was coupled simultaneously onto the surface from the mixture solution. In the two step method, one silane was first coupled onto the surface,
followed by deposition of the second silane. The former method was suitable for silanes of similar size and chemical properties to control the preferential adsorption of one silane over the other. The relationship between the surface concentration and initial solution concentration ratio still was not absolutely predictable [128]. In order to make a mixed film for probes and oligomer immobilization, the active functional group on one of the silanes has to be protected in order to prevent them from reacting with each other. A mixed film of 3-aminopropyltrimethoxysilane (APTMS) and its benzaldehyde protected derivative was silanized onto a glass surface [129]. The benzaldehyde derivative was synthesized using an equimolar ratio of benzaldehyde and APTMS in toluene, and refluxed under argon for 24 hours [129]. The product was used in subsequent silanization along with APTMS onto the surface without purification because the fundamental reactivity of both silanes was the same. If the protected silane was made from a different silane, not the same one mixed in for silanization, then purification would be required.

The two step method involved the deposition of a second silane into the intentional defect sites made in the first silane on the surface [128]. This allowed control over the size of the defect sites by varying the time of reaction, and different combinations of silanes could be exploited. Choi and coworkers investigated a mixed film from an alkylsilane, CH$_3$(CH$_2$)$_{17}$Si(OCH$_3$)$_3$, with APTMS on hydroxylated silicon using this method [128]. A short reaction time of APTMS followed by long deposition time for the alkylsilane showed aggregated APTMS islands that were densely surrounded by the alkylsilane [128]. However, long reaction time with the alkylsilane followed by short deposition time for APTMS resulted in APTMS filling the small areas not occupied by the alkylsilane [128]. Thus, the deposition of the second silane depends on the surface morphology of the predeposited silane [128]. By controlling the reaction time and the order of the silane deposition, a structurally controlled surface of silanes with different chain lengths and chemical functionalities can be obtained.
3.1.3 Polymers

Polymers with two or three functional groups have been introduced for biomolecule attachment onto surfaces. Such polymers are designed to contain at least one functional group for surface coupling and an active functional group for immobilization of biomolecules. A third component is usually introduced as a spacer, or as a site for surface capping. The polymer backbone adjacent to and buried under the surface is often hydrophobic, which provides an advantage for reducing non-specific adsorption. There are two routes to couple probe oligonucleotides onto polymers: by grafting directly onto the polymer; or by synthesizing a polymer with multiple functional groups for post-site specific immobilization. Amine and carboxylic acid active surfaces have been made and studied since they are two of the most versatile functional groups for further functionalization and their charge can be tuned by adjustment of the pH of solution. The polymer content depends on the reactivity and concentration of the monomers in the synthesis. The monomer ratios in the resulting polymers are important as they govern the sites available for subsequent attachments. The challenges in synthesis include the choice of monomers, compatibility of the monomers with each other, efficient purification techniques and, solubility and stability of the polymer.

Taira and Yokoyama introduced DNA-conjugated polymers for immobilization of gold. An amine DNA-conjugated polymer, shown in Figure 1.11(a) was made by first reacting 10:1 polyallylamine hydrochloride and thiocytic acid in EDC/NHS, followed by coupling of NHS activated amine 30-mer ssDNA [130]. The ratio of ssDNA and thiocytic acid to the amines on the surface was found to be 1/424 and 1/42, respectively [131]. A carboxylic acid DNA-conjugated polymer, shown in Figure 1.11(b), was synthesized by reacting polyacrylic acid with NHS activated amine 20-mer DNA, followed by coupling with 3-(2-pyridyldithiol)propionyl hydrazide [132]. The ratio of ssDNA and the dithiol to the carboxylic acid groups was found to
be 1/1500 and 1/93, respectively [131]. Both polymers were assembled onto gold through the thiol groups. Comparison between the two polymers on surfaces towards target hybridization showed less non-specific adsorption on the carboxylic acid DNA-conjugated polymer due to electrostatic repulsion [130]. The advantage of these polymers is that the amino or carboxylic groups are available for coupling to other oligomers.

![Figure 1.11](image)

**Figure 1.11.** Structures of (a) amine DNA-conjugated polymer and (b) carboxylic acid DNA-conjugated polymer introduced by Taira and Yokoyama [131] for probe immobilization on gold surfaces.

Terpolymers are polymers with three functional groups. A photolabile amine protected terpolymer was introduced by Braun and coworkers for covalent coupling onto glass substrates [113]. The terpolymer, shown in Figure 1.12(a), was synthesized using equimolar of NVOC protected N-(3-aminopropyl)methacrylamide, methyl methacrylate (MMA) and 3-(trimethoxysilyl)propylmethacrylate (TMSPA) in the presence of the free radical initiator, 2,2’-azoisobutyronitrile (AIBN). The synthesized terpolymer was found to contain 18 mol-% of
NVOC protected amine, 46 mol-% MMA and 36 mol-% TMSPA [113]. The silyl group served as the anchoring group to the glass surface, whereas the MMA acted as a spacer. The terpolymer was spin coated onto the glass surface, followed by baking. A smooth film of 30 nm in thickness with less than 1 nm surface roughness was obtained [133]. Deprotection of NVOC with a long wavelength UV lamp gave primary amines, which was the surface active group. By changing the solution pH, the positive charge density on the surface could be tuned. At lower solution pH, higher positive surface charge density was achieved for adsorption of negatively charged materials [133]. As shown in Figure 1.12(b), a similar photolabile carboxylic acid protected terpolymer with p-methoxyphenacyl protected methacrylic acid, MMA and TMSPA was introduced by Millaruelo and coworkers [134]. The terpolymer was synthesized and assembled onto glass surfaces using the same methods as just described. The terpolymer composition with equimolar feed was 32 mol-% protected methacrylic acid, 39 mol-% MMA and 29 mol-% TMSPA [134]. The film thickness was found to be less than 20 nm with surface roughness of 5.4 nm [135]. From the composition of both polymers, it was determined that the incorporation of monomers into the terpolymer depended on size and reactivity. Since all the monomers were methacrylate derivatives, their reactivity was similar. Thus, their size was the governing factor, relating to mobility in solution. Monomers larger in size had lower incorporation in the polymer because the mobility was less compared to smaller monomers. NVOC protected amine was largest in size, followed by TMSPA and MAA, resulting in increasing mol-% in the polymer. As for the carboxylic acid DNA-conjugated polymer, the MMA was smaller than the other two monomers, resulting in higher incorporation ratio.
A terpolymer with dimethyl-11-methacryloyloxyundecyl phosphonates, poly(ethylene glycol)methyl ether methacrylate and n-butyl methacrylate was synthesized by free radical chain transfer polymerization with 1-dodecanthiol in the presence of benzoyl peroxide. The poly(ethylene glycol) monomer had a molecular weight of 2000 g/mol, containing 44 repeating units of ethylene glycol [136]. The resulting terpolymer contained a ratio of 1:1:8 for the phosphonate, poly(ethylene glycol) and n-butyl methacrylate monomers with input molar feed of 1:1:7, respectively [136]. The degree of polymerization was reported as 30 monomers [136]. The terpolymer was used as an antifouling coating on titanium oxide surfaces due to the poly(ethylene glycol) brushes situated at the outermost layer of the film. The terpolymer was coupled onto the surface through the phosphonate groups.
3.2 Methods for Synthesizing Oligomers for Matrix Isolation

Oligomers can be introduced to the surface by “grafting from” or “grafting to” techniques. The former involves growing oligomers from the surface by stepwise solid phase synthesis or surface initiated polymerization reactions, whereas the latter utilizes pre-synthesized end group functionalized oligomers for specific attachment of termini onto the surface. Grafting from techniques yield high surface density oligomers because the monomer molecules can diffuse easily to the growing chains without hindrance by the already grafted chains on the surface [137,138]. However, the molecular weight and chain length distributions formed in grafting from techniques cannot always be accurately controlled and measured [138]. This can be overcome by grafting to methods because the ex-situ synthesized oligomers can be purified and characterized prior to introduction to the surface. Although suffering from concentration gradient and diffusion barrier of the oligomers to the surface, well characterized chain length oligomers are assembled [138]. Thus, grafting from techniques yield films with thickness from a few nanometers to greater than a micrometer, whereas grafting to methods are more suitable for films in the 1-10 nm range [139]. The main challenge in oligomer synthesis in this thesis is to control the length so that the oligomers do not overshadow the probe oligonucleotides on the surface. The probe oligonucleotides of interest are usually of approximately 20-mer length, which corresponds to approximately 70 Å in length.

The order of immobilization of probe oligonucleotides being before or after the immobilization of oligomer on the surface should be considered. In grafting from techniques, immobilization of probes may be hindered after the oligomer synthesis due to high grafting density of the oligomers on the surface. However, immobilization of probes prior to oligomer synthesis poses issues regarding probe stability to the radicals and polymerization conditions.
These issues are less prominent in grafting to methods due to the lower surface density on the mixed film surface with site specific reactive groups for immobilization.

3.2.1 Grafting From the Surface

Grafting from the surface with controlled oligomer length can be achieved by solid phase synthesis or surface initiated polymerization. Solid phase synthesis involves coupling of the monomer stepwise onto the surface, where ideally the number of cycles determines the number of repeating units and the length of the oligomer. The monomers consist of two reactive groups, with one of them protected. This is to ensure that the monomers are coupled onto the surface one per cycle. Thus, the grafting process is dependent on the efficiency of removal of the protecting group and the accessibility of monomers to the binding sites. This technique is widely used in oligonucleotide [110,117] and peptide synthesis [140]. A mixed film of 20:1 mole ratio of ethylene glycol phosphate oligomer and oligonucleotides was fabricated using this technique [110]. Since it is a stepwise synthesis technique, it offers the opportunity of incorporation of different monomers into the oligomer synthesis. With the use of multiple protecting groups, different oligomers can be grafted from the surface.

Surface initiated polymerization requires the immobilization of the initiator onto the surface where the polymerization reaction propagates, or immobilization of a dormant species for nitroxide mediated processes (NMP) and atom transfer radical polymerization (ATRP) [137]. The immobilization of initiators onto surfaces involves multistep synthesis to modify the initiators for covalent attachment to the surface. Asymmetrical azo initiators and related derivatives have been synthesized with a chlorosilane head group for covalent attachment onto surfaces [141]. Polymerization was thermally initiated, producing two initiation sites per
initiator; one attached to the surface and the other in solution [141]. Polymerization of the monomers occurred on both initiation sites. The density of the surface bound polymer was observed to be dependent on the silane concentration, which corresponded to the number of initiator sites [142]. The polymer length could be controlled by the monomer concentration, polymerization time and temperature [142]. Polystyrenes [141] and polymethacrylic acids [143] have been synthesized and studied using this method. Photoinitiated polymerization can be achieved with derivatives of AIBN, peroxides and benzoin ethers attached to the surface, or with photosensitizers in bulk solutions to abstract hydrogen atoms from the substrate, which initiates the reaction [139]. In comparison, photoinitation is faster than thermal initiation and can accommodate thermally unstable functional groups or monomers since photoinitiation can be activated at room temperature [139].

Both NMP and ATRP are controlled living polymerization reactions that give products with predetermined molecular weights and low polydispersity [144]. However, these methods are specific for certain groups of monomers and rate of polymerization, thus the length of the oligomer is controlled by a set of reaction conditions. NMP utilizes a reversible homolytic cleavage of a weak covalent bond that results in a propagating radical and a stable nitroxide free radical [144]. NMP is suitable for the synthesis of linear homo- and co-polymers of styrene based monomers. TEMPO was used as the mediator due to its ability to bond reversibly to the chain ends of polystyryls at temperatures greater than 120 °C [144]. New nitroxides have been introduced to incorporate dienes and acrylates into copolymers, but methacrylates are still problematic [144].

ATRP offers a more versatile strategy to synthesize oligomers of controlled length by controlling the polymerization time since a linear relationship exists between the length and
polymerization time [129,137]. These relationships are specific for each combination of monomer, transition metal ligand complex, reaction temperature and solvent used in the polymerization reaction. ATRP is controlled by a reversible redox reaction between alkyl halides and transition metal complexes [144]. The alkyl halides serve as the dormant species that is immobilized on the surface. Cu(I) complexes abstract the halogen atom from the dormant species, resulting in a radical for attachment for alkene monomers and subsequently propagating the radical to the end of the newly formed monomer [144]. Cu(I) is oxidized in the process. The radical then abstracts the halogen from Cu(II) to revert back to the dormant species with Cu(I) regenerated [144]. ATRP relies on fast initiation and fast deactivation by the higher oxidation state metal complex [144]. Capping agents such as CuBr₂ are added to control the surface initiated living radical polymerization under low overall concentration of the dormant species [137]. ATRP can accommodate monomers through the tuning of the transition metal and ligand of the catalyst system [144]. ATRP catalyst systems can tolerate several different types of impurities, but monomers with acid groups may poison the catalyst and result in no polymerization [144]. Thus, protecting groups are required when acid groups are present.

ATRP was used in synthesizing polymerized 2-hydroxyethyl methacrylate (PHEMA) oligomers from APTMS surfaces to build a mixed film of PHEMA oligomers and probe oligonucleotides [129]. It was found that a linear growth rate of 17.2 nm/hr and 17.8 nm/hr was obtained from benzaldehyde capped aminosilanes as opposed to 12.8 nm/hr and 20.2 nm/hr for APTMS films synthesized from mole ratios of 1 : 0.3 : 0.29 for CuCl : CuBr₂ : 2,2’-dipyridyl in equal volumes of 20 mL of monomer and water [129]. Low probe immobilization efficiency was observed after the oligomer was synthesized on the surface [129]. It was found that immobilization of probe oligonucleotides on the surface prior to the ATRP oligomer synthesis did not affect the ATRP processes, and that the probes were stable to the acidic treatment that is
used for removal of benzaldehyde, initiator coupling and the oligomer synthesis conditions [129].

3.2.2 Grafting To the Surface

Grafting to the surface requires a functionalized end group on the oligomer for surface attachment. The oligomers are synthesized ex-situ, purified and characterized before coupling onto the surface. The challenges in synthesis include the oligomer length and the coupling of the reactive end group, typically an amine, at the terminal of the oligomer. Solution phase polymerization reactions are optimized for making macromolecules, thus the reaction conditions have to be modified for early termination. This often involves the tuning of monomer concentration, reaction time and polymerization temperature. ATRP is favourable since it is a controlled living polymerization method with relatively slow polymerization rate [139] and the length of the product increases linearly with polymerization time. This permits control of oligomer length. Moreover, after termination, the halide remains attached at the end of the chain, thereby allowing end group modifications to the desired active groups for surface coupling [139]. However, this technique is not compatible with all monomers. It is suitable with alkene monomers and cannot tolerate acidic groups. The catalyst, ligand, solvents and temperature are variables for the polymerization reaction. Thus, these variables have to be tuned to accommodate polymerization of other monomers.

Oligomers synthesized on solid supports using the grafting from techniques discussed in the previous section can be cleaved off the support and grafted to the surface. An additional step is required to add the active end group to the synthesized oligomer. For solid phase stepwise synthesis, the active end group can be added to the last sequence in the synthesis. An ester or
amide functionality can be incorporated as a spacer into the surface bound initiators between the chlorosilane and the initiator. This allows hydrolysis for oligomer cleavage and provides an amine, hydroxyl or carboxylic acid end group for further modification or direct surface attachment. NMP and ATRP yield nitroxides or halides, respectively, at the end of the chain, which can subsequently be derivatized.

Oligomers can also be made using solution phase multistep synthesis by joining monomers together; a technique similar to the solid phase stepwise synthesis. However, this requires repetitive protection and deprotection steps, along with purifications to ensure the purity of the product throughout the synthesis. The oligomer is built slowly, but in a controlled fashion. However, with subsequent synthesis steps, fewer products will be obtained due to purification and characterization of the product. This is useful when a molecule containing several repeating units of the desired monomer is available and only several repeated couplings are required to make the oligomer.

Templates can be used to control the oligomer synthesis and its length. Beta-cyclodextrin has been introduced as template for methacrylic acid oligomer synthesis. Beta-cyclodextrin is a stereochemically pure macrocyclic compound with 7 primary hydroxyl groups and 14 secondary hydroxyl groups along the rims of the molecule [145]. Complete esterification of the hydroxyl groups with methacrylic anhydride allowed the polymerization of the vinyl groups along the rims of beta-cyclodextrin. Polymerized product was hydrolyzed to give methacrylic acid oligomers. The polymerization reaction was accomplished by ATRP with methyl 2-bromopropionate, CuBr and 2,2’-dipyridyl as the initiator, catalyst and ligand respectively [145]. Solvent systems and reaction temperatures were investigated by Saito and Yamaguchi to optimize the polymerization reaction and control the degree of polymerization of the oligomers [145]. It was found that
oligomers with 7 and 14 repeating units were achieved using the 20.4 methacryloyl substituted beta-cyclodextrin in a methanol and water mixture at 50 °C for 4 hours [145]. MALDI-ToF results indicated that the hydrolyzed oligomers have populations of 6, 7, 13 and 14 repeating units, whereas gel permeation chromatography analysis showed two peaks centered at 700 g/mol and 1300 g/mol that corresponded to 7 and 14 repeating units respectively [145].

### 3.3 Effects of Mixed Film to DNA Immobilization and Hybridization

Mixed film platforms reduce the probe density on the surface while maintaining the orientation of the probe for target hybridization. This reduces the charge and steric effects on the surface for target hybridization, which in turn increases the hybridization efficiency. This overcomes the problems encountered for surfaces of high probe density where low hybridization efficiencies were observed due to the high charge and steric effects on the surface that limit the access of the incoming target [11]. Matrix isolation surfaces offer some control over probe orientation and the surface chemistry with non-probe oligomers. These non-probe oligomers can serve to reduce non-specific adsorption.

Selectivity of the surface can be tuned depending on the chemistry of the oligomer, the ionic strength of the solution and the hybridization temperature. The oligomers control the environment of the oligonucleotides by reducing probe-to-probe interactions and probe-to-surface interaction. Ionic strength controls the amount of counterions present to reduce the repulsion of negative charges on the oligonucleotides. Higher ionic strength results in greater stability of the hybridized duplex. At low ionic strength, electrostatic repulsions are more prominent, which destabilize the duplexes. However, some oligomers are also affected by ionic
strength in terms of swelling, thereby changing the conformation of the oligomers on the surface and possibly affecting oligonucleotides. Thus, ionic strength is an important variable in controlling the selectivity of mixed films. Increased hybridization temperature can reduce non-specific adsorption of oligonucleotides and can destabilize mismatched targets in preference to fully complementary targets.

Two matrix isolation platforms have been reported; a surface with permanent negatively charged polyethylene glycol phosphate oligomers [110], and another with neutral PHEMA oligomers [109,129]. It was found that the melting temperature of fully complementary and mismatched targets were lowered on the mixed film surfaces in comparison to the pure oligonucleotide surfaces [110,129]. This suggested that the interfacial environment of the nucleic acid duplex has a direct effect on its thermodynamic stability [110]. Sharper melt curves were observed for the mixed films, which indicated that the selectivity of the surface was improved [109,129]. This is likely due to the minimization of oligonucleotide probe adsorption on the mixed film surface [109].

The difference in melting temperature ($\Delta T_m$) between fully complementary hybrids and single base pair mismatches for the polyethylene glycol phosphate mixed film and the pure oligonucleotide surface were in good agreement and no significant change in $\Delta T_m$ was observed with increasing ionic strength of hybridization solution from 0.1 M to 1.0 M [110]. This is because polyethylene glycol phosphates have permanent negative charges that offered an electrostatic environment that was similar to that for pure oligonucleotides.

The neutral PHEMA mixed film was shown to improve signal-to-noise ratio by reducing non-specific adsorption in comparison to APTMS surfaces [129]. Investigations with SNPs resulted in a $\Delta T_m$ as large as 15.7 ±1.7 °C for the mixed film and 4.7 ± 2.6 °C for the pure
oligonucleotide surface in 0.5xPBS buffer [109]. The observed increase in $\Delta T_m$ for the mixed film compared to the pure oligonucleotide surface was due to the decrease in $T_m$ for the SNP targets and an increase in $T_m$ for the fully complementary targets in the mixed film [109]. This suggested that the uncharged PHEMA destabilized the SNP targets, but did not compromise the stability of the fully complementary targets at this ionic strength [109]. A $\Delta T_m$ twice as large for the mixed film in 0.1xPBS with respect to the pure oligonucleotide surface was obtained, and similar $\Delta T_m$ was observed for both surfaces at 1.0xPBS [109]. Thus, the ionic strength affected the PHEMA mixed film differently in comparison to the mixed film with charged polyethylene glycol phosphates. At high salt concentrations, 1.0xPBS, the ionic screening counteracted the repulsion of the negative charges on the oligonucleotides and destabilization forces of PHEMA, which resulted in high $T_m$ for fully complementary and SNP targets [109]. Low salt concentration of 0.1xPBS was insufficient to suppress the negative charge repulsion, resulting in lower $T_m$ for both targets [109]. Thus, 0.5xPBS gave greatest selectivity between the targets for the PHEMA mixed film [109].

4 Research Contributions

In the development of DNA biosensors, it has been suggested that the surface chemistry for probe oligonucleotide immobilization, and the interfacial environment of the probes, play a significant role in hybridization performance of biosensors. In the work of this thesis, the surface chemistry was used to determine the sites for probe immobilization and organization of the probes on the surface. The local environment of the probes is influenced by the method of probe immobilization, probe density and ionic strength. Recently, non-probes oligomers have been introduced to surfaces in order to control the local environment of the probes by reducing probe-
to-probe and probe-to-surface interactions. The surface was designed so that the probes are on average surrounded by non-probe oligomers, thus the term ‘matrix isolation’ was applied. Two non-probe oligomers, charged polyethylene glycol phosphate [110] and neutral PHEMA [129], have been used in ‘matrix isolation’ platforms. In comparison to surfaces made of pure oligonucleotides, such mixed films of probe oligonucleotides and non-probe oligomers have shown improved selectivity towards SNP detection. The melting temperature of hybridized duplexes in mixed films was found to be lowered and sharper melt curves were obtained.

The work in this thesis explored the design of a surface chemistry suitable for constructing a ‘matrix isolation’ DNA biosensor. The work involved the synthesis and characterization of a terpolymer, synthesis and characterization amine terminated methacrylic acid oligomer, fabrication of selective films using oligonucleotides, characterization of the surfaces and examination of the performances of the surfaces in the presence of different non-probe oligomers. The non-probe oligomers are referred to as ‘oligomers’ in the remainder of this thesis. Figure 1.13 depicts the differences between conventional oligonucleotide surface, PHEMA mixed film and terpolymer templated mixed film that was constructed in the work of this thesis.
Figure 1.13. Illustration of types of surfaces for immobilization of probe oligonucleotides, (a) conventional surfaces with probe oligonucleotide only, (b) PHEMA mixed film with the structure of the PHEMA oligomer [129], and (c) terpolymer mixed film with the structure of the terpolymer fabricated in the work of this thesis.
A photolabile terpolymer with three active functional groups was synthesized as the template for site directed immobilization of probe oligonucleotides and oligomers. The terpolymer was synthesized from equal molar quantities of NVOC protected aminopropylmethacrylamide (NVOC-amine), glycidyl methacrylate and 3-(trimethoxysilyl)propylmethacrylate by free radical polymerization. It was determined that the terpolymer contained 1 : 3 : 2 ratio of NVOC-amine : epoxide : silyl groups. The terpolymer was anchored onto the substrate through trimethoxysilyl groups, and amine terminated oligomers were directed to the epoxide sites. Amines were recovered from NVOC deprotection, and through activation with the heterobifunctional sulfo-SMCC linker, thiolated probe oligonucleotides were immobilized. Since the probes and oligomers were site directed onto the reactive sites on the terpolymer, the ratio of the functional groups on the terpolymer determined the ratio of probes and oligomers on the surface. In addition, the probe density can be controlled by selective removal of NVOC by UV irradiation. Thus, the terpolymer was a suitable for the development of a ‘matrix isolation’ DNA biosensor.

The goal of this thesis is to investigate whether mixed films of negatively charged oligomers would improve selectivity in discriminating between fully complementary and partially-mismatched targets, and how this construct compares to mixed films of neutral oligomers and conventional spotted oligonucleotides. The neutral oligomer used was polyethylene glycol (PEG) because it is known to ameliorate non-specific adsorption of nucleic acids on surfaces. The negatively charged oligomers were methacrylic acid (MAA) oligomer and dC\textsubscript{20} oligonucleotide. MAA was of interest because the charges on the carboxylic group could be tuned by adjustment of pH and ionic strength of the solution. This offered an additional factor in controlling the energetics and dynamics at the surface. The dC\textsubscript{20} oligonucleotide was used in order to mimic conventional nucleic acid surfaces coated only with probe oligonucleotides. Two
different lengths of PEG were used to evaluate the effects of oligomer length on probe immobilization and fully complementary target hybridization. The results were similar and showed no significant differences between the two lengths examined.

The probe oligonucleotide used was a mixed base 19-mer SMN1 probe. The SMN1 sequence coded for survival motor neuron proteins [146].

The mixed film surfaces with neutral and negatively charged oligomers were evaluated in hybridization experiments. Hybridization profiles and efficiencies of the various oligomer surfaces were compared to determine how the properties of various oligomers played a role in the local environment of the probes in terms of hybrid stability. The surfaces were further investigated for selectivity towards partially-mismatched targets at different solution pH, ionic strengths and temperatures.

It was demonstrated that pH can be tuned to ameliorate non-specific adsorption and ionic strength governed the selectivity of the surfaces. Improved selectivity was achieved at high salt concentration (1 M NaCl) on PEG and dC$_{20}$ mixed films at room temperature. The MAA surface did not show significant improvements in selectivity. This indicated that charge of the oligomers does not dominate control of selectivity. The results suggested that the terpolymer construct depressed the T$_{m}$ of the hybridized duplex to within 5 to 10 °C of room temperature. With the T$_{m}$ shifted closer to room temperature, operation of the films at room temperature was sufficient for discrimination of hybridization between fully complementary and single base pair mismatch targets.
Chapter 2
Materials and Methods

1 Chemicals and Materials

All chemicals were reagent grade or better and were used as received without further purification. 6-Nitroveratryloxycarbonyl chloride, NaHCO$_3$, Na$_2$SO$_4$, glycidyl methacrylate, 3-(trimethoxysilyl)propylmethacrylate, 1,4-dioxane, CDCl$_3$, beta-cyclodextrin, methacrylic anhydride, hydroquinone, pyridine, methyl 2-bromopropionate, 2,2’-bipyridyl, copper(I) bromide, 2-bromoethylamine hydrobromide, potassium iodide, benzoyl peroxide, VAZO 88, DMSO, d$_6$-DMSO, SDS and DTT were obtained from Sigma-Aldrich (Oakville, ON, Canada). N-(3-aminopropyl)methacrylamide hydrochloride was from Polysciences Inc. (Warrington, PA, USA). Ethyl acetate, n-hexane, diethyl ether, H$_2$SO$_4$, methanol, ethanol, toluene, CH$_2$Cl$_2$, sodium hydroxide, potassium hydroxide, HNO$_3$, HCl, hydrogen peroxide, ammonium hydroxide and Tris hydrochloride were from EM Science (Gibbstown, NJ, USA). Sodium hydrogen phosphate, ammonium molybdate and ceric ammonium sulfate were from BDH Chemicals (Toronto, ON, Canada). AIBN was provided by GL Chemtec International Ltd. (Oakville, ON, Canada).

Polyethylene glycol oligomers were from Fluka (Oakville, ON, Canada).

PEG 1: (750) \[\text{NH}_2\text{CH}_2\text{CH}_2\{\text{OCH}_2\text{CH}_2\}_n\text{-OCH}_3\]

PEG 2: (3000) \[\text{NH}_2\{\text{OCH}_2\text{CH}_2\}_n\text{-H}\]

Oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA, USA). All sequences underwent HPLC purification. Thiol modified probes were reduced with DTT before use.
Probes:

Probe 1: SH-SMN1-Cy5  5’ SH-C6-ATT TTG TCT GAA ACC CTG T-Cy5-3’
Probe 2: SH-SMN1  5’ SH-C6-ATT TTG TCT GAA ACC CTG T-3’

Oligomer:

NH₂-dC₂₀-Cy₃:  5’ NH₂-C₁₂-CCC CCC CCC CCC CCC CCC CCC CC-Cy₃-3’
NH₂-dC₂₀:  5’ NH₂-C₁₂-CCC CCC CCC CCC CCC CCC CCC CC-3’

Targets:

SMN1 Fully Complementary:  5’ Cy₅-ACA GGG TTT CAG ACA AAA T-3’
SMN1 1bp Mismatch:  5’ Cy₅-ACA GGG TTT TAG ACA AAA T-3’
SMN1 2bp Mismatch:  5’ Cy₅-ACA GGG TTC TAG ACA AAA T-3’
dA₂₀-Cy₅ Non-Complementary:  5’ Cy₅-AAA AAA AAA AAA AAA AAA AA-3’

PBS buffers were made from 50 mM Na₂HPO₄ and corresponding NaCl concentrations. 1xPBS buffer was made by dissolving 52.60 g NaCl and 6.39 g Na₂HPO₄ in 450 mL sterile water with heat. The pH of the solution was adjusted to pH 7 with HCl to lower pH or NaOH to increase pH. The solution was transferred into a graduated cylinder and sterile water was added to the 900 mL mark. The solution was then poured into a 1 L media bottle and autoclaved. TRIS-SDS buffer was made in the same manner by dissolving 5.26 g NaCl, 1.42 g Tris hydrochloride and 0.09 g SDS in 450 mL sterile water with heat, adjustment to pH 7.5, and added sterile water to a total volume of 900mL. All bottles containing buffers were autoclaved.
Purified water was obtained using a Milli-Q five-stage cartridge purification system supplied by Millipore Corporation (Mississauga, ON, Canada) with a resistance of 18 MΩ·cm. Argon gas was from BOC Gases (Oakville, ON, Canada), which was used in reactions that required an inert environment. Glass slides were Fisherbrand microscope slides with dimensions 25 mm x 75 mm x 1 mm from Fisher Scientific (Ottawa, ON, Canada). P/B doped silicon wafers with diameter of 100.0 mm and thicknesses of 425-475μm were from International Wafer Service (Limerick, PA, USA).

2 Apparatus

All NMR spectra were acquired using an Innova 500 spectrometer (Varian, Palp Alto, CA) or a Varian Mercury 300 spectrometer.

Elemental analysis was done in a Perkin Elmer Model 2400II CHN analyzer with Perkin Elmer AD-6 autobalance, calibrated with thermal standard acetanilide (C:71.09, H:6.71, N:10.36) both before and after a sample run. Results are given in ± 0.3 % unless otherwise specified.

Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-ToF) samples were analyzed with a Waters MALDI micro MX mass spectrometer equipped with a 337 nm Nitrogen laser. All samples were placed in α-cyano-4-hydroxycinnamic acid (α-CHCA) matrix.

The UV Lamp used for photodeprotection was a UVP-B100A (UVP, CA, USA) high intensity, 350 nm long wave UV lamp equipped with a 100 W spot bulb.
UV-Vis absorbance spectra were collected using a QE65000 scientific grade spectrometer from Ocean Optics (FL, USA) equipped with a DH-2000 deuterium tungsten halogen light source.

The X-ray Photoelectron Spectroscopy (XPS) analysis was done with a Thermo Scientific K-Alpha (East Grinstead, UK) instrument equipped with a monochromatic Al Kα X-ray source. A 50 eV pass energy was used over a sample size of 1 x 1 cm². Samples were analyzed at take-off angles of 20 ° and 90 °. The software used to process the XPS data was the Avantage Data System.

Time of Flight-Secondary Ion Mass Spectrometry (ToF-SIMS) samples were run in an Ion-ToF ToF-SIMS IV (Muenster, Germany) instrument using a static operating condition. The ion source was a ⁶⁹Ga liquid metal ion gun and an area of 500 x 500 µm² was analyzed. Both positive and negative ion mass spectra were collected over a mass range of m/z of 0 to 500 amu.

Film thickness on silicon wafers were measured using a Rudolph Research Ellipsometer, Auto EL III Rev.0.508, equipped with a He-Ne laser with emission at 632.8 nm with an incident angle of 70 °.

The wettability of the surface was measured using a light microscope that was mounted horizontally, and that included a protractor in the ocular lense. The angle of curvature of the droplet of water to the surface was measured.

Fluorescence detection on glass samples was obtained by scanning with a Bio-Rad VersArray ChipReader confocal microscope (Hercules, CA, USA). The ChipReader was designed to excite and detect two specific fluorescent dyes, Cy 3 and Cy 5. The excitation
wavelengths were 532 nm and 635 nm, whereas the fluorescence emission was detected at 570 nm and 670 nm, for respective dyes.

3 Synthesis of Terpolymer

The synthesis of the photolabile group and the terpolymer was done following the procedures described by Braun and coworkers [113]. The terpolymer was modified by replacing the methyl methacrylate monomer with glycidyl methacrylate. The reaction scheme for the terpolymer synthesis is shown in Figure 2.1. Reaction and purification of the product was monitored and checked by TLC under UV exposure.
Figure 2.1. Reaction scheme for the synthesis of the NVOC-amine, glycidyl methacrylate and 3-(trimethoxysilyl)propylmethacrylate terpolymer.
3.1 Synthesis of N-(N-6-Nitroveratyloxy carbonylaminopropyl)methacrylamide (NVOC-amine)

3.218 g (11.67 mmol) 6-Nitroveratyloxy carbonyl chloride (NVOC-Cl) in 203.5 mL 1,4-dioxane was added to a solution of 1.897 g (10.62 mmol) N-(3-aminopropyl)methacrylamide hydrochloride (NAPMAAH) and 2.977 g (37.15 mmol) NaHCO$_3$ in 265 mL water. The mixture was stirred at room temperature for 24 hours. The clear yellow solution was extracted three times with 177 mL ethyl acetate and the organic phase was washed twice with 150 mL water. The combined organic phase was dried over Na$_2$SO$_4$, filtered, and concentrated to form yellow oil with crystals. The crystals were filtered and discarded. The yellow oil was allowed to cool to yield pale yellow solids. Purification was done by recrystallization with ethyl acetate/n-hexane. (3.295 g, 81.4 % yield) $R_f = 0.2$, n-hexane:ethyl acetate (1:4 v/v).

Melting point : 121-122°C

$^1$H NMR (500 MHz, CDCl$_3$) : δ 1.71 (m, 1H, -CH$_2$-CH$_2$-CH$_2$-), 1.97 (s, 3H, -CH$_3$), 3.27 (q, 2H, -COO-NH-CH$_2$-), 3.41 (q, 2H, -CH$_2$-NH-CO-), 3.96 (s, 3H, Ar-OCH$_3$), 3.99 (s, 3H, Ar-OCH$_3$), 5.35 (s, 1H, H-CH=C-), 5.52 (s, 2H, Ar-CH$_2$-O-), 5.74 (s, 1H, H-CH=C-), 6.37 (s, 1H, -NH- ), 7.04 (s, 1H, Ar-H), 7.72 (s, 1H, Ar-H).

3.2 Synthesis of NVOC-Amine, Glycidyl Methacrylate and 3-(Trimethoxysilyl)propylmethacrylate Terpolymer

Under Argon, 0.048 g (0.30 mmol) AIBN was added into a solution of 0.142 g (1.0 mmol, 0.132 mL) glycidyl methacrylate, 0.248 g (1.0 mmol, 0.24 mL) 3-(trimethoxysilyl)propylmethacrylate and 0.382 g (1.0 mmol) NVOC-Amine in
7.8 mL 1,4-dioxane. The mixture was stirred at 75 °C for 72 hours. The solution was concentrated and precipitated in cold diethyl ether. Recrystallization was done in 1,4-dioxane/cold diethyl ether. The precipitate was isolated and dried under vacuum to give a pale yellow product (0.365 g). R_t = 0, n-hexane:ethyl acetate (1:4 v/v).

\[ [(C_{17}H_{23}N_{3}O_7)x/(C_{7}H_{10}O_3)y/(C_{10}H_{20}O_5Si)z]_n \]

Elemental analysis calculated: C 53.38, H 7.22, N 3.22 (with x = 1, y = 3, z = 2, from 

\[^{1}H\text{ NMR}\] (500 MHz, CDCl_3): δ 1.71 (s, 6H, Silyl-CH_3), 2.64 (s, 3H, Epoxide-CH-H), 2.85 (s, 3H, Epoxide-CH-H), 3.23 (s, 4H, NVOC-CH_2-CH_2-CH_2- & 3H, Epoxide-CH-CH_2-), 3.57 (s, 18H, Silyl-OCH_3), 3.81 (s, 3H, Epoxide-COO-CH_2-), 3.96 (s, 3H, NVOC-Ar-OCH_3), 3.99 (s, 3H, NVOC-Ar-OCH_3), 4.29 (s, 3H, Epoxide-COO-CH_2-), 5.49 (s, 2H, NVOC-Ar-CH_2-), 7.03 (s, 1H, NVOC-Ar-H), 7.70 (s, 1H, NVOC-Ar-H).

\[^{13}C\text{ NMR}\] (500 MHz, CDCl_3): δ 5.36 (Silyl-Si-CH_2-), 16.75 (Epoxide-CH_3), 18.56 (Silyl-CH_3), 21.54 (Silyl-CH_2-CH_2-CH_2-), 29.07 (NVOC-CH_2-CH_2-CH_2-), 36.61 (NVOC-CH_2-CH_2-CH_2-), 37.97 (NVOC-CH_2-CH_2-CH_2-), 44.82 (Epoxide ring-CH_2-O), 48.93 (Epoxide-O-CH-CH_2-), 50.54 (Silyl-OCH_3), 54.16 (Polymer CH_2, NVOC-CH_2-O-), 56.46 (NVOC-OCH_3), 63.55 (Epoxide-CH_2O-CO-), 65.36 (Silyl-CH_2O-CO-), 108.04 (NVOC-o Ar CH), 110.29 (NVOC-m Ar CH), 128.09 (NVOC-Ar C-CH_2), 139.73 (NVOC-Ar C-NO_2), 147.95 (NVOC-p Ar C-OCH_3), 153.67 (NVOC-m Ar C-OCH_3), 156.65 (NVOC-COO), 176.70 (C=O), 177.38 (C=O).
4 Synthesis of Amine Terminated Methacrylic Acid Oligomer

The synthesis of methacrylic acid oligomer using beta-cyclodextrin as a template was done following the procedures suggested by Saito and Yamaguchi [145]. The coupling of 2-bromoethylamine onto the hydroxyl terminated methacrylic acid oligomer was done using a conventional ether linkage reaction. The reaction scheme is shown in Figure 2.2.

All reactions and purifications were monitored and checked by TLC. TLC spots not visible under UV light were sprayed with ceric ammonium molybdate solution and heated for visualization. The solution used for spraying was made from 12 g ammonium molybdate, 0.5 g ceric ammonium sulfate, 15 mL concentrated H₂SO₄ and 235 mL water.
Figure 2.2. Reaction scheme for the synthesis of amine terminated methacrylic acid using beta-cyclodextrin as the template.
4.1 Synthesis of Methacrylated Beta-Cyclodextrin

14.9 mL (10.07 mmol) Methacrylic anhydride was added to a solution of 2.70 g (2.379 mmol) beta-cyclodextrin and 0.03 g (0.273 mmol) hydroquinone in 15 mL pyridine, stirred and refluxed at 50 °C for 5 hours. The product was precipitated in 300 mL cold water. Recrystallization was done in 1:5 (v/v) methanol:cold water. The solid was dissolved in warm CH$_2$Cl$_2$, dried over Na$_2$SO$_4$, filtered and precipitated in n-hexane with vigorous stirring to give white powder (3.77g). R$_f$ = 0, CH$_2$Cl$_2$. R$_f$ = 0.5, CH$_2$Cl$_2$:EtOH (95:5 v/v).

$^1$H NMR (500 MHz, CDCl$_3$) : δ 1.96 (s, 21H, -CH$_3$), 3.55 (t, 7H, -CH$_2$-CH-CH-), 4.07 (m, 14H, -CH-CH$_2$-O-), 4.20 (m, 7H, -O-CH-CH$_2$-), 4.64 (d, 7H, -COO-CH-CH-CH-O-), 4.85 (d, 7H, -COO-CH-CH-O-), 5.14 (d, 7H, -O-CH-O-), 5.68 (s, 6H, =CH), 6.14 (s, 6H, =CH).

$^{13}$C NMR (500 MHz, CDCl$_3$) : δ 13.31 (CH$_3$), 57.63 (-CH$_2$-O-), 64.46 (-O-CH-CH-CH-O-), 65.13 (-O-CH-CH-O-), 67.79 (-CH$_2$-CH-O-), 77.46 (-CH$_2$-CH-CH-), 94.44 (-O-CH-O-), 121.43 (=CH$_2$), 130.43 (-C=CH$_2$), 161.41 (C=O).

4.2 Synthesis of Polymerized Methacrylate on Beta-Cyclodextrin

1.0 g (0.0396 mmol) Methacrylated beta-cyclodextrin, 0.091 mL (0.792 mmol) methyl 2-bromopropionate, 0.309 g (1.98 mmol) 2,2'-bipyridyl, 0.114 g (0.792 mmol) copper(I) bromide, 0.2 mL (1.98 mmol) toluene, 90 mL methanol and 10 mL water was added to a sealable Pyrex reactor. Three freeze-thaw cycles was done, sealed under vacuum and stirred at 50 °C for 4 hours. A colour change in the reaction mixture was observed from dark brown to dark green, to green, to blue green, as seen in 20 minutes intervals, respectively. The product was filtered, concentrated and precipitated in 200 mL water. Recrystallization was done in 1:2.5 (v/v)
methanol:water twice. The solid was dissolved in warm CH₂Cl₂, dried over Na₂SO₄, filtered, and precipitated in n-hexane to give powder with an off-white colour (0.41 g). Rᵣ = 0, CH₂Cl₂.

Rᵣ = 0.24, CH₂Cl₂:EtOH (95:5 v/v).

¹H NMR (500 MHz, CDCl₃) : δ 1.65 (s, -CH₂), 1.96 (s, -CH₃), 3.57 (s, -CH₂-CH-CH-), 3.71 (s, -OCH₂), 4.10 (s, -CH-CH₂-O-), 4.23 (s, -O-CH-CH₂-), 4.61 (s, -COO-CH-CH-CH-O-), 4.93 (m, -COO-CH-CH-O-), 5.13 (s, -O-CH-O-), 5.63 (s, =CH), 6.14 (s, =CH).

4.3 Hydrolysis of Methacrylic Acid Oligomer from Beta-Cyclodextrin

0.04 g (1.0 mmol) Sodium hydroxide was added to a solution of 0.1 g (0.037 mmol) polymerized methacrylate beta-cyclodextrin in 4.0 mL methanol and stirred at room temperature for 4 hours. The product was poured into 20 mL acetone. The precipitate was collected and washed with a mixture of 1:1 (v/v) CH₂Cl₂:EtOH and 0.1N HNO₃ twice. The white solid was collected and dried under vacuum (0.010 g). Rᵣ = 0.05, CH₂Cl₂:EtOH (1:1 v/v).

¹H NMR (300MHz, d₆-DMSO) : δ 3.27-3.39 (m, -CH₂-), 3.52-3.71 (m, -CH₃), 3.87 (s, -OH), 4.83 (s, -CH).

4.4 Synthesis of Amine Terminated Methacrylic Acid

0.0197 g (0.352 mmol) Potassium hydroxide, 0.0901 g (0.44 mmol) 2-bromoethylamine hydrobromide, 0.0029 g (0.0176 mmol) potassium iodide was added to a solution of 0.1000 g (0.088 mmol) synthesized methacrylic acid in 5mL water. The mixture was stirred under Argon at 75 °C for 72 hours. Adjusted pH of the product to around 7.5, washed with CH₂Cl₂ three times
and poured into 20 mL acetone. The precipitate was collected and washed with a mixture of 1:1 (v/v) CH₂Cl₂:EtOH and 0.1N HNO₃ twice to give white solid. Rᵣ = 0, CH₂Cl₂:EtOH (1:1 v/v), Rᵣ = 0.11, EtOH.

Elemental analysis was calculated to be : C 86.8, H 11.4, N 1.8 ( with n=13 ); and was experimentally determined to be : C 83.24, H 14.05, N 2.7 ( with ± 0.7% error ).

¹H NMR (500 MHz, d₆-DMSO) : δ 2.09 (s, -CH₂-NH₂), 3.29-3.38 (m, -CH₂-), 3.54-3.69 (m, -CH₃), 4.83 (d, -CH), 5.67 (sb, -NH₂).

¹³C NMR (500 MHz, d₆-DMSO) : δ 46.54 (-CH₃), 59.97(-CH₂-NH₂), 72.05(-O-CH₂-CH₂-NH₂), 72.41(-C-CH₂-O-CH₂), 73.13(-C-CH₂-C-), 81.54(-C-COOH), 101.95(C=O).

5 Surface Modifications
5.1 Substrate Cleaning

Glass and silicon wafers were first cleaned with 1:1:5 (v/v/v) solution of ammonium hydroxide:30% hydrogen peroxide:water at 80 °C for 10 minutes, washed thoroughly with sterile water. Then the wafers were cleaned with 1:1:5 (v/v/v) solution of HCl:30 % hydrogen peroxide:water at 80 °C for 10 minutes. The substrates were then washed with isopropanol, dichloromethane and diethyl ether twice, dried and kept in 120 °C oven until required.
5.2 Surface Functionalization with Terpolymer

Cleaned substrates were spin coated with 2 % w/w terpolymer solution in anhydrous DMSO at 2000 rpm for 1 minute, followed by baking at 80 °C for 30 minutes and at 120 °C for 2 hours. Absorptive materials were removed by sonication in toluene for 10 minutes and the substrates were washed in dichloromethane, dried and kept under vacuum until required.

5.3 Immobilization of Oligomer

2 μM solutions of polyethylene glycol and methacrylic acid oligomers were made in 50 mM PBS buffer, whereas dC_{20} was made in 1xPBS buffer. Immobilization involved dispensing a 5 μL solution to form each spot. The slide was placed in a humid environment for 2 hours at room temperature, and then was washed and sonicated in sterile water for 10 minutes. An average spot size of 6.38 ± 0.43 mm^2 with an average diameter of 2.85 ± 0.09 mm was obtained as determined from Cy 3 fluorescence signals of immobilized Cy 3 labeled dC_{20}.

5.4 Photodeprotection of NVOC Photolabile Group

Deprotection of the NVOC group was done by immersing the sample in DMSO and irradiating with a 350 nm UV lamp at a distance of 5 cm follow by rinsing in CH_2Cl_2. The time of irradiation was 5 minutes unless specified.
5.5 Immobilization of Linker

Droplets of 2 μL 500 μM sulfo-SMCC linker made in 50 mM PBS buffer were spotted onto the surface and let stand for 1 hour in a humid environment at room temperature. The surface was then washed with 50 mM PBS buffer and sterile water three times; respectively.

5.6 Immobilization of Probe Oligonucleotide

Thiolated probe oligonucleotides were first treated with DDT before use. 1 μM of probe oligonucleotides (unless otherwise specified) in 1xPBS were spotted onto the surface with 2 μL per spot. Spotted slides were kept in a humid and dark environment for 2 hours. Slides were then washed in Tris-SDS and sterile water twice, respectively, and were boiled in sterile water for 5 minutes. Subsequently, the slides were dried and kept under vacuum.

6 UV-Vis Absorption Study on Photodeprotection on NVOC Photolabile Group

6.1 Terpolymer in DMSO Solution

A solution of 0.05 % w/w terpolymer in DMSO was irradiated at a distance 5 cm from the 100 W 350 nm UV lamp. The UV-Vis absorbance was measured at irradiation time of 0 s, 15 s, 30 s, 60 s, 90 s, 120 s, 150 s, 180 s, 240 s, 300 s, 420 s and 600 s.
6.2 Terpolymer on Glass

The 1.1 cm x 3.5 cm glass slide functionalized with terpolymer was placed in a cuvette with DMSO and was irradiated at a distance of 5 cm from the UV lamp. The UV-Vis absorbance of the glass slide was measured at irradiation time of 0 s, 15 s, 30 s, 60 s, 90 s, 120 s, 180 s and 300 s.

7 Surface Characterization of Terpolymer and NVOC removed Terpolymer

7.1 X-ray Photoelectron Spectroscopy (XPS)

Two samples were made with the NVOC-amine, epoxide and silyl terpolymer bonded to cleaned silicon wafers of 1 cm x 1 cm dimensions. One sample was retained without further processing, while the other was subjected to 10 minutes of UV irradiation to remove the NVOC protecting group from the surface. The NVOC protected and deprotected samples were analyzed at take-off angles of 20° and 90°.

7.2 Time of Flight Secondary Ion Mass Spectrometry (ToF-SIMS)

Two samples were made with the NVOC-amine, epoxide and silyl terpolymer bonded to cleaned silicon wafers of 1 cm x 1 cm. One sample was kept as it was, while the other was subjected to 10 minutes of UV irradiation to remove the NVOC protecting group from the surface. The NVOC protected and deprotected samples were analyzed in both positive and negative ion mode.
7.3 Ellipsometry

The surface coating thickness of 12 silicon wafers of 1 cm x 1 cm was measured following each of the processing steps; cleaned silicon wafers, functionalized NVOC-amine, epoxide and silyl terpolymer silicon wafers, immobilization of oligomers, deprotection of NVOC and immobilization of sulfo-SMCC linker. The four different oligomers that were investigated included PEG 1, PEG 2, MAA and dC\textsubscript{20}, with each oligomer immobilized on three different silicon wafers. Each silicon wafer was measured at 3 different locations for each of the processing steps.

7.4 Static Contact Angle

The 12 silicon wafers of dimensions 1 cm x 1 cm that were investigated using ellipsometry, were further studied by measuring the shape of a droplet of 2 μL water on each surface after each processing step. Four different oligomers were investigated, PEG 1, PEG 2, MAA and dC\textsubscript{20}, with each oligomer immobilized on three different silicon wafers.

8 Hybridization Experiments

Each sample was exposed to 0.1 μM targets in 1.0xPBS buffer at pH 7, unless otherwise specified, for 30 minutes in a humid and dark environment. The samples were then washed with PBS buffer and dried, followed by scanning using the confocal microscope.
9 Denaturation Experiments

Hybridized samples were immersed in PBS buffers equilibrated at 25 °C, 30 °C, 35 °C, 40 °C, 45 °C and 50 °C for 3 minutes each step. The samples were dried, followed by scanning using the confocal microscope after treatment at each temperature.

10 Surface Regeneration

Hybridized samples were placed in a 60 °C sterile water bath for 5 minutes. The sample was then dried, followed by scanning using the confocal microscope.
Chapter 3
Results and Discussion

There are four sections that describe the work of the thesis in this chapter. The first section includes the synthesis and characterization of the terpolymer that was used to template the surface for the matrix isolation platform. The second section addresses the synthesis and characterization of the amine terminated methacrylic acid oligomer, which served to reduce probe-to-probe and probe-to-surface interactions on the biosensor surface. The third section presents surface characterization of the terpolymer film and the immobilized non-probe oligomers. The fourth section analyzes the probe oligonucleotide immobilization and the effects of the various surface designs on target hybridization.

1 Synthesis and Characterization of Terpolymer

The terpolymer consisted of three different active functional groups; amine, epoxide and silyl. These functional groups were chosen so that the silyl group served as an anchoring site for covalent bonding to glass or silicon substrates, and the amine and epoxide provided reactive sites for site directed covalent attachment of the molecules of interest. Since the amine and epoxide could react with each other, the amine was first protected with NVOC (81.4 % yield). NVOC is a photolabile protecting group that is stable under room light conditions but dissociates readily under UV irradiation at 350 nm.

The three monomers chosen to construct the terpolymer were methacrylate derivatives, N-(N-6-nitroveratryloxycarbonyl-aminopropyl) methacrylamide, glycidyl methacrylate and
3-(trimethoxysilyl)propylmethacrylate. The intention was to have monomers with similar reactivity for polymerization. One of the factors that governs the composition of the terpolymer is the mobility of each monomer in the solution during the polymerization reaction. A smaller monomer will have a greater average velocity relative to a larger monomer, causing it to have a higher collisional frequency and thus a higher probability of being incorporated into the polymer. Among the three monomers, NVOC-amine is the largest in size and the epoxide monomer is the smallest. Therefore, the composition of the terpolymer synthesized with equal molar monomer feed will have more epoxide groups incorporated into the terpolymer in comparison to other monomers, which was supported by the results from NMR characterization. This is important for the development of matrix isolation platforms where the probe oligonucleotides are on average surrounded by non-probe oligomers. The mixed film is achieved by directing the oligomers to the epoxide sites and probe oligonucleotides to the amine sites, respectively. Thus, the ratio between the probe and oligomer is controlled by the composition of the terpolymer and the accessibility of the coupling sites. To achieve a matrix isolation platform, the oligomer should be in excess relative to the probes. Since the terpolymer composition is determined by selection of the monomer size in synthesis, the terpolymer can be used for surface templating and design of the mixed film.

The terpolymer was synthesized with equal molar monomer feed in the presence of the free radical initiator, AIBN, for 72 hours at 75 °C in 1,4-dioxane under inert condition. The half-life of AIBN at 70 °C is 5 hours [147]. Therefore, with 72 hours of reaction time at 75 °C, the polymerization reaction was driven to completion. The length and composition of the terpolymer was reproducible under these conditions.
The terpolymer was a pale yellow product that was purified by repeated precipitation in dry, cold diethyl ether from 1,4-dioxane. The purification efficiency was monitored by TLC since the NVOC-amine was UV active. Further confirmation of the removal of monomers was determined by $^1$H and $^{13}$C NMR. Due to the sensitivity of the trimethoxysilane group to moisture, and to protect the epoxy group, the terpolymer was stored as solid under vacuum. In the presence of moisture, self silanization of the silyl groups occurred, forming a complex polymer network.

Substantial effort was required for the development of a reliable method to reproducibly prepare a suitable terpolymer. Once this was achieved, other permutations of the terpolymer were not explored. The focus of this research was to investigate the influence of mixtures of charged oligomers and oligonucleotides on the stability of hybrids. The terpolymer that was developed served as a suitable platform for investigation of the primary hypothesis of the work presented in this thesis, namely that charged oligomers could be used to advantageously tune selectivity of hybridization.

Characterization of the terpolymer was done with $^1$H and $^{13}$C NMR, MALDI-ToF, elemental analysis and UV deprotection profile. Effort has also been made to find a suitable replacement of the AIBN initiator for the synthesis.
1.1 NMR, MALDI-TOF and Elemental Analysis

The $^1$H NMR of NVOC-amine in CDCl$_3$ is shown in Figure 3.1. The alkene proton signals were observed at 5.35 ppm and 5.74 ppm. The distinct aromatic proton signals ($H_a$, $H_b$) at 7.04 ppm and 7.72 ppm, aromatic CH$_2$ signal ($H_c$) at 5.52 ppm, and methoxy proton signals ($H_c$, $H_d$) at 3.96 ppm and 3.99 ppm were used to identify the presence and ratio of NVOC-amine in the terpolymer composition.

Figure 3.1. $^1$H NMR of purified NVOC-Amine in CDCl$_3$. The peaks for the aromatic protons ($H_a$, $H_b$), the methoxy protons ($H_c$, $H_d$) and the aromatic CH$_2$ ($H_e$) are the key identification peaks for this monomer.
The $^1$H NMR of the synthesized and purified terpolymer in CDCl$_3$ is shown in Figure 3.2. The distinct signals that indicated the presence of each monomer, and the lack of alkene hydrogen signals near 5.4 ppm and 5.7 ppm, suggested that the terpolymer was free of unreacted monomers. The twin signals of methoxy protons (H$_a$, H$_b$) in NVOC-amine were again observed at 3.96 ppm and 3.99 ppm, as were the aromatic CH$_2$ (H$_e$) at 5.49 ppm, and the aromatic protons (H$_a$, H$_b$) at 7.03 ppm and 7.70 ppm. For the epoxide functional group, each of the five protons should provide distinct signals. This was confirmed for the pure monomer (data not shown). Useful signals for identification of the epoxy monomer were the split signals of CH$_2$ (H$_k$) at 2.64 ppm and 2.85 ppm, and the split signals of the CH$_2$ adjacent to the oxygen (H$_m$) at 3.81 ppm and 4.29 ppm. The tertiary CH on the epoxide ring (H$_l$) was masked by the larger signals from NVOC CH$_2$ at 3.23 ppm. For the trimethoxysilyl group, the prominent signals include the intense silyl methoxy protons (H$_o$) at 3.57 ppm and the methyl protons (H$_p$) at 1.71 ppm. From the integration of these signals, as summarized in Table 3.1, it can be deduced that the composition of the terpolymer was in 1 : 3 : 2 ratio of NVOC-amine : epoxide : silyl groups. The NVOC methoxy proton integration was higher than expected, resulting from inclusion of a portion of the adjacent peak due to incomplete spectral separation.
Figure 3.2. $^1$H NMR of the synthesized NVOC-amine, epoxy and trimethoxysilyl terpolymer in CDCl$_3$.

Table 3.1. Summary of proton signals for respective monomers, and integrations for calculation of the ratio of each monomer in the terpolymer composition.

<table>
<thead>
<tr>
<th>$\delta$ (ppm)</th>
<th>Hydrogen Identification</th>
<th>Integration</th>
<th>Number of Hydrogens per monomer</th>
<th>Rounded Ratio</th>
<th>Respective Monomer</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.96, 3.99</td>
<td>NVOC OCH$_3$</td>
<td>8.7</td>
<td>6</td>
<td>1</td>
<td>NVOC-Amine</td>
</tr>
<tr>
<td>5.49</td>
<td>NVOC Ar-CH$_2$</td>
<td>1.9</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>7.03</td>
<td>NVOC Ar-H</td>
<td>1.1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>7.70</td>
<td>NVOC Ar-H</td>
<td>1.0</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2.64</td>
<td>Epoxide CH$_2$</td>
<td>2.6</td>
<td>1</td>
<td>3</td>
<td>Epoxide</td>
</tr>
<tr>
<td>2.85</td>
<td>Epoxide CH$_2$</td>
<td>2.8</td>
<td>1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>1.71</td>
<td>Silyl CH$_3$</td>
<td>5.9</td>
<td>3</td>
<td>2</td>
<td>Silyl</td>
</tr>
<tr>
<td>3.57</td>
<td>Silyl OCH$_3$</td>
<td>18.6</td>
<td>9</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>
The $^{13}$C NMR of the terpolymer in CDCl$_3$ is shown in Figure 3.3. The individual signals were assigned with reference to the pure monomer $^{13}$C NMR spectra (data not shown). The aromatic carbon peaks were observed in the 105 ppm to 155 ppm region and the carbonyl carbon signals were further downfield at 157 ppm and 177 ppm.

Figure 3.3. $^{13}$C NMR of NVOC-amine, epoxy and trimethoxysilyl terpolymer in CDCl$_3$.

A MALDI-ToF run was done to determine the distribution and length of the terpolymer. A 337 nm nitrogen pulse laser and the α-CHCA matrix were used. The full mass spectrum, where a mass sweep was done up to 22000 m/z, showed two peak distributions. Both were lower than 6000 m/z, suggesting that the terpolymer synthesized was relatively short. However, polyethylene glycol contaminants were present because the peaks were 44 m/z apart from each
other and the pattern was distinctive. The contamination originated from sample preparation for MALDI-ToF and was not from the terpolymer sample. Due to the low concentration and volatility of the terpolymer, the contaminant signal dominated. The terpolymer peak was shown in Figure 3.4. The peak itself was noisy mainly due to the complexity of the terpolymer itself and a huge variety of possible fragmentation sites. However, major peaks were identified using the composition ratio of the monomers in the terpolymer determined from NMR. The fragmentation patterns of the terpolymer are summarized in Table 3.2 and outlined in Figure 3.5.

Figure 3.4. Expanded MALDI-ToF mass spectrum showing only the terpolymer region, using α-CHCA matrix and excitation with 337 nm pulsed N₂ laser.
Table 3.2. Summary of MALDI-ToF peaks for the terpolymer with the number of monomers represented as M(NVOC-Amine, epoxide, silyl) and CHCA is the matrix.

<table>
<thead>
<tr>
<th>Observed Peak (m/z)</th>
<th>Calculated Peak (m/z)</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>4361.2</td>
<td>4361.5</td>
<td>[M(4,12,8) - 4NVOC - (-CH₂-Epoxide) - (-OCH₃) + CHCA + H]^*</td>
</tr>
<tr>
<td>4459.5</td>
<td>4459.5</td>
<td>[M(4,12,8) - 4NVOC - (-CH₂-Epoxide) - 4(-OCH₃) + 2CHCA + 3H]^*</td>
</tr>
<tr>
<td>4500.6</td>
<td>4500.5</td>
<td>[M(4,12,8) - 4NVOC - 3(-CH₂-Epoxide) - 5(-OCH₃) + 3CHCA]^*</td>
</tr>
<tr>
<td>4557.3</td>
<td>4557.6</td>
<td>[M(4,12,8) - 4NVOC - 2(-CH₂-Epoxide) - 5(-OCH₃) + 3CHCA]^*</td>
</tr>
<tr>
<td>4595.6</td>
<td>4595.6</td>
<td>[M(4,12,8) - 4NVOC - 3(-CH₂-Epoxide) - 2(-OCH₃) + 3CHCA + 2H]^*</td>
</tr>
<tr>
<td>4726.8</td>
<td>4726.8</td>
<td>[M(4,12,8) - 4NVOC - (-COO-CH₂-Epoxide) + 3CHCA + H]^*</td>
</tr>
<tr>
<td>4862.3</td>
<td>4862.9</td>
<td>[M(4,12,8) - 4NVOC - 5(-OCH₃) + 4CHCA + 2H]^*</td>
</tr>
<tr>
<td>4926.9</td>
<td>4927.0</td>
<td>[M(4,12,8) - 4NVOC - 3(-OCH₃) + 4CHCA + 4H]^*</td>
</tr>
<tr>
<td>5056.6</td>
<td>5056.1</td>
<td>[M(4,12,8) - 4NVOC - (-CH₂-Epoxide) - 3(-OCH₃) + 5CHCA + H]^*</td>
</tr>
</tbody>
</table>
Figure 3.5. Outline of major fragments of terpolymer in MALDI-ToF and the structure of \( \alpha \)-CHCA.
The peaks below 4361 m/z were not included in the terpolymer identification because they overlapped with the strong polyethylene glycol signals. This limited the determination of low molecular weight terpolymer populations that could be present in the sample. Since the nitrogen laser that was used for excitation was fairly close to the wavelength of NVOC deprotection and due to the strong intensity of the laser, it is very likely that the NVOC groups were lost during the excitation step. The terpolymer composition that fit the fragmentation profile was determined to have NVOC-amine : epoxide : silyl in a ratio of 4 : 12 : 8 with molecular weight of 5220 g/mol. Therefore, the chain length of the terpolymer could vary between 6 to 24 while maintaining the composition ratio of 1 : 3 : 2 for NVOC-amine : epoxide : silyl.

Elemental analysis of the terpolymer found that it contained 52.63 %C, 6.81 %H and 4.29 %N (standard deviation ± 0.3 %). Using the monomer ratio of 1 : 3 : 2 for NVOC-amine : epoxide : silyl monomers, the calculated percentages were 53.38 %C, 7.22 %H and 3.22 %N. This is consistent with the conclusion that the NVOC-amine monomer was successfully incorporated into the terpolymer.

1.2 Investigation of Alternative Initiators

AIBN is a low temperature free radical initiator, which is widely used for linear polymer synthesis. However, due to concerns about tetramethy succinonitrile toxicity, supply of AIBN is prohibited in Canada. Benzoyl peroxide and 1,1’-azobis(cyclohexanecarbonitrile) (Vazo 88) free radical initiators were investigated in order to find a suitable replacement for the terpolymer synthesis. Benzoyl peroxide was chosen because it is a commonly used initiator in polymer synthesis and is widely available. However, it is a strong oxidizing agent that is highly flammable and explosive. Vazo 88 is a milder reagent that is more stable and not shock sensitive.
It is in the same family as AIBN, thus having the same property in yielding linear polymers. Both of these initiators are activated at higher temperatures compared to AIBN. The polymerization temperature was kept at 85°C for benzoyl peroxide and 95°C for Vazo 88. The reaction time was maintained for 72 hours with equal monomer feed and the solvent was 1,4-dioxane. One of the main concerns associated with the change of initiator was whether the trimethoxysilyl group would be compatible because gelation often occurs with this group, which interferes with the isolation process [113].

Benzoyl peroxide was tested in the synthesis of the terpolymer containing NVOC-amine, methyl methacrylate and 3-(trimethoxysilyl)propylmethacrylate. This terpolymer was also made by using AIBN as the initiator in a separate synthesis. Comparing the product from both reactions, the product from benzoyl peroxide was difficult to purify by precipitation, even though a series of organic solvents were screened. Moreover, its solubility was much lower than the product made from AIBN. This indicated the presence of crosslinked product since the solubility decreases as the polymer network forms. The NMR spectrum (not shown) was complex, which further suggested that the purification was not successful.

Vazo 88 was used in the copolymerization of glycidyl methacrylate and 3-(trimethoxysilyl)propylmethacrylate. The NVOC-amine monomer was not included in this polymerization because the monomer and the NVOC protecting group are known to be stable at the reaction temperature and any unreacted monomer can be easily removed in the precipitation step. Therefore, the focus was an examination of the compatibility of the silyl group with the initiator, and ensuring that the reactivity of the epoxide group was maintained after polymerization. Using the same precipitation method as in the terpolymer synthesis with AIBN, the isolated product was pure, and formed as a white solid. The product was readily soluble in
CDCl₃ and DMSO, thus suggesting the absence of crosslinked products and the retention of the silyl group. The epoxide hydrogens were observed in the ¹H-NMR (not shown), which confirmed that the epoxide ring was unaffected by the initiator and the polymerization conditions. Thus, Vazo 88 is a suitable replacement for AIBN in the terpolymer synthesis.

1.3 UV Deprotection Profile of NVOC in Solution

The deprotection of NVOC in the terpolymer was done in a solution of 0.05 % w/w terpolymer in DMSO with the sample contained in a fused silica cuvette. A 100 W 350 nm UV source was placed 5 cm away from the sample. The UV-Vis absorbance of the sample was measured as a function of deprotection time as shown in Figure 3.6. Without irradiation, a peak was observed at 350 nm, and could be used to monitor the presence of NVOC. Upon irradiation, this peak decreased with time, indicating the removal of NVOC from the terpolymer. A weak absorption was observed at about 400 nm with irradiation time, corresponding to the removal product of NVOC, 4,5-dimethoxy-6-nitrosoaldehyde [113]. Figure 3.7 shows the plot of absorbance at 350 nm against deprotection time. It was found that under these conditions, full deprotection was achieved in 5 minutes. This agreed with results observed by Braun and coworkers [113]. The deprotection reaction followed a first order reaction, as reported by Chan and Yousaf [114], with a rate constant of 0.14 min⁻¹.
Figure 3.6. UV-Vis absorbance profile of the NVOC group deprotection in a solution of 0.05 % w/w terpolymer in DMSO. Irradiation was done at 5 cm from the 100 W 350 nm UV source for (a) 0 s, (b) 30 s, (c) 60 s, (d) 90 s, (e) 120 s, (f) 180 s and (g) 300 s.
Figure 3.7. UV-Vis absorbance at 350 nm for NVOC deprotection in a solution of 0.05 % w/w terpolymer in DMSO. Irradiation was done at 5 cm from the 100 W 350 nm UV source. It followed a first order reaction with a rate constant of 0.14 min$^{-1}$.

2 Synthesis and Characterization of Methacrylic Acid Oligomer

Methacrylic acid oligomer is of interest because it has partial negative charges. These charges can be tuned by ionic strength and pH of the solution. Thus, in the mixed film, it served to provide the necessary charges for repulsion and orientation of the probe oligonucleotides on the surface. Moreover, it does not have groups that will interact strongly with oligonucleotides, which would result in reduction of non-specific adsorption. Since the oligomer is directed to the epoxide sites on the terpolymer, an amine terminus on the oligomer is preferred for covalent coupling. The challenge in the synthesis was to control the length of the oligomer and the incorporation of the amine terminus.
Controlled length synthesis of methacrylic acid oligomers was achieved by using beta-cyclodextrin as the template by Saito and Yamaguchi [145]. Beta-cyclodextrin has two rims, with 7 primary and 14 secondary hydroxyl groups, respectively. The multistep synthesis involved the esterification of the hydroxyl groups on beta-cyclodextrin with methacrylic anhydride, followed by polymerization with ATRP. Hydrolysis of the polymerized product gave methacrylic acid oligomers with a hydroxyl group on one terminal, which was subsequently derivatized to an amine. The esterification step of the hydroxyl groups on beta-cyclodextrin was crucial because the number of vinyl groups on the template determines the length of repeating units in the oligomer. Two populations of methacrylic acid oligomers with 7 and 14 repeating units were obtained from beta-cyclodextrin templates with an average of 20.4 vinyl groups [145]. This indicated that complete esterification of all 21 hydroxyl groups on beta-cyclodextrin was achieved, along with some partially derivatized product. ATRP polymerization with methyl 2-bromopropionate as the initiator and copper(I) bromide/2,2'-dipyridyl as the catalyst system allowed polymerization of adjacent vinyl groups along each rim of the template. Thus, the maximum number of repeating units of methacrylic acid that can be synthesized is limited to 14. Colour change was observed in the reaction mixture during the course of polymerization due to the copper catalyst, and provided an indication of reaction progress. The polymerized product was then hydrolyzed with sodium hydroxide in methanol. The purified methacrylic acid oligomer was subsequently reacted with 2-bromoethylamine to form an ether linkage with an amine terminal.

2.1 NMR, MALDI-ToF and Elemental Analysis

From the $^1$H NMR of the methacrylated beta-cyclodextrin, shown in Figure 3.8, the vinyl proton signals were observed at 5.68 ppm and 6.14 ppm, whereas the methyl proton signal was at
1.96 ppm. Individual proton signals from the glucose monomer of beta-cyclodextrin were also distinctive in the spectrum. By integrating these signals, it was found that an average of 15 methacrylates was coupled on the template. This was supported by the MALDI-ToF result shown in Figure 3.9. Four related fragmentation signals that were spaced 68 m/z apart were observed, corresponding to the loss of methacrylate group. It was determined that the peaks indicated the presence of 14 to 16 methacrylate groups on the template as summarized in Table 3.3.

Figure 3.8. $^1$H NMR of methacrylated beta-cyclodextrin in CDCl$_3$. The vinyl proton signals (H$_b$) were observed at 5.68 ppm and 6.14 ppm. The number of methacrylate groups on the beta-cyclodextrin template was calculated by using the integrated signal of the vinyl (H$_b$) and methyl protons (H$_a$) in comparison to that of the proton signal from the glucose ring (H$_c$). An average of 15 methacrylates was coupled onto the template.
Figure 3.9. MALDI-ToF of methacrylated beta-cyclodextrin in α-CHCA matrix. Series of peaks 68 m/z apart indicated loss of methacrylate groups, CH$_3$C(=CH$_2$)CO.
Table 3.3. Summary of MALDI-ToF fragmentation patterns for methacrylated beta-cyclodextrin in α-CHCA matrix. Loss of 68 m/z was observed between the peaks in each series.

<table>
<thead>
<tr>
<th>Number of Methacrylates</th>
<th>Observed Peaks (m/z)</th>
<th>Calculated Peak (m/z)*</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>1842.5, 1910.6, 1979.5, 2047.6</td>
<td>2046.9</td>
<td>Beta-cyclodextrin with 14 Methacrylates, 7 Hydroxyls and loss of a CH₃C=CH₂ group</td>
</tr>
<tr>
<td>15</td>
<td>1937.6, 2005.6, 2073.6, 2141.6</td>
<td>2141.0</td>
<td>Beta-cyclodextrin with 15 Methacrylates, 6 Hydroxyls and loss of a CH₃ group</td>
</tr>
<tr>
<td>15</td>
<td>2254.6, 2323.7</td>
<td>2322.2</td>
<td>Beta-cyclodextrin with 15 Methacrylates, 6 Hydroxyls, CHCA, H₂O and loss of a CH₃C=CH₂ group</td>
</tr>
<tr>
<td>16</td>
<td>2099.6, 2167.6, 2235.7</td>
<td>2242.1</td>
<td>Beta-cyclodextrin with 16 Methacrylates, 5 Hydroxyls and H₂O</td>
</tr>
</tbody>
</table>

* the calculated peaks are based on the proposed structure.

The polymerization of the methacrylate groups on the template was done by ATRP. Since the initiator used was methyl 2-bromopropionate, the methyl propionate group was attached to one terminal of the synthesized oligomer. Two new peaks in the $^1$H NMR of the polymerized product were observed at 1.65 ppm and 3.71 ppm, which corresponds to the polymerized alkyl CH₂ and the methyl group on the propionate ester, respectively. The vinyl protons at 5.63 ppm and 6.14 ppm were present in the polymerized product. This indicated that not all methacrylate groups on beta-cyclodextrin were polymerized. This can be explained by the relative proximity of the vinyl groups on the template. ATRP relied on fast transfer of the halide onto the next monomer for polymerization. In this case, the monomer was bound to the template, which
limited its accessibility. Thus, if the monomer was not in close proximity of the propagating chain, it would not be polymerized.

The hydrolyzed methacrylic acid oligomer was identified by $^1$H NMR. The coupling of the ethylamine onto the hydroxyl terminal was confirmed by $^1$H NMR, MALDI-ToF and elemental analysis. The broad hydroxyl peak near 3.87 ppm was replaced by the amine proton signal at 5.67 ppm, and a new peak for CH$_2$ was observed at 2.09 ppm, as shown in Figure 3.10. From MALDI-ToF (data not shown), two peaks at 1173 m/z and 1296 m/z were observed indicating that the ether linkage was successful, and that the oligomers contained 12 and 13 repeating units of methacrylic acid, respectively. This suggested that the polymerization reaction took place along the rim with 14 hydroxyl groups, where the methacrylates were in close proximity for ATRP.
Figure 3.10. $^1$H NMR of (a) hydrolyzed methacrylic acid and (b) amine terminated methacrylic acid in $d_6$-DMSO. The broad hydroxyl peak near 3.87 ppm in (a) was absent in (b), and the presence of amine proton signal at 5.67 ppm along with a new CH$_2$ peak at 2.09 ppm confirmed the attachment of ethylamine.

Experimentally determined elemental analysis confirmed that the amine was incorporated into the compound with 83.24 %C, 14.05 %H and 2.7 %N (with error of ± 0.7 %). The calculated percentages were 86.8 %C, 11.4 %H and 1.8 %N.
3 Characterization of Terpolymer on Substrates

The terpolymer was covalently coupled onto silicon wafers and glass slides for surface characterization via the trimethoxysilyl groups. The terpolymer was spin coated in 2 % w/w terpolymer solution in anhydrous DMSO onto the substrate, followed by baking at 80 °C for 30 minutes and at 120 °C for 2 hours. The coated substrate was then sonicated in toluene and washed with dichloromethane to remove absorbed materials.

Once the terpolymer was bound to the substrate, amine terminated oligomers were immobilized onto the epoxide sites. The photolabile NVOC groups were then deprotected by UV irradiation to yield amines for probe oligonucleotide immobilization. The heterobifunctional linker sulfo-SMCC was used because it contained a succinimidyl group that reacted with the amine on the surface and provided the maleimido group for immobilization of thiol terminated probe oligonucleotides. Figure 3.11 illustrates the fabrication steps and structure of the mixed film on the substrate surface.
Figure 3.11. Schematic representation of the mixed film fabricated on silicon based substrates. The terpolymer was anchored onto the surface through the trimethoxysilyl group. Amine terminated oligomers were coupled onto the epoxide sites and thiolated ssDNA was immobilized onto the amine sites via sulfo-SMCC linker. The ratio of the groups on the surface was experimentally determined to be 1 : 3 : 2 for NVOC-amine : epoxide : silyl, respectively.
3.1 XPS and ToF-SIMS

XPS and ToF-SIMS were used to confirm the attachment of the terpolymer onto the substrate and the recovery of the amine functionality after UV deprotection. Samples of terpolymer on silicon wafers before and after NVOC deprotection were analyzed. The samples were measured at take-off angles of 20° and 90° using XPS. At 20°, the sensitivity of the experiment is such that a thinner depth of the film on the surface was measured. The elemental composition from low resolution data and binding energies from high resolution data for the NVOC terpolymer and deprotected sample are summarized in Table 3.4.
Table 3.4. XPS low and high resolution data for terpolymer samples on silicon wafers before and after NVOC deprotection at take-off angles of 20° and 90°.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Take-Off Angle (degree)</th>
<th>Low Resolution Data Elemental Composition (%)</th>
<th>High Resolution Data Binding energies (eV) of deconvoluted peaks (% area)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C (1s)</td>
<td>N (1s)</td>
</tr>
<tr>
<td>NVOC Sample</td>
<td>20</td>
<td>66.6</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>64.9</td>
<td>3.0</td>
</tr>
<tr>
<td>Deprotected</td>
<td>20</td>
<td>67.6</td>
<td>2.4</td>
</tr>
<tr>
<td>Sample</td>
<td>90</td>
<td>63.7</td>
<td>3.0</td>
</tr>
</tbody>
</table>
The differences that are seen for the two types of samples are small at both of the take-off angles, suggesting the presence of a relatively thick film on the surfaces. This is confirmed by the low Si (2p) signals and ellipsometry data on page 119. The ratio between C (1s) and N (1s) at 90° take-off angle for the NVOC sample and deprotected sample was 18:1 and 21:1, respectively. These values are close to the theoretical ratios of 18:1 and 22:1 calculated from the monomer ratios of the terpolymer as determined from NMR. This is consistent with the presence of the terpolymer on the surface.

Five carbon peaks were deconvoluted between binding energies of 284 eV to 289 eV. Aliphatic carbons have binding energy of ~ 285 eV and the carbonyl carbons have higher binding energy at ~ 289 eV. Carbonyl carbons are electron deficient because electrons are drawn towards the electronegative oxygen, thus more energy is required to eject the core carbon electron resulting in higher binding energy. For the ether C-O bond, the extent of oxygen withdrawal of electrons from carbon is less prominent than that observed for the C=O carbonyl bond, therefore the ether carbons have intermediate binding energies at ~ 286.5 eV. From the high resolution C(1s) spectra in Figure 3.12, a shoulder was observed at ~ 287 eV for the NVOC terpolymer sample. This peak was reduced in the deprotected sample, which suggested the removal of the NVOC group.
Figure 3.12. High resolution XPS spectra of C (1s) signals from terpolymer on silicon wafers before deprotection at (a) 20 ° and (b) 90 °, and after NVOC deprotection at (c) 20 ° and (d) 90 °. The shoulder peak at 287 eV was reduced after deprotection.

There were two nitrogen populations observed in the NVOC terpolymer sample. They represented the presence of two different nitrogens; the aliphatic NH at ~ 399 eV and the NO$_2$ on the NVOC group at ~ 405 eV as seen in Figure 3.13. The NO$_2$ peak was absent in the deprotected sample and a new shoulder was seen at ~ 401 eV. This confirmed the quantitative removal of the NVOC group from the surface and the amine functionality was recovered. It was
noted that the elemental percentages of the NH nitrogens were identical for the same take-off angles both before and after NVOC deprotection, indicating that this nitrogen population was maintained through the deprotection step.

![Figure 3.13](image)

Figure 3.13. High resolution XPS spectra of N (1s) signals from terpolymer on silicon wafers before deprotection at (a) 20° and (b) 90°, and after NVOC deprotection at (c) 20° and (d) 90°. The NO₂ nitrogen peak at ~ 405 eV was absent in the deprotected sample, indicative of quantitative removal of NVOC from the terpolymer on the surface.
The high resolution O (1s) spectra is shown in Figure 3.14. There were no significant changes in peak shape between the samples. As seen in the Si (2p) spectra in Figure 3.15, two peaks were observed at the 90° take-off angle spectra because elemental silicon from the bulk silicon wafer was sampled. The elemental silicon and silicon oxide signals occurred at ~99 eV and ~102 eV, respectively. Comparing the NVOC terpolymer and the deprotected sample, higher elemental silicon signal was observed in the deprotected sample.

Figure 3.14. High resolution XPS spectra of O (1s) signals from terpolymer on silicon wafers before deprotection at (a) 20° and (b) 90°, and after NVOC deprotection at (c) 20° and (d) 90°.
Figure 3.15. XPS spectra of Si (2p) signals from terpolymer on silicon wafers before deprotection at (a) 20 ° and (b) 90 °, and after NVOC deprotection at (c) 20 ° and (d) 90 °.

ToF-SIMS measurements in positive and negative ion mode, shown in Figs 3.16 and 3.17, respectively, were obtained for the terpolymer and NVOC deprotected sample on silicon wafers. The top row labeled Si_49 refers to the terpolymer sample whereas the bottom row labeled Si_50 corresponds to the deprotected sample. In the positive ion mass spectrum, two peaks at 180 m/z and 196 m/z were contributed by the NVOC aromatic group. These peaks were
absent in the deprotected sample. The presence of the epoxide group was identified by the peaks at 43 m/z and 59 m/z. In the negative ion mass spectrum, evidence for the removal of NVOC was supported by the absence of 181 m/z and 196 m/z peaks for the deprotected sample. The presence of the epoxide group was indicated by signals of 31, 41 and 43 m/z, which correlated with peaks observed in 3-glycidoxypropyltrimethoxysilane films [66]. Two peaks at 95 m/z and 97 m/z were absent in the terpolymer but present in the NVOC deprotected spectra. They could be fragments of the aminopropyl group that linked the NVOC group to the polymer backbone. Since the NVOC group was deprotected, it allowed the aminopropyl group to fragment. The possible positive and negative ion fragments are summarized in Figure 3.18.
Figure 3.16. Positive ion ToF-SIMS mass spectrum for Si_49 (terpolymer) and Si_50 (deprotected sample).
Figure 3.17. Negative ion ToF-SIMS mass spectrum for Si_49 (terpolymer) and Si_50 (deprotected sample).
Both XPS and ToF-SIMS results confirmed the coupling of the terpolymer onto the surface and the quantitative removal of NVOC from the terpolymer surface. The amine functionality was recovered, and the epoxide groups that were evident in the ToF-SIMS spectra...
were not affected by the deprotection process. Thus, the surface is shown to contain both the amine and epoxide groups that are available for subsequent immobilizations.

3.2 Ellipsometry and Static Contact Angle Experiments

Ellipsometry was used to measure the thickness of the terpolymer films coupled to the silicon wafers that were spin coated at different speeds. The terpolymer was spin coated onto the substrates for 1 minute at different speeds, followed by the coupling process. Thinner films were expected at higher speeds, which agreed with the results gathered. Three samples were made at each speed, and three thickness measurements were taken on each sample. Average film thicknesses of 199 ± 29 Å, 134 ± 6 Å and 87 ± 11 Å, were obtained with coating speeds of 2000, 3000 and 4000 rpm respectively. These silicon wafers were then subjected to NVOC deprotection and the resulting film thickness was measured. An average decrease of 14 Å was found after NVOC removal.

Static contact angle of the surface was measured with a 2 µL droplet of water. The precision of the angle measurements was ± 2 °. The terpolymer surface is expected to be relatively hydrophobic, and the static contact angle was 60°. After NVOC deprotection, the bulky aromatic group was removed from the surface, thus exposing the more hydrophilic amines. The deprotected surface had a static contact angle of 40 °. The 2000 rpm setting was used in the fabrication of surfaces in order to ensure terpolymer coverage on the surface.

The fabrication process was monitored by ellipsometry and static contact angle after each of the modifications. Measurements were taken at each step after the silicon wafers were cleaned, functionalized with terpolymer, covered with oligomers and NVOC deprotected. Four amine terminated oligomers were used, as were two polyethylene glycol oligomers (PEG1 and
PEG2), the synthesized methacrylic acid oligomer (MAA) and dC20. PEG1 was shorter than PEG2 with an average molecular weight of 750 g/mol giving an average of 15 ethylene glycol units. PEG2 had an average molecular weight of 3000 g/mol with an average of 67 ethylene glycol units. Change in film thickness was observed after each step. Different solvents ranging from organic solvents such as DMSO and dichloromethane to buffer and water were used. Therefore, the measured thickness does not correspond to the absolute film thicknesses on the surface in each case. However, the trend and consistency of the data obtained in each modification step for the various surfaces provided confidence that modification of each surface had been achieved. The wettability measurements are summarized in Table 3.5.

Table 3.5. Static contact angle measurements of respective surfaces after each processing step with a precision of ± 2 °.

<table>
<thead>
<tr>
<th>Oligomer Surfaces</th>
<th>Cleaned Substrate</th>
<th>Terpolymer</th>
<th>Oligomer Immobilization</th>
<th>NVOC Deprotection</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG 1</td>
<td>0 °</td>
<td>60 °</td>
<td>50 °</td>
<td>40 °</td>
</tr>
<tr>
<td>PEG 2</td>
<td>0 °</td>
<td>60 °</td>
<td>51 °</td>
<td>40 °</td>
</tr>
<tr>
<td>MAA</td>
<td>0 °</td>
<td>60 °</td>
<td>50 ° – 60 °</td>
<td>38 ° – 40 °</td>
</tr>
<tr>
<td>dC20</td>
<td>0 °</td>
<td>60 °</td>
<td>45 °</td>
<td>35 ° – 38 °</td>
</tr>
</tbody>
</table>

The cleaned silicon surface was hydrophilic due to the silanol groups, and provided a 0 ° angle. In the presence of the terpolymer, with the alkyl backbone and bulky NVOC groups, the static contact angle changed to 60 °. A decrease in measured static contact angle was observed after oligomer immobilization because all four oligomers introduced hydrophilic groups to the
surface. The polyethylene glycols contained ether linkages that contributed to the static contact angle of the surface, and provided a static contact angle of 50°. The addition of MAA yielded a range of static contact angle between 50° to 60°, whereas the presence of dC$_{20}$ resulted in an angle of 45° due to the phosphate backbone. After NVOC deprotection, the bulky aromatic group was removed with the amine exposed, and a general trend of decrease of the measured static contact angle was observed.

3.3 UV Deprotection Profile of NVOC on Surfaces

Terpolymer coupled glass slides with dimensions of 1.1 cm x 3.5 cm were subjected to UV irradiation 5 cm distance from the UV lamp. The UV-Vis absorbance on the glass slide was measured as a function of deprotection time, as shown in Figure 3.19. The broad peak at ~350 nm decreased with irradiation time, which corresponded to the removal of NVOC from the surface. As shown in Figure 3.20, the deprotection reaction followed a first order reaction, which agreed with the results obtained from the deprotection profile of the terpolymer in solution. Shorter deprotection time was required for the complete removal of the NVOC groups from the surface because there was less NVOC groups on the surface compared to the 0.05 % w/w terpolymer solution as in Figure 3.6. On surfaces, complete deprotection was achieved in 2 minutes. A similar absorption profile (data not shown) was observed from surfaces coated with PEG1, PEG2, MAA and dC$_{20}$ oligomer. The presence of the oligomers on the surface did not affect the NVOC deprotection process.
Figure 3.19. UV-Vis absorbance profile for NVOC deprotection on a terpolymer functionalized glass surface in DMSO. Irradiation was done at 5 cm from the 100 W 350 nm UV source for (a) 0 s, (b) 15 s, (c) 30 s, and (d) 120 s.
Figure 3.20. UV-Vis absorbance at 350 nm for NVOC deprotection on a terpolymer functionalized glass surface in DMSO. Irradiation was done at 5 cm from the 100 W 350 nm UV source. It followed a first order reaction, similar to the NVOC deprotection in solution. Full deprotection can be achieved in 2 minutes.

4 Evaluation of the Functionalized Surfaces

The mixed films that were evaluated were fabricated on glass slides. Amine terminated oligomers of PEG1, PEG2, MAA and dC$_{20}$ were spotted onto the terpolymer surface in grids. A droplet of 5 µL of 2 µM solution of the respective oligomers was used for each spot. Cy 3 labeled amine terminated dC$_{20}$ served as a control on the surface to monitor the presence of the oligomers over the course of the experiments. Following NVOC deprotection, a 500 µM solution of sulfo-SMCC was introduced to the surface with 2 µL per spot. Sulfo-SMCC served to activate the amine sites for coupling of thiolated 19-mer SMN1 probe oligonucleotides. The probes were prepared in 1 M NaCl PBS (1xPBS) buffer and introduced to the surface as 2 µL spots. The spot size was controlled by the volume of the immobilized solution. The immobilization buffer used in all of the coupling steps was PBS buffered to pH 7.
As determined by wettability experiments, the terpolymer surface was more hydrophobic than the oligomer surface after NVOC deprotection. Thus, a greater volume was used for the coupling of the amine terminated oligomers to ensure coverage over the spot. From the Cy 3 fluorescence signal of dC_{20}, an average spot size of 6.38 ± 0.43 mm$^2$ with an average diameter of 2.85 ± 0.09 mm was obtained. Sulfo-SMCC was introduced at a lower volume within the oligomer spot, followed by the immobilization of probe oligonucleotides. Since the probes were bound to the surface through the linker, only the area containing linker determined the spot size that contained immobilized probes. Using Cy 5 labeled probes, an average spot size of 3.60 ± 0.18 mm$^2$ with an average diameter of 2.07 ± 0.10 mm was obtained. The smaller spot size associated with Cy 5 fluorescence from the probes in comparison to the Cy 3 signal from the oligomers was consistent with the intended design and indicated that the probes were immobilized on the oligomer surface.

The effectiveness of the linker for probe immobilization was investigated and the mixed films were evaluated with fully complementary (FC), single base pair mismatch (1bp), two base pair mismatch (2bp) and non-complementary (NC) targets. The selectivity of the surfaces towards fully complementary targets was studied by controlling the stringency conditions using pH, ionic strength and temperature. The regeneration of the surfaces after hybridization for repeated use was examined.
4.1 Immobilization of Probe Oligonucleotides

Cy 5 labeled thiolated SMN1 probes were immobilized onto the oligomer surfaces via the sulfo-SMCC heterobifunctional linker. The succinimidyl group on the linker reacted with the deprotected amine sites on the terpolymer. This activated the surface with maleimido groups for the immobilization of thiolated probes. The linker and probes were spotted onto the surface as shown in Figure 3.21. Each of the oligomers, PEG1, PEG2, MAA and dC20, were localized to 3 rows on the surface, respectively. The last row contained no oligomer and served as a control. No linker was introduced to the first column. However, the spots were subjected to 1.0 µM thiolated probes. The remaining 4 columns had linker attached except for the last spot in the last column. Probe concentrations of 0.3 µM, 0.5 µM, 0.8 µM and 1.0 µM were immobilized in the columns, respectively. A probe concentration of 1.0 µM was spotted onto all spots in the last row which did not contain oligomers. The spotted surface was kept in a humid and dark environment at room temperature for 2 hours for reaction to proceed.

The immobilized surfaces were boiled in sterile water in order to remove adsorbed materials. Decrease in fluorescence signal was observed after 5 minutes of boiling, but subsequent boiling for another 5 minutes did not show significant changes in fluorescence intensities. Thus, 5 minutes of boiling was sufficient to prepare the surface for further analysis.
Figure 3.21. Immobilization of probes on PEG1, PEG2, MAA, dC20 and without oligomer surfaces in 1xPBS buffer for 2 hours in humid environment. (a) Confocal fluorescence image of Cy 5 signal from the probes in the mixed films on glass slide after boiling twice in sterile water, for 5 minutes each time. (b) Showing materials introduced to corresponding spots in each column.
4.1.1 Site Directed Immobilization of Probes on Sulfo-SMCC Linker

The effectiveness of the sulfo-SMCC linker was examined by spotting 1.0 µM solution of probe oligonucleotides in 1xPBS buffer onto sites where the linker was present and absent on the oligomer surfaces. The fluorescence intensity of the probes associated with the various oligomer surfaces is shown in Figure 3.22. It is evident that the probes were immobilized onto the surface only in the presence of the linker. Thus, the results suggest that the probes are site directed onto the amines of the terpolymer.

![Fluorescence intensity of Cy 5 associated with SMN1 probes that were immobilized in 1xPBS buffer on various oligomer surfaces, in the presence and absence of the sulfo-SMCC linker after boiling in sterile water twice, for 5 minutes each time.](image-url)
PEG1 and PEG2 are neutral oligomers with an average chain length of 15 and 67 repeating units of ethylene glycol, corresponding to 69 Å and 294 Å in length, respectively. MAA and dC20 are negatively charged oligomers with approximately 13 and 20 monomer units with chain lengths of 45 Å and 88 Å, in length respectively. The lengths of PEG1, PEG2 and MAA were estimated from the lengths of individual bonds [148] present in the chain. The surface without oligomer served as the control for determination of efficiency of immobilization. It was expected that the presence and properties of the oligomers on the surface would affect probe immobilization efficiency because the accessibility of probes to the surface would be hindered in comparison to a surface without oligomer. Also, amines on nucleotide sequences can bind to the uncapped epoxide sites on the surface without oligomer, which would reduce the probe immobilization efficiency. As for the PEG surfaces, polyethylene glycols are well known for reducing non-specific adsorptions of nucleic acids on surfaces. The interaction of the probe oligonucleotides with the surface would be ameliorated. PEG2 has a longer chain, which could hinder the accessibility of probe oligonucleotides to the surface for immobilization as compared to the shorter PEG1. This would give a reduction in fluorescence intensity relative to PEG1. Considering the negatively charged surfaces, MAA has a lower charge density than dC20, and a reduction in net charge should reduce the charge repulsion effect on the probes in terms of access to the surface. A higher fluorescence signal was expected for a larger quantity of probe molecules. However, such a trend was not observed in these experiments, and no significant differences in probe fluorescence signal were observed for these oligomer surfaces. The results suggested that the probe immobilization efficiency was governed predominantly by the surface activation of the sulfo-SMCC, where the thiolated probes coupled to the reactive maleimido groups for site directed probe immobilization.
4.1.2 Relative Efficiency of Immobilization of Probes

Immobilization using different solution concentrations of probes was done on PEG1, PEG2, MAA and dC$_{20}$. The relative fluorescence intensity of the labeled probe oligonucleotides is shown in Figure 3.23. Increased fluorescence intensities were observed with increasing probe concentrations. There are no significant differences in the trend for immobilization on the neutral PEG surfaces (Figure 3.23(a)). This was expected because the two PEG systems have the same chemical functionality, and that length differences did not dominate the probe immobilization efficiency. For the negatively charged surfaces, the dC$_{20}$ surfaces showed a similar trend to those for MAA (Figure 3.23(b)). However at higher probe concentrations (1.0 µM) the resulting fluorescence intensity for dC$_{20}$ was lower than that of MAA. This may be due to the effect of negative charge repulsion that reduced the accessibility of probes to the surface for binding. Since dC$_{20}$ has more negative charges than MAA, the effect would be greater.
Figure 3.23. Immobilization of SMN1 probes at different concentrations in 1xPBS buffer for 2 hours on (a) neutral PEG1 and PEG2, and (b) negatively charged MAA and dC20 oligomer surfaces.
Average probe density on the surface was estimated from a calibration curve, shown in Figure 3.24, obtained from data collected by Omair Noor of the Chemical Sensors Group at University of Toronto Mississauga. The calibration curve was constructed by spotting known volumes of various concentrations of Cy 5 labeled SMN1 probes onto glutaraldehyde functionalized glass slide. The spots were air dried without rinsing or washing. The slide was scanned with the confocal microscope used in the work of this thesis. It was assumed that the background subtracted fluorescence intensity from each spot corresponded to the amount of probes spotted onto the surface. The surface chemistry was not a factor in this case because the intention was to determine the correlation between Cy 5 fluorescence intensities to the number of labeled probe molecules present on the surface. Self quenching of Cy 5 was observed when probe density was greater than $4.1 \times 10^{12}$ molecules/cm$^2$ (data not shown). The average probe density on the oligomer surfaces was estimated to be $2.2 \pm 0.3 \times 10^{12}$ molecules/cm$^2$, which was within the linear region of the calibration curve.
Figure 3.24. Calibration curve of background subtracted Cy 5 fluorescence intensity per cm$^2$ to probe density of single stranded probe oligonucleotides in cm$^2$ estimated from data collected by Omair Noor of the Chemical Sensors Group at UTM. The equation and $R^2$ for the line of best fit are shown.

On surfaces with monolayer coverage of pure probe oligonucleotides, high probe density is in the order of $10^{12}$ molecules/cm$^2$ or greater and low probe density is on the order of $10^{10}$ molecules/cm$^2$ or less [149]. Since the estimated average probe density was $2.2 \pm 0.3 \times 10^{12}$ molecules/cm$^2$ on the oligomer surfaces, it is in the high probe density regime compared to surfaces with monolayer coverage of pure oligonucleotides. The terpolymer design diluted the sites for probe immobilization with sites for oligomer binding and surface anchoring of the
terpolymer. The terpolymer contains 1 probe immobilization site in every 6 functional sites. The probe density was greater than that for mixed films of PHEMA and probe oligonucleotides made by ‘grafting from’ technique. The reported probe density was \(8.4 \times 10^9\) molecules/cm\(^2\) for 1:1 ratio of the surface functional sites for probe immobilization and PHEMA synthesis \([129]\). This indicated that the terpolymer film was likely a multilayer providing more sites for probe immobilization in comparison to the PHEMA mixed film.

4.2 Sensitivity of the Mixed Film in Target Hybridization

Mixed film surfaces prepared for target hybridization were made using solutions of 1.0 \(\mu\)M probe. The resulting surfaces were boiled in sterile water for 5 minutes. Cy 5 labeled targets were used to track hybridization. The target solutions were prepared in 1.0xPBS buffer at pH 7 and were introduced to the surface as 2 \(\mu\)L spots. Hybridization was done at room temperature in a dark and humid environment for 30 minutes. The surfaces were washed with hybridization buffer and were then scanned with the confocal microscope. The sensitivity of response of the mixed films was investigated using submicromolar target concentrations to reflect the concentration regime of typical analytical devices.

4.2.1 Hybridization of Fully Complementary and Non-Complementary Targets

The oligomer surfaces containing PEG1, PEG2, MAA and dC\(_{20}\) with non-labeled probe oligonucleotides were subjected to 0.1 \(\mu\)M fully complementary (FC) and non-complementary (NC) targets that were both labeled with Cy 5. The fluorescence intensities of the targets in Figure 3.25(a) showed that the probes immobilized on the surfaces were available for target
hybridization. The low signal intensities from non-complementary targets indicated low non-specific adsorption on all the surfaces, and confirmed that the mixed films had selectivity towards fully complementary targets. Both of the different PEG surfaces showed similar fluorescence intensities for fully complementary target, and so did the MAA and dC_{20} surfaces. However, the negatively charged surfaces gave higher fluorescence intensities than the neutral PEG surfaces.

The difference in signals between the neutral and charged films can be reasoned by deviation in probe density, availability of probes for target hybridization and stability of the hybrids in the mixed films. As shown in Figure 3.2, no significant differences in probe density were observed between the mixed films. The neutrality of PEG may have allowed more PEG molecules to be immobilized onto the terpolymer surface in comparison to the charged oligomers. This would reduce the availability of probes on PEG surfaces due to steric effects. However, the neutrality of PEG could also contribute to stabilization of the hybrids. Although electrostatic repulsion from the charged films may have increased the availability of probes, it is likely that an increase of charge density also resulted in destabilization of hybrids. Therefore, the results are a combined effect of the factors.
Figure 3.25. (a) Fluorescence signals from 0.1 µM Cy 5 labeled fully complementary (FC) and non-complementary (NC) targets hybridized on various mixed film oligomer surfaces containing immobilized probe. (1.0xPBS, 30 minutes hybridization time) (b) Integrated fluorescence intensities from Cy 3 labeled FC and NC targets on PHEMA mixed films, adapted from reference [109] with permission from Elsevier.
The averaged ratios of fluorescence intensities of FC in comparison to NC for the neutral PEG surfaces was 6.7 and for the negatively charged surfaces was 12.2. The integrated fluorescence intensities of FC and NC targets introduced to PHEMA mixed films is shown in Figure 3.25(b), with a FC/NC ratio of 30.5 [109]. This value is much greater than those obtained from the terpolymer film.

4.2.2 Hybridization of Fully Complementary Targets at Different Concentrations

Fully complementary targets were introduced to the surface in concentrations of 0.01 µM, 0.05 µM and 0.10 µM. These concentrations were tested in order to find an appropriate working target concentration for the mixed films that would be suitable for detection of mismatched targets in later experiments. Fluorescence intensities of the targets on the mixed films of PEG1, PEG2, MAA, dC20 and without oligomer are shown in Figure 3.26. The results showed that the surfaces were functional for target hybridization and confirmed that the presence of different oligomers on the surface modified the hybridization profiles. The surface without oligomer was included as a control. A target concentration of 0.1 µM was used for subsequent hybridization experiments. This maximized the fluorescence signal from mixed films, and represented a concentration that is encountered in practical assays of oligonucleotides.
As expected, the fluorescence intensity observed for PEG1 and PEG2 showed similar trends for target hybridization. Saturation was observed at 0.5 µM target solution on PEG surfaces. The negatively charged mixed films of MAA and dC_{20} showed small differences in hybridization profile. The fluorescence intensity of targets for the MAA surface followed a linear trend with increasing target concentration, whereas the dC_{20} surface showed curvature and a greater slope for hybridization for samples at lower concentration. Since there is a GGG sequence on the targets, it may have associated with the dC_{20} oligomers and facilitated hybridization with the probes. Although the MAA surface can form hydrogen bonds with the nucleobases on the targets, it did not hinder target hybridization. This was supported by the
response profile from the surface without oligomer, where there are no functional groups on the surface that could interact with the targets.

4.2.3 Hybridization Efficiencies of the Mixed Films

Hybridization efficiency experiments used Cy 5 labelled probes and targets. Cy 5 labeled probes were spotted on the first column of slides similar to that shown in Figure 3.21(a). The remaining columns contained non-labeled probes. Cy 5 labeled fully complementary targets of 0.1 µM in 1.0xPBS buffer were introduced to the sites that were occupied with non-labeled probes. The surface was rinsed with hybridization buffer and dried, followed by scanning with confocal microscope. Hybridization efficiencies for each surface were determined by taking the ratio of Cy 5 fluorescence intensity of the labeled targets over the average fluorescence intensities gathered from the Cy 5 labeled probes.

Similar hybridization efficiencies of 33 ± 5 % and 28 ± 4 % were obtained for the neutral surfaces of PEG1 and PEG2 respectively. The negatively charged surfaces, MAA and dC20 gave hybridization efficiencies of 54 ± 4 % and 43 ± 4 % respectively. The negatively charged surfaces showed higher hybridization efficiencies than the neutral surfaces.

For monolayer coverage of short probes on gold [98] and glass surfaces [102], hybridization efficiencies were found to increase with decreasing probe densities. This is because both electrostatic and steric effects were reduced at lower probe densities [98]. High probe densities with ~10^{12} molecules/cm^{2} or greater were reported to give less than 20 % hybridization efficiencies [98], whereas low probe densities with ~10^{10} molecules/cm^{2} or lower yielded hybridization efficiencies of 80 % to 100 % [102]. Comparing the hybridization efficiency of the
mixed films to the reported ranges, the mixed film surface is consistent with having intermediate probe densities, which is the regime between the defined high and low probe densities. However, the estimated average probe density on the mixed film was $2.2 \pm 0.3 \times 10^{12}$ molecules/cm$^2$. Since there are three times as much of the oligomer on the surface in comparison to the probes, the overall surface density of probes and oligomer is $\sim 9 \times 10^{12}$ molecules/cm$^2$. This fell in the high density regime, which indicated that the hybridization efficiency was higher than expected due to the multilayer mixed film. Hybridization efficiency of 71% was reported for mixed film made from surface initiated PHEMA [129]. This is greater than the hybridization efficiency obtain from the neutral PEG1 and PEG2 surfaces because the probe density on PHEMA mixed film was $8.4 \times 10^9$ molecules/cm$^2$ [129], which is lower than those on the PEG surfaces.

Hybridization efficiency is not expected to change significantly if the probe density is lowered. This is because the probes are assumed to be isolated from one another by the oligomers, and the environment of the probes remained unchanged. However, a change in overall density of oligonucleotides and oligomers on the surface would affect hybridization efficiency.

It may be possible to better control the deposition of a monolayer of the terpolymer on the surface by controlling the moisture content in the coupling reaction. This could be done by refluxing the terpolymer in a low moisture content solvent at elevated temperature. However, in comparison to spin coating and baking technique, greater amount of terpolymer is needed in order to immerse the slides in the coupling solution. There is a possibility that incomplete coverage of the surface may occur with a monolayer of terpolymer, which would lead to non-specific adsorption.
4.3 Selectivity of the Mixed Film with Mismatch Targets

The mixed films investigated were PEG1, MAA and dC$_{20}$. PEG2 was not studied due to its similarity to PEG1. PEG1, MAA and dC$_{20}$ were short oligomers with 20 repeating units or less. These oligomers served to control the environment of the probes on the surface by reducing probe-to-probe and probe-to-surface interactions. This would increase the selectivity of the surface towards target capture. The surface charge densities among the mixed films are different. PEG1 is neutral, MAA is partially negatively charged dependent on pH, and dC$_{20}$ is permanent negatively charged. The degree of deprotonation of MAA affects the oligomer brush height. A chain becomes more extended with a greater number of negative charges on the oligomer. This is due to charge repulsion between carboxylates. Thus, it is expected that the properties of the oligomers would affect target hybridization and duplex stability under different conditions. Mixed films of neutral PHEMA [109] and negatively charged polyethylene glycol phosphate oligomers [110] grown by “grafting from” methods were reported to increase target selectivity in comparison to pure oligonucleotide films.

It has been shown in previous sections that the oligomers and probes are site directed onto the terpolymer surface. The mixed films were functional for hybridization with fully complementary targets and were able to discriminate non-complementary targets. This section investigates the selectivity of the mixed films with 19-mer fully complementary, single base pair mismatch, two base pair mismatch and non-complementary targets. The mismatched position is located in the middle of the targets. The selectivity of the surfaces was investigated in terms of solution pH, ionic strength and temperature.
4.3.1 Effect of pH on PEG1 and MAA Surfaces

The selectivity of PEG1 and MAA surfaces were studied at pH 5, 7 and 9 with 0.1 µM fully complementary, single base pair mismatch and non-complementary targets. The hybridization solutions were adjusted to a selected pH using diluted hydrochloric acid and sodium hydroxide.

The fluorescence intensities of Cy 5 labeled targets introduced onto PEG1 and MAA surface at pH 5, 7 and 9 are shown in Figs. 3.27 and 3.28, respectively. Cy 5 fluorescence emission was not affected by change in solution pH between pH 3 and 10 as reported in reference [150]. Therefore, the fluorescence signal observed was indicative of target binding on the surface. Selectivity ratios calculated from the ratio of fluorescence intensities of the targets to that of the fully complementary target on the various surfaces are summarized in Table 3.6.
Figure 3.27. Fluorescence intensity of Cy 5 labeled fully complementary (FC), single base pair mismatch (1bp) and non-complementary (NC) targets hybridized on PEG1 surfaces from 0.1 µM target concentration in pH 5, 7 and 9, 1.0xPBS hybridization buffer.
Figure 3.28. Fluorescence intensity of Cy 5 labeled fully complementary (FC), single base pair mismatch (1bp) and non-complementary (NC) targets hybridized on MAA surfaces from 0.1 µM target concentration in pH 5, 7 and 9, 1.0xPBS hybridization buffer.

Table 3.6. Summary of selectivity ratios shown in percentages calculated from the fluorescence intensities of non-complementary (NC) and single base pair mismatch (1bp) targets to that of the fully complementary targets at pH 5, 7 and 9 on PEG1 and MAA surfaces.

<table>
<thead>
<tr>
<th></th>
<th>pH 5</th>
<th></th>
<th>pH 7</th>
<th></th>
<th>pH 9</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>1bp/FC (%)</td>
<td>NC/FC (%)</td>
<td>1bp/FC (%)</td>
<td>NC/FC (%)</td>
<td>1bp/FC (%)</td>
<td>NC/FC (%)</td>
</tr>
<tr>
<td>PEG1</td>
<td>73 ± 4</td>
<td>48 ± 10</td>
<td>63 ± 12</td>
<td>13 ± 4</td>
<td>66 ± 13</td>
<td>9 ± 4</td>
</tr>
<tr>
<td>MAA</td>
<td>76 ± 8</td>
<td>33 ± 2</td>
<td>62 ± 6</td>
<td>12 ± 1</td>
<td>74 ± 2</td>
<td>6 ± 2</td>
</tr>
</tbody>
</table>
The selectivity towards single base pair mismatch targets on PEG1 surfaces did not show significant difference between the three pH levels. This was expected as a change in solution pH would not affect the neutral PEG1 surface because the oligomer does not contain ionizable functional groups. At pH 5, increased fluorescence signal from all three targets was observed in comparison to signals from pH 7 and 9. This suggested that non-specific adsorption was more prominent at pH 5.

MAA is sensitive to pH, where the carboxylic acid groups can be driven between protonated and deprotonated form. The pK<sub>a</sub> of MAA oligomers deviate with its length, conformation and environment. Using combined quantum mechanical and continuum solvation methods, Dong and coworkers predicted that the pK<sub>a</sub> of MAA dimer and trimer are 0.8 pK<sub>a</sub> units higher than the predicted monomer value of 4.83 [151]. They later used the continuum dielectric model of the Poisson-Boltzmann equation to predict the intrinsic pK<sub>a</sub> of MAA oligomers with 2 to 20 repeating units [152]. Taking the conformations of MAA oligomers into account, the pK<sub>a</sub> value was found to converge at 7.5 when the chain length is greater than 12 units [152]. Surface bound polymethacrylic acids have been reported to have pK<sub>a</sub> values ranging from 4 to 5 [137]. In multilayers of polymethacrylic acids and highly charged polycations, the pK<sub>a</sub> was reported to be around 6 to 7 [153]. From the study of interactions of globular proteins with polymethacrylic acids in multilayer configuration, it was found that at pH 5, the protein was stabilized through extensive hydrogen bondings that involved the protonated carboxylic groups [153]. The hydrogen bonds could not be disrupted by high ionic strength conditions. However, at higher pH levels, where the carboxylic groups are deprotonated and electrostatic charges dominate, the stability of the proteins decreased dramatically with increasing ionic strength [153].
The selectivity on the MAA surface was affected by the change in solution pH. The charges on the surface played a role in reducing non-specific adsorption. High non-specific adsorption was observed at pH 5 relative to signals from pH 7 and 9. This could be a result of hydrogen bondings between the protonated MAA oligomer and DNA targets [153]. The differences in signals between pH 5 and 7 reflected that the pK$_a$ of the MAA oligomer on the mixed film is between 5 and 7. This is higher than the reported value for surface bound polymethacrylic acid. The pK$_a$ was shifted because the mixed film surface is not homogenous and the presence of probe oligonucleotides may have changed the environment for MAA ionization. Therefore, at pH 7 and 9, MAA is deprotonated and charged, giving similar results within precision error.

Between pH 7 and 9, the PEG1 surface showed comparable selectivity with higher target fluorescence intensities observed at pH 9. As for MAA surface, the selectivity was better at pH 7, and non-specific adsorption was reduced at pH 9. Since pH 7 is closer to physiological pH and compatible with bioanalytes, pH 7 was used in subsequent hybridization experiments.

4.3.2 Effect of Ionic Strength on PEG1, MAA and dC$_{20}$ Surfaces

The selectivity of PEG1, MAA and dC$_{20}$ surfaces were investigated with fully complementary, single base pair mismatch and two base pair mismatch targets in hybridization buffers containing ionic strengths of 0.1xPBS, 0.5xPBS and 1.0xPBS at room temperature. Ionic strength of the hybridization solutions provided counterions to screen the negative charges on probes, targets and the oligomers.
Figure 3.29. Fully complementary (FC), single base pair mismatch (1bp) and two base pair mismatch (2bp) targets hybridized on PEG1 surfaces at pH 7 in hybridization buffers with ionic strengths of 0.1xPBS, 0.5xPBS and 1.0XPBS.

Figure 3.30. Fully complementary (FC), single base pair mismatch (1bp) and two base pair mismatch (2bp) targets hybridized on MAA surfaces at pH 7 in hybridization buffers with ionic strengths of 0.1xPBS, 0.5xPBS and 1.0XPBS.
Figure 3.31. Fully complementary (FC), single base pair mismatch (1bp) and two base pair mismatch (2bp) targets hybridized on dC20 surfaces at pH 7 in hybridization buffers with ionic strengths of 0.1xPBS, 0.5xPBS and 1.0xPBS.

Table 3.7. Selectivity ratio of fluorescence intensities of single base pair mismatch (1bp) and two base pair mismatch (2bp) targets in comparison to that of fully complementary (FC) targets on PEG1, MAA and dC20 surfaces. The ratio for PHEMA mixed film was calculated from results in reference [109]. Hybridization was performed at room temperature in 1.0xPBS buffer.

<table>
<thead>
<tr>
<th>Mixed Film</th>
<th>1bp/FC (%)</th>
<th>2bp/FC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG1</td>
<td>49 ± 7</td>
<td>41 ± 6</td>
</tr>
<tr>
<td>MAA</td>
<td>67 ± 10</td>
<td>70 ± 12</td>
</tr>
<tr>
<td>dC20</td>
<td>59 ± 10</td>
<td>56 ± 4</td>
</tr>
<tr>
<td>PHEMA</td>
<td>33 ± 24</td>
<td>-</td>
</tr>
</tbody>
</table>
It was observed in Figs. 3.29, 3.30 and 3.31 that the hybridization signal and target selectivity were dependent on the ionic strength of the hybridization buffer. Increased fluorescence signals were obtained from targets in buffers with higher ionic strength. Hybrids with greater stability were formed due to reduced electrostatic repulsions between the surface and targets due to charge screening [149]. The magnitude of increased signals for the fully complementary targets was greater than those from mismatched targets. This indicated that the hybrids of fully complementary targets were more stabilized than those with mismatched targets.

The selectivity of the surface can be tuned by ionic strength. In comparison to pH experiments shown in the previous section, the effect of ionic strength on target selectivity was more significant. At low ionic strength, a change in pH between 4 and 9 would not affect the activity coefficient significantly. In the range of ionic strengths investigated, the best selectivity was observed at high ionic strength with 1 M NaCl for all three surfaces. Although the trend shows improved selectivity with higher ionic strength, salt concentrations greater than 1 M were not used. Background fluorescence intensity from the salt increases with salt concentration, and the usual working range for PBS buffers is within 0.1 to 1.0 M salt concentration in nucleic acid assays. The high salt dominance for target selectivity is consistent with results observed by Watterson and coworkers [154].

The selectivity ratios in percentages for PEG1, MAA and dC\textsubscript{20} calculated from the ratio of fluorescence intensities of single base pair mismatch and two base pair mismatch targets to that of the fully complementary target hybridized in 1.0xPBS buffer at room temperature are summarized in Table 3.7. The ratio for PHEMA mixed film was calculated from results in reference [109]. The probe and target sequences used on the PHEMA mixed film are identical to the sequences used in the work of this thesis. PEG1 surface shows comparable selectivity to the
PHEMA mixed film for distinguishing fully complementary from single base pair mismatch targets.

Among the surfaces examined, PEG1 and dC\textsubscript{20} showed improved target selectivity at 1.0xPBS. The MAA surface did not show such improvements. This suggested that the oligomers in the mixed film may have participated in the stabilization of the fully complementary duplex and destabilization of the mismatched hybrids. The resultant of these effects was not enough to improve selectivity on the MAA surface. This confirms that charge of the oligomers does not dominate control of selectivity, and answers one of the primary questions posed in this thesis.

4.3.3 Effect of Temperature on PEG1, MAA and dC\textsubscript{20} Surfaces

The effect of temperature on target selectivity is studied by melt curve experiments. Conventional melt curve experiments are done by monitoring the fluorescence intensity of the hybridized targets over temperature ranges with a ramp rate of 0.3 °C/min [110]. This is to ensure equilibrium is reached at the surface. However, the surfaces investigated in this thesis do not allow conventional melt curve determination. Therefore, a modified procedure was employed. Hybridization of Cy 5 labeled targets was done by appropriate sequential spotting at room temperature, with reactants kept for 30 minutes in a humid and dark chamber. Denaturation of the hybridized targets was done by immersing the surfaces in buffer solutions equilibrated at 25 °C, 30 °C, 35 °C, 40 °C, 45 °C and 50 °C for 3 minutes each. The fluorescence signals from the targets were obtained by scanning dried samples with the confocal microscope after treatment at each temperature. Since the experiments were done under the same conditions, the results are comparable. This is a procedure that has previously been used in studying temperature effects on stability and selectivity of hybridized duplexes [109].
The investigation used fully complementary, single base pair mismatch and two base pair mismatch targets. Denaturation profiles were collected using hybridization buffers with ionic strengths of 0.1xPBS, 0.5xPBS and 1.0xPBS. The normalized fluorescence intensities for targets in 0.1xPBS, 0.5xPBS and 1.0xPBS for PEG1 and MAA are shown in Figure 3.32. The results for dC_{20} are shown in Figure 3.33. It can be seen that as temperature increased, the hybridized duplexes became destabilized, resulting in reduction of fluorescence intensity. Full melt curve profiles could not be obtained using the mixed films prepared in this thesis work. The melt profile may have extended to temperatures below room temperature. However, these temperatures were not attempted because they are not within typical working temperature range of DNA biosensor assays. Complete denaturation of the duplexes from the surfaces was achieved at 40 °C.

The selectivity ratios determined from the fluorescence intensities of single base pair mismatch and two base pair mismatch targets in comparison to the fluorescence intensities of fully complementary targets at 25 °C, 30 °C and 35 °C in 0.1xPBS, 0.5xPBS and 1.0xPBS on PEG1, MAA and dC_{20} surfaces are summarized in Table 3.8.
Figure 3.32. Denaturation profile of fully complementary (FC), single base pair mismatch (1bp) and two base pair mismatch (2bp) targets in (a) 0.1xPBS, (b) 0.5xPBS and (c) 1.0xPBS on PEG1, and (d) 0.1xPBS, (e) 0.5xPBS and (f) 1.0xPBS on MAA surfaces. The associated statistical errors are showed in Table 3.8.
Figure 3.33. Denaturation profile of fully complementary (FC), single base pair mismatch (1bp) and two base pair mismatch (2bp) targets in (a) 0.1xPBS, (b) 0.5xPBS and (c) 1.0xPBS on dC_{20} surface. The associated statistical errors are showed in Table 3.8.
Table 3.8. Summary of selectivity ratios for 1bp/FC and 2bp/FC in percentages in 0.1xPBS, 0.5xPBS and 1.0xPBS buffers at 25 °C, 30 °C and 35 °C on (a) PEG1, (b) MAA and (c) dC20 surfaces.

(a) PEG1

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>0.1 x PBS</th>
<th></th>
<th>0.5 x PBS</th>
<th></th>
<th>1.0 x PBS</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1bp/FC (%)</td>
<td>2bp/FC (%)</td>
<td>1bp/FC (%)</td>
<td>2bp/FC (%)</td>
<td>1bp/FC (%)</td>
<td>2bp/FC (%)</td>
</tr>
<tr>
<td>25</td>
<td>47 ± 25</td>
<td>33 ± 5</td>
<td>51 ± 16</td>
<td>82 ± 23</td>
<td>41 ± 12</td>
<td>36 ± 3</td>
</tr>
<tr>
<td>30</td>
<td>54 ± 15</td>
<td>35 ± 5</td>
<td>44 ± 15</td>
<td>60 ± 2</td>
<td>38 ± 18</td>
<td>33 ± 8</td>
</tr>
<tr>
<td>35</td>
<td>68 ± 7</td>
<td>43 ± 21</td>
<td>71 ± 40</td>
<td>79 ± 14</td>
<td>36 ± 5</td>
<td>35 ± 1</td>
</tr>
</tbody>
</table>

(b) MAA

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
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<th></th>
<th>0.5 x PBS</th>
<th></th>
<th>1.0 x PBS</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1bp/FC (%)</td>
<td>2bp/FC (%)</td>
<td>1bp/FC (%)</td>
<td>2bp/FC (%)</td>
<td>1bp/FC (%)</td>
<td>2bp/FC (%)</td>
</tr>
<tr>
<td>25</td>
<td>51 ± 7</td>
<td>68 ± 3</td>
<td>73 ± 11</td>
<td>89 ± 3</td>
<td>61 ± 9</td>
<td>59 ± 15</td>
</tr>
<tr>
<td>30</td>
<td>47 ± 7</td>
<td>70 ± 18</td>
<td>64 ± 10</td>
<td>75 ± 4</td>
<td>54 ± 3</td>
<td>57 ± 4</td>
</tr>
<tr>
<td>35</td>
<td>32 ± 20</td>
<td>55 ± 4</td>
<td>62 ± 4</td>
<td>75 ± 2</td>
<td>41 ± 10</td>
<td>61 ± 8</td>
</tr>
</tbody>
</table>

(c) dC20

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>0.1 x PBS</th>
<th></th>
<th>0.5 x PBS</th>
<th></th>
<th>1.0 x PBS</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1bp/FC (%)</td>
<td>2bp/FC (%)</td>
<td>1bp/FC (%)</td>
<td>2bp/FC (%)</td>
<td>1bp/FC (%)</td>
<td>2bp/FC (%)</td>
</tr>
<tr>
<td>25</td>
<td>72 ± 21</td>
<td>72 ± 23</td>
<td>71 ± 10</td>
<td>75 ± 21</td>
<td>57 ± 3</td>
<td>49 ± 11</td>
</tr>
<tr>
<td>30</td>
<td>69 ± 25</td>
<td>75 ± 30</td>
<td>62 ± 33</td>
<td>60 ± 7</td>
<td>49 ± 5</td>
<td>49 ± 4</td>
</tr>
<tr>
<td>35</td>
<td>75 ± 38</td>
<td>78 ± 11</td>
<td>47 ± 20</td>
<td>57 ± 19</td>
<td>55 ± 9</td>
<td>47 ± 1</td>
</tr>
</tbody>
</table>
The melting temperature ($T_m$) is sensitive to the nature of substrate, immobilization chemistry and interfacial environment of the probes [155]. Various values of $T_m$ for SMN1 duplexes with different probe immobilization strategies are summarized in Table 3.9. A general trend in $T_m$ depression was observed for target hybridization with surface bound probes in comparison to hybridization in bulk solution. From the data collected in the temperature profiles, the melt temperature of SMN1 duplexes on PEG1, MAA and dC$_{20}$ mixed films can be estimated to be within 5 to 10 °C of room temperature. This is consistent with the results observed by Liu and Krull [156] and it is possible to shift the $T_m$ closer to room temperature. Single nucleotide polymorphism detection is typically done at elevated temperature in order to maximize selectivity. With the $T_m$ shifted closer to room temperature, it is possible to improve selectivity at room temperature.

Table 3.9. Summary of $T_m$ values for 19-mer SMN1 fully complementary (FC) and single base pair mismatch (1bp) targets on glass surfaces with probes immobilized on silica microbeads [156], probes synthesized in situ [157], PHEMA mixed film [109], and in bulk solution [109]. The ratio and temperature that represented the best selectivity on the surfaces are included. The types of hybridization solutions used in the experiments are listed.

<table>
<thead>
<tr>
<th>Immobilization Methods</th>
<th>$T_m$ of FC (°C)</th>
<th>$T_m$ of 1bp(°C)</th>
<th>1bp/FC (%)</th>
<th>Hybridization Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probes on Silica Microbeads</td>
<td>28$^{a}$</td>
<td>27$^{a}$</td>
<td>19.3 (30 °C)$^{d}$</td>
<td>1xSSC, 0.1 % PVP</td>
</tr>
<tr>
<td>In Situ Probe Synthesis</td>
<td>48$^{b}$</td>
<td>32$^{b}$</td>
<td>17.8 (40 °C)$^{c}$</td>
<td>1xSSPE, 0.1 % PVP</td>
</tr>
<tr>
<td>PHEMA Mixed Film</td>
<td>48.5 ± 0.8</td>
<td>43.9 ± 0.3</td>
<td>26.7 (40 °C)$^{e}$</td>
<td>1xPBS</td>
</tr>
<tr>
<td>Bulk Solution</td>
<td>65.9 ± 0.1</td>
<td>48.0 ± 1.3</td>
<td>-</td>
<td>1xPBS</td>
</tr>
</tbody>
</table>

$^a$ Estimated from partial melt curve, $^b$ Estimated from full melt curve, $^c$ Estimated from normalized data, $^d$ Estimated from raw fluorescence intensities, $^e$ Selectivity ratio in 0.5xPBS
Among the ionic strengths surveyed, selectivity was observed at 1.0xPBS. This suggested that charge screening was important to maximize differences in thermal stability. PEG1 surfaces also showed selectivity at 0.1xPBS, but not at 0.5xPBS. MAA and dC$_{20}$ surfaces did not show significant selectivity at 0.1xPBS and 0.5xPBS.

The affinity constant of a DNA duplex is dependent on the on rate and off rate of DNA association. For a 19-mer mixed base hybrid on gold, the affinity constant was found to be $1.47 \times 10^8$ M$^{-1}$ by electrochemical impedance spectroscopy [158]. It was deduced from on-rate of $4.93 \times 10^5$ M$^{-1}$min$^{-1}$ and off-rate of $3.36 \times 10^3$ min$^{-1}$ [158]. The rates vary with interfacial properties such as probe length, probe density and immobilization method [159], [160], [158]. Therefore, it is expected that the association and dissociation constants of DNA hybrids would change on different surfaces.

4.4 Regeneration of Surfaces

The mixed film surfaces were investigated for repeated use using 0.1 $\mu$M targets in 1.0xPBS. The surface was regenerated by denaturation of the hybridized duplex in 60 °C sterile water for 5 minutes. The surface was scanned after each hybridization and denaturation cycle.

4.4.1 Cycles of Regeneration

The PEG1, PEG2, MAA and dC$_{20}$ mixed film surfaces were investigated using fully complementary targets. The fluorescence intensities of the mixed films for 5 subsequent hybridization and denaturation cycles are shown in Figure 3.34. Among the surfaces, PEG1 showed a decrease to half of the initial target fluorescence signal in the second hybridization
cycle. There are no other significant changes for cycles of hybridization [129]. Thus, the surfaces can be regenerated at least 4 times for detection of fully complementary targets.

Figure 3.34. The oligomer surfaces, PEG1, PEG2, MAA and dC\textsubscript{20}, hybridized (H) with fully complementary targets for 30 minutes at room temperature and denatured (D) by immersing in 60 °C sterile water bath for 5 minutes.
4.4.2 Selectivity of Hybridization of Targets on PEG1 and MAA Surfaces

The selectivity of fully complementary, single base pair mismatch and non-complementary targets on PEG1 and MAA surfaces was investigated in two consecutive hybridization cycles. The fluorescence intensities of fully complementary, single base pair mismatch and non-complementary targets in two cycles of hybridization are shown in Figure 3.35.

![Bar chart showing fluorescence intensities of Cy 5 labeled targets on PEG1 and MAA surfaces](image)

Figure 3.35. Subsequent hybridization fluorescence intensities of Cy 5 labeled 0.1 µM fully complementary (FC), single base pair mismatch (1bp) and non-complementary (NC) targets in pH 7 1.0xPBS hybridization buffer at room temperature on PEG1 and MAA mixed film surface.
Regeneration of surfaces by thermal denaturation freed up binding sites on the surface [154]. In subsequent hybridization, these sites become available for both target hybridization and non-specific adsorption, which are two competing processes. Target hybridization was indicated by the difference in fluorescence signals from the targets on PEG1 and MAA surfaces. Non-specific adsorption was observed on the MAA surface as suggested by the increase in non-complementary target fluorescence intensity in the 2nd hybridization. However, PEG1 surface showed similar non-complementary target signals in both hybridizations. This demonstrated that the regenerated MAA surface was more susceptible to non-specific adsorption than the regenerated PEG1 surface.

A decrease in fluorescence intensity for fully complementary and single base pair mismatch targets was observed in the 2nd hybridization on the PEG1 surface. However, the MAA surface showed an opposite trend, with an increase in fluorescence intensities for all three targets in the 2nd hybridization. This is consistent with results obtained from regenerated surfaces that were coated with oligonucleotides only [154]. It was suggested that on charged surfaces, the presence of fully complementary targets provided a driving force to release adsorbed materials on the surface and activate the surface for subsequent hybridization [154].

The selectivity ratios of fluorescence intensities of single base pair mismatch and non-complementary targets to that of fully complementary targets for PEG1 and MAA surfaces in the two hybridization cycles are summarized in Table 3.10. The numerical data indicated that the selectivity towards single base pair mismatch target on PEG1 surface was improved on the regenerated surface, whereas the selectivity on MAA surface was reduced due to non-specific adsorption.
Table 3.10. Summary of selectivity ratios shown in percentages calculated from the fluorescence intensities of non-complementary (NC) and single base pair mismatch (1bp) targets to that of the fully complementary targets on PEG1 and MAA surfaces for subsequent hybridizations.

<table>
<thead>
<tr>
<th></th>
<th>1bp / FC (%)</th>
<th>NC / FC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG1 1st Hybridization</td>
<td>68 ± 15</td>
<td>22 ± 2</td>
</tr>
<tr>
<td>PEG1 2nd Hybridization</td>
<td>44 ± 9</td>
<td>46 ± 6</td>
</tr>
<tr>
<td>MAA 1st Hybridization</td>
<td>41 ± 6</td>
<td>22 ± 1</td>
</tr>
<tr>
<td>MAA 2nd Hybridization</td>
<td>68 ± 1</td>
<td>37 ± 1</td>
</tr>
</tbody>
</table>

MAA oligomer can be a suitable substitute for dC\textsubscript{20} when considering development of surfaces of nucleic acid biosensors. Although the experimental results did not show significant improvements in selectivity on the MAA surface in comparison to dC\textsubscript{20}, they are comparable. The advantage of MAA over dC\textsubscript{20} is that MAA is not oligonucleotide based, which will reduce possible non-specific interactions that can occur between oligonucleotides. Moreover, the dC\textsubscript{20} mixed film will be limited from analyzing targets with sequences having significant content of G. Therefore, the dC\textsubscript{20} mixed film was used for comparison of a negatively charged surface, and represents the surface with pure oligonucleotides only.
Chapter 4
Conclusions and Future Directions

1 Conclusions

The intent of this thesis was to fabricate a film at a surface that contained a mixture of oligonucleotide probes and oligomers to examine whether charged oligomers would be beneficial in controlling the selectivity of oligonucleotide hybridization. The surface was constructed using a terpolymer as a template for site directed immobilization of probes and oligomers. The synthesized photolabile terpolymer was found to contain a ratio of 1 : 3 : 2 of NVOC-amine : epoxide : silyl groups, respectively, determined from NMR, MALDI-ToF and elemental analysis. Characterization of surface bound terpolymer by XPS and ellipsometry suggested a thick film on the surface. Quantitative removal of NVOC on surface bound terpolymer was indicated in XPS and ToF-SIMS results.

The environment experienced by the probes on the surface was modified by selection of neutral and negatively charged oligomers represented by PEG, MAA and dC20. It was shown that probes and oligomers could be immobilized onto the amine and epoxide sites, respectively, on the terpolymer. The presence of oligomers on the surface did not significantly affect the efficiency of probe immobilization. However, the oligomers were found to modify the hybridization behavior of probes.

It was possible to tune selectivity to distinguish fully complementary and single base pair mismatch targets. The oligomers in the mixed film may have participated in the stabilization of the fully complementary duplex and destabilization of the mismatched hybrids. Selectivity is the result of competing processes of target hybridization and non-specific adsorption. It was shown
that at pH 7 and high salt condition (1 M NaCl), hybridization selectivity was improved on PEG1 and dC₂₀ mixed films at room temperature. The MAA surface did not show significant improvements in selectivity, which indicated that charge of the oligomers does not dominate control of selectivity. Non-specific adsorption could be ameliorated by adjusting the pH of the solution to 7 or 9.

Interestingly, it was estimated that the melting temperature of the 19-mer mixed base duplex was perhaps within 5 to 10 °C of room temperature. The results suggested that the terpolymer construct played a role in depression of meting temperature of the hybridized duplex. With the melting temperature shifted closer to room temperature, operation of the films at room temperature was sufficient for discrimination of hybridization between fully complementary and single base pair mismatch targets.

Surface regeneration studies showed that target signal was increased on the regenerated MAA surface. This is consistent with results from surfaces coated with oligonucleotides only. The results achieved using MAA as the oligomer was comparable to those using dC₂₀. This indicated that MAA was a suitable non-nucleotide oligomer that could be used to control surface charge while avoiding undesired interactions with target nucleic acids that could arise through interaction with guanine nucleobases when using dC₂₀.

2 Future Directions

Future work should include investigation of hybridization of long nucleic acid sequence targets from real samples on the mixed films. The charge density in the mixed films is expected to be lower in comparison to pure surfaces constructed from only oligonucleotides (as in the case
of microarrays and suggested for most biosensors), and the result may be that long targets can be better accommodated for hybridization. It is also the case that real samples may expose the sensing surface to proteins and other biological compounds. The degree of non-specific adsorptions onto the surface and the efficiency of target hybridization in the presence of these types of non-specific interactions should be evaluated. The work of this thesis suggests that amelioration of non-selective adsorption using these mixed films may be achieved by operating at basic pH conditions.

A further opportunity lies in the photolabile reagent that is presented in the synthesis methods of this thesis. The probe-to-oligomer ratio can be tuned by selective deprotection of the NVOC group on the terpolymer. As shown in the work of this thesis, selective deprotection can be achieved by reducing the time of UV irradiation. A change in probe-to-oligomer ratio will alter the probe and charge density on the surface. This may change target hybridization and selectivity of the mixed films. A reduction in the ratio can also reduce steric hindrance to accommodate the longer targets mentioned above.

The orientation of the probes in the mixed film, whether they are extended into solution or buried in the oligomer film, should be studied. This can be achieved with molecular modeling simulations. It can provide insights to whether and how the oligomers influenced the orientation of the probes in the mixed film. This affects the selectivity and performance of the surface. In addition, interactions between different oligomers and probes, targets or duplex can be explored for better understanding on the mixed film.

Since the probe length, surface structure and nature of the oligomers shift the melting temperature of hybridized duplex and selectivity of the surface, by optimizing the lengths of the probes on different oligomer surfaces it is possible to design a surface containing different probe
sequences that have the same melting temperature. This offers optimization of selectivity for fully complementary targets at all probe sites on the surface at one temperature, which is beneficial for quantitative analyses.
References


