Ternary sensing surface with DNA-based spacer group : characterization, comparison and optimization

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2013

Yang, Y. (2013). Ternary sensing surface with DNA-based spacer group: characterization, comparison and optimization. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/54958>

<https://doi.org/10.32657/10356/54958>

NANYANG
TECHNOLOGICAL
UNIVERSITY

TERNARY SENSING SURFACE WITH DNA-BASED SPACER

GROUP:

CHARACTERIZATION, COMPARISON AND OPTIMIZATION

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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2013

**TERNARY SENSING SURFACE WITH DNA-BASED SPACER GROUP:
CHARACTERIZATION, COMPARISON AND OPTIMIZATION**

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A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2013

ACKNOWLEDGEMENT

First of all, I would like to thank Prof Bo Liedberg for his invaluable knowledge and guidance that has benefited me tremendously during this period of time. I would also have to thank Prof Freddy Boey who has fully supported me from the start of my PhD years, without it I would not have come this far.

I also greatly appreciate all the help I get from all the research staffs who I have worked with during these 4 years. I would like to especially express my heartfelt gratitude towards Dr Umit Hakan Yildiz for his help and guidance for the past 2 years.

Lastly, to all my family and friends, thanks for supporting and assisting me in one way or another.

In the past decade, outbreaks of new diseases have brought much fear in the people worldwide. Many lives were lost due mainly to the late detection of these diseases as well as the lack of knowledge to prevent or cure them. To allow fast response to curb the spread of such infections, real-time sensors with high sensitivity and selectivity are required. With the advancement in technology and the need for miniaturization, biosensors based on electrochemistry has proven to be a powerful detection method due to the ease-of-use, low instrumentation cost, possible non-labeling and fast target detection. In an electrochemical detection, the sensitivity of the biosensors is mostly affected by the accessibility of the specific target towards the recognition site of the receptors immobilized on the electrode surface. To reduce the steric hindrances of the target molecules to the receptors, in this case the single-stranded DNA (ssDNA) probes, short organic spacer groups are normally used to modulate the ssDNA probe density on the electrode. In this report, a ternary sensing surface optimized using DNA-based spacer group for the detection of Methicillin-resistant *Staphylococcus aureus* (MRSA) has been created. As compared to commonly-used organic spacer group, such as 2-mercaptoethanol (ME), 3-mercaptoprop-1-panol (MP) and 6-mercapto-1-hexanol (MCH), thymine-based spacer groups (T9) displayed a 10-fold improvement of signal-to-noise in discriminating between complementary DNA (cDNA) and non-complementary DNA (NcDNA) hybridization. Analysis from Surface Plasmon Resonance (SPR), Quartz Crystal Microbalance (QCM-D) and electrochemistry showed a sensing surface of excellent selectivity, optimized at a ratio of 1:1 (probe:T9). On this surface, the ssDNA probes are aligned by the T9 spacer groups and thereby capable of maximizing cDNA hybridization and differentiating with non-specific NcDNA binding. Single-mismatch (SMM) detections have shown to be possible at this optimized ratio, with the ability to differentiate between the SMM at different positions. By creating similar sensing surfaces on gold-deposited microelectrodes, an improvement of the S/N by a factor of 8 was observed compared to the detection using planar gold electrodes, showing capabilities of creating a highly selective and sensitive biosensor with microelectrodes. Attempts were also made to create binary sensing surfaces and comparisons were made with using shorter thymine spacer

groups (T6) on both planar and microelectrodes. While all DNA-based spacer lengths were capable of modulating probe density and reducing steric hindrances, sensing surfaces created using the longer T9 spacer groups performed better in terms of S/N at their respective optimized ratio. Improvement in detection was observed for neutral PNA strands as compared to negatively-charged DNA strands at the same optimized ratio using microelectrodes.

Despite the general idea that a compact and uncharged layer is more desirable to form a highly selective biosensor based on electrochemistry, I have shown that as compared to the use of organic spacer groups, a more highly selective ternary sensing layer can be formed by using thymine-based spacer groups. Thymine spacers allow the assembly of a less compact sensing surface (with easily accessible domains in between the upright ssDNA probes) for efficient transfer of electrons across the surface, which is important for signal enhancement in an electrochemical biosensor. Furthermore, similar to one of the functions of the organic spacer groups, thymine spacers are also capable of removing the non-specific interactions between the DNA bases and gold due to their highly negative charged nature. In addition, thymine spacers having similar hydrophilicity to the ssDNA probe is likely to form a more homogenous sensing surface than that using the hydrophobic organic spacer groups. Lastly and most importantly, this work initiates and brings insights into the use of an alternative spacer group for the assembly of a sensing layer.

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1 Introduction

1.1 Background

In the past decade, outbreaks of new diseases have brought much fear in the people worldwide. To allow fast response to curb the spread of such infections, real-time sensors with high sensitivity and selectivity are required. In recent years, sensors based on electrochemistry have also been reported widely due to the ease-of-use, low cost, possible non-labeling and fast detection. The use of spacer groups is one of the methods used by many research groups to reduce steric hindrances and non-specific interactions^(1, 2), which are the two main factors that can affect the sensitivity and selectivity of the biosensors. In this thesis, in-depth investigation has been carried out on how DNA-based spacer groups will alter the surface receptor density on the electrode surface, and how this can eventually affect the sensitivity and selectivity of the biosensor.

1.2 Objectives

- To determine the capability of DNA-based spacer groups to improve sensor performance
- To understand how DNA-based spacer groups affect surface receptor densities
- To design an optimized platform for creating a highly sensitive and selective biosensor based on electrochemistry
- To apply DNA-based spacer groups on the sensing surface of planar and sonochemically-fabricated microelectrodes
- Compare the performance of DNA-based spacer groups with commonly-used organic spacers

1.3 Scope

The scope of study includes the use of spacer groups to form an optimized platform for the detection of 30mers linear Methicillin-resistant *Staphylococcus aureus* (MRSA) oligonucleotides. Spacer groups include thymine bases and commonly-used organic groups such as 2-

mercaptoethanol (ME), 3-mercaptop-1-propanol (MP) and 6-mercapto-1-hexanol (MCH). Detection capabilities will be based solely on electrochemistry and other techniques will only serve as surface characterization tools.

2 Literature Review

In the past decade, outbreaks of new diseases have brought much fear in the people worldwide. Epidemics which came as a surprise include the H1N1 virus infection in 2009 and the Severe Acute Respiratory Syndrome (SARS) in 2003. Many lives were lost due mainly to the lack of awareness, the late detection of these viruses, as well as the lack of knowledge to prevent or cure them.

To allow fast response to curb the spread of such viruses, real-time portable sensors with high sensitivity and selectivity are required. However, the development of such portable biosensors is facing many challenges which impede the commercialization of laboratory-based sensors. While most successful laboratory-based sensors depend on detections in an ideal environment, real samples are made up of complex compositions, such as the human body fluids, which make detections using real samples inaccurate or non-reproducible. Furthermore, the complexity of the platforms for developing some of the sensors also makes them difficult or impossible for unskilled people to use. Nevertheless, much effort has continuously been placed in developing easy-to-use sensors to accurately detect real samples ^(3, 4).

Currently, many different sensing techniques are used for the detection of various disease biomarkers. The available techniques generally include optical and physical sensing schemes, where signals change upon interaction with the target species due to the difference in, for example, refractive index ⁽⁵⁾ and mass ⁽⁶⁾.

One of the most common optical sensing techniques in biosensing is that using the surface plasmon resonance (SPR). Ever since gas sensing and selective antibody binding were first shown possible in 1983 ⁽⁷⁾, there has been a rapid increase in research pertaining to the potential of SPR in the field of biosensors. Sophisticated equipments using the SPR technique have since been build and commercialized, which allow numerous types of low level detections in applications such as medical diagnostics ⁽⁸⁾ and food safety ^(3, 9).

As its name implies, the SPR technique is based on the detection of a shift in the angle of the incident light occurring upon interaction between the surface plasmon evanescent wave and the surrounding medium. The shift in angle incidence is proportional to the change in refractive index η on the surrounding solution, so long as the changes in η are small. Shift in the resonance conditions is caused by changes in refractive indexes when an additional layer of biomolecules is allowed to attach on the sensing surface, eg. thin layer of gold. By monitoring the changes in refractive index of the sensing surface, kinetics in terms of the association and dissociation rate of the analyte can be determined in real-time. Depending on the type of biomolecular interactions used between the receptors on the sensing surface and the analyte introduced, highly selective and sensitive biosensors can be created ⁽¹⁰⁻¹⁴⁾.

Among the detection techniques based on physical sensing, the use of quartz-crystal microbalance (QCM) has garnered the most attention in the research field ^(15, 16). This is due to the direct quantification of analyte attachment by monitoring the change in frequency, which is directly related to mass by the Sauerbrey relation. In order for the Sauerbrey equation to be valid, several assumptions, such as the formation of a homogenous and rigid layer, have to be fulfilled. However, in most cases, the formed layer is not rigid, which will result in an overestimation of frequency (mass) change.

In order to ensure an accurate quantification of biomolecules on the quartz surface, QCM has also been equipped with simultaneous monitoring of dissipation changes (QCM-D) ^(17, 18). While dissipation changes are negligible ($D \approx 0$) for rigid films, significant dissipation changes will be observed for non-rigid films due to viscoelastic drag. The ability of QCM-D to calculate both the mass changes and the viscoelastic properties makes it an important tool to study the assembly of, for example, DNA layers ⁽¹⁹⁾.

In recent years, sensors based on electrochemistry have also been used widely due to the ease-of-use, low cost, possible non-labeling and fast detection ⁽²⁰⁻²⁴⁾. Electrochemistry studies the chemical

reactions that occur on or near the electrode surface in the solution (electrolyte), which result in a transfer of electrons between the working electrode and the redox species in the electrolyte. Redox species can either be the actual target species of detection or redox probes, such as $[\text{Fe}(\text{CN})_6]^{3-/4-}$, which are deliberately added into the electrolyte. Depending on whether electrons are being absorbed (reduction) or are released (oxidation) during the reaction, a difference in signal will be observed, which enables the detection of the target species.

Within the field of electrochemistry, one can broadly divide the methods of detection to voltammetric, amperometric and impedimetric sensing. In voltammetric and amperometric sensing, current is measured as a function of applied voltage and time, respectively. In the case of impedimetric sensing, an AC potential at a particular frequency is usually applied and the current is being measured, which eventually gives impedance (current/voltage). This approach is also known as the Electrochemical Impedance Spectroscopy (EIS), where measurements are repeated over a range of frequencies. EIS is sensitive to changes at the electrode-solution interface and hence probe–target surface interactions can be easily distinguished.

Electrochemical sensors started out as membrane-based electrode devices. With advancement in technology, miniaturization coupled to greater sensitivity and selectivity is desired to meet market demands. A strategy that can be employed to address these issues is to use microelectrode array-based sensors.

Microelectrodes come in different shapes and sizes, typically with at least one dimension less than $100\text{ }\mu\text{m}$ ⁽²⁵⁾. Commonly seen microelectrodes include the microdisks and the microbands, which can either be bought commercially or fabricated via lithographic methods. Individual microelectrodes can also be fabricated into regular arrays⁽²⁶⁾, random arrays^(27, 28) or interdigitated arrays⁽²⁹⁾.

As compared to planar electrodes, microelectrodes possess many attractive properties which are desirable in the field of sensing. Having a hemispherical solute diffusional profile, the microelectrode

arrays allow greater sensitivity and faster response time ⁽³⁰⁾. This is due to the enhanced mass transport and the higher rate of steady-state diffusion of reactants towards the electrodes, and products, if any, away from the electrode. The individual microelectrodes also results in a lower charging current, commonly also referred to as “noise” ⁽³¹⁾. Accumulated charging current is undesirable since it distorts the experimental signals that are being measured. When individual microelectrodes are spaced sufficiently apart to form an array such that the diffusion layer of each microelectrode overlap and behave planar-like, an overall surface area equivalent to the whole array ⁽³²⁾ will allow a larger cumulative signal response to be measured ⁽³²⁾, as compared to that from an individual microelectrode. Hence, with a reduced noise due to the smaller size of the microelectrode, and a higher measured signal due to the enhanced mass transport of the microelectrode array, signal-to-noise ratio can be improved significantly.

Various types of interactions can be used for electrochemical detection, such as enzymatic reactions ^(27, 33-37), antigen-antibody interactions ^(28, 38, 39) and DNA hybridization ^(4, 29, 40-48), to mention a few. These highly-specific interactions allow easy target recognition and are therefore widely used in biosensors.

In enzymatic biosensors, by-products produced by the catalytic reactions between the enzyme and substrate are usually the ones responsible for the change in the signal of detection. A well-known enzymatic reaction is that between glucose oxidase (usually anchored on the working electrode) and glucose (target species). Amperometric monitoring of its by-product, hydrogen peroxide (H_2O_2), shows the presence of glucose. Based on similar detection process, various enzymatic biosensors also can be fabricated to detect other targets ^(33, 35-37).

The strong affinity between an antigen and its specific antibody has also been employed in electrochemical biosensors. The antibody molecule is usually attached onto the substrate, while the antigen acts as the target molecule for detection. With a Y-shaped configuration, it is necessary that the antibody is being immobilized on the substrate in favourable orientation such that the binding

site for antigen is readily exposed. Even when oriented at the correct position, a closely packed layer of immobilized antibody can reduce the binding affinity due to steric hindrance ⁽⁴⁹⁾ and this is especially pronounced for high molecular weight targets. Hence, immobilized antibodies need to be oriented correctly and spaced sufficiently apart to achieve a low detection limit of the sensor.

DNA hybridization occurs when a single-stranded DNA (ssDNA) binds specifically to its target complementary strand. For biosensor development, one of the DNA strands, generally known as the DNA probe, is immobilized onto the working electrode before the addition of the target DNA for hybridization. This DNA probe can either be a linear or a structured single-stranded DNA.

An example of a structured DNA probe is the stem-loop DNA. This stem-loop DNA is basically made up of a linear single-stranded oligonucleotide, with a few deliberately included complementary bases at both ends of the actual sensing sequence for hybridization. These complementary bases will hybridize with each other to form a stem structure, resulting in the formation of a loop structure of the actual sequence for hybridization. Since first developed by Tyagi and Kramer in 1996 ⁽⁵⁰⁾, much effort has been done to further develop this type of structured DNA and to incorporate it into the biosensor system ⁽⁵¹⁻⁵⁵⁾. The initial stem-loop DNA structure includes a quencher and a fluorophore that are attached at the extreme ends of the whole oligonucleotide. The formation of the stem and loop structure will bring the quencher and fluorophore in close proximity, resulting in the quenching of the fluorescence. When a target complementary DNA hybridizes with the sequence at the loop portion, a more stable and rigid double-stranded structure will form. This will force “unzipping” of the stem sequences, bringing the quencher and fluorophore apart and hence, a measurable fluorescence signal is obtained.

The most common type of linker that is used between the DNA probe and the substrate is the thiol group (-SH). As compared to the other linkers, such as -OH and -NH₂, the -SH group has the advantage of forming a strongly attached and oriented layer on the gold electrode. The -SH group forms a strong metal-thiolate bond without the formation of any oxides. Highly specific interactions

between two different species can also be employed to act as linkers. A commonly seen interaction is that of the biotin and avidin. These two species attract specifically and mutually to each other. The advantages of using interaction-based linkers are that there is no formation of by-products and they are very specific to each other. Another possible interaction-based linker is the bacteriophage-bacteria interaction ^(56, 57). Bacteriophage can be attached easily to the substrate via amide-bonds and it also attracts specifically to a certain type of bacteria.

To-date, while some groups report an increase in electron transfer due to the enhanced accessibility of the redox probes to the electrode surface, some reported a decrease in electron transfer due to the increased charge repulsion with the higher interfacial negative charges. The improved accessibility of the redox probes to the electrode surface was believed to be due to the formation of the more rigid and rod-like double-stranded DNA, which exposed the electrode surface for efficient electron transfer ^(29, 43). The increase in the number of negatively charges at the electrode surface due to the additional amount of DNA after hybridization, on the other hand, result in an increased in electrostatic repulsion with the negatively-charged redox probes, hence reducing electron transfer ^(40, 58).

A sufficiently-packed thiolated single-stranded DNA (ssDNA) probe density immobilized on the gold electrode surface is known to be an important factor to attain a biosensor with high sensitivity. While it is convenient to think that sensitivity of the biosensor increase with the number of immobilized DNA present for hybridization, it is widely known, that a 100% closed-packed DNA probe can have a detrimental effect on DNA hybridization.⁽²³⁾ When the DNA probe is too densely packed, DNA hybridization decrease, since the target complementary DNA is unable to penetrate efficiently in-between the probe DNAs due to steric hindrance ^(59, 60). The change in electrochemical signals after DNA hybridization may also be negligible as the closely-packed ssDNA would have already blocked the electrode surface for efficient electron transfers ⁽⁴³⁾. On the other hand, if the ssDNA probe density is too low, the detection efficiency will also be low. Thus, maximum

hybridization will only occur at the optimum surface probe density. Shorter spacers groups have been employed to modulate the ssDNA probe density on the electrode. Besides reducing steric hindrance, the use of spacer groups also help to remove non-specific interaction between the nucleotide groups and the gold surface, such that the ssDNA probe is only attached to the electrode surface via the thiol-gold interaction ^(1, 61-63). Commonly used spacer groups include 2-mercaptoethanol (ME), 6-mercapto-1-hexanol (MCH) and 3-mercaptopropionic acid (MPA). Even though these short spacers groups can effectively reduce steric hindrance and non-specific interactions ^(2, 64), it also has been shown that electron tunneling through, for example, a ME packed layer, is relatively easy due to the presence of multiple pinholes ^(43, 65, 66).

For a couple of years, Dharuman and co-workers have worked actively on the ability for DNA hybridization discrimination using different sensing platforms comprising of binary and ternary mixed layer of short alkane spacer groups and single-stranded DNA (ssDNA) probes ⁽⁶⁷⁻⁶⁹⁾. They have successfully showed that, while hydrophobic spacer groups are required for the formation of a compact sensing platform, some hydrophilicity from the spacer groups is also needed for ssDNA rearrangement on the surface by electrostatic repulsion. From XPS analysis, hydrophobic MCH has shown to effectively fill up the exposed Au surface after ssDNA immobilization, whereas hydrophilic MPA has the ability to remove the non-specific interactions between the DNA bases and Au surface ⁽⁶⁷⁾. By observing the voltammetric reductive desorption pattern, the hydrophilic MPA has also shown to cause phase separation with the hydrophobic ssDNA when immobilized together ⁽⁶⁸⁾. Their work is, however, focused mainly on DNA hybridization discrimination on the different DNA platforms in the presence of cationic intercalators (eg. methylene blue), which can have the problem of non-specific interactions with other negatively-charged groups present on the sensing platform. Unwanted charge compensation between proflavine (a cationic intercalator) and the ssDNA probes has also shown to result in false electrochemical signal. ⁽⁶⁰⁾ Nevertheless, based on their sensing methodology, a ternary sensing platform comprising of two different spacer groups and the ssDNA

probe will be required to show greater DNA hybridization discriminations. By using both MCH and MPA as backfillers, the ternary sensing surface (ssDNA/MCH/MPA) displayed an improved discrimination efficiency of 1.79 before and after hybridization with 1 μ M target, as compared to using binary sensing surface (ssDNA/MCH) ⁽⁶⁷⁾. Besides the use of conventional linear organic thiols, Campuzano *et al* has also created a ternary sensing surface made up of co-immobilizing ssDNA and linear dithiol (eg. hexanedithiol), followed by backfilling with MCH. Such a sensing surface has also shown to improve the signal-to-noise ratio before and after hybridization with 1 nM target, as compared to a binary sensing surface ⁽⁷⁰⁾. Once again, however, this finding is based on chronoamperometric measurements using a sandwich hybridization method, which is indirect and cumbersome.

In this study, DNA-based spacer group has been employed as an alternative to conventional organic spacer group to reduce steric hindrances of DNA probes and improve target recognition. In-depth investigation on the capabilities of DNA-based spacer group has been carried out. Particular interest has been devoted to the use of strands of thymine bases. Very few studies have been reported on their ability as efficient spacer groups for obtaining a suitable sensing platform. With structures of a nucleotide, strands of thymine bases which contain both the hydrophilic phosphate backbone and hydrophobic methyl group are likely to satisfy the requirement of spacer groups having both hydrophobic and hydrophilic properties, as concluded by Dharuman *et al* ⁽⁶⁷⁻⁶⁹⁾. Thymine bases also have shown to adsorb most weakly to gold surface as compared to the other three DNA bases (cytosine, adenine and guanine) ⁽⁷¹⁾, which is important to ensure minimal non-specific interaction with the gold surface by the spacer group. Higher probe coverage was also achieved when thymine bases are present near the anchoring sites for DNA immobilization on gold substrate ⁽⁷²⁾. This is an important factor for electrochemical biosensor, since compactness of the SAM formed on the surface will affect the measured current flow before and after hybridization.

Strands of thymine bases will be compared with commonly-used organic spacer groups in terms of their ability to form single-phase sensing platform (eg .compactness of formed SAM), the ability to orientate the different receptors for effective target hybridization and the ability to discriminate against non-target bindings.

Ultimately, this report aims to have a deeper understanding of how DNA-based spacer group can perform in comparison to organic spacer group to reduce steric hindrances of the target molecules to the receptors. Various characterization tools, such as SPR, QCM-D and Atomic Force Microscope (AFM), have been used to complement the electrochemical methods to provide new insights on the assembly of DNA strands as spacer groups.

3 Experimental Section

3.1 Materials

Aniline ($\text{C}_6\text{H}_5\text{NH}_2$), 2-mercaptoethanol (ME), 3-mercapto-1-propanol (MP), 6-mercapto-1-hexanol (MCH), Potassium Ferricyanide (III) ($\text{K}_3[\text{Fe}(\text{CN})_6]$), Sodium Chloride (NaCl), Magnesium Chloride (MgCl_2), Sulphuric acid (H_2SO_4) and Gold (III) Chloride (HAuCl_4) were obtained from Sigma Aldrich.

Potassium Hexacyanoferrate (II)-3-hydrate ($\text{K}_4[\text{Fe}(\text{CN})_6] \cdot 3\text{H}_2\text{O}$) was obtained from Riedel-deHaën.

10 X Phosphate Buffer Saline (PBS) solution was obtained from 1st BASE Pte. Ltd., Singapore.

Saline sodium citrate (SSC) buffer dry blend was obtained from Fluka, USA.

Ammonia solution (25% NH_4OH), Hydrogen peroxide (30% H_2O_2) and Hydrochloric Acid (HCl) were obtained from Merck-Chemical.

1 μm , 0.3 μm and 0.05 μm alumina (Al_2O_3) suspensions were obtained from Allied High Tech Products, Inc., Singapore.

Planar gold electrodes (diameter 2 mm), platinum wire electrode (diameter 2 mm) and Ag/AgCl reference electrodes were obtained from CH Instruments, Inc., USA.

5 MHz AT-cut piezoelectric quartz crystal coated with 100 nm gold were obtained from Q-Sense.

SPR gold chips were obtained from GE Healthcare, Biacore division.

Commercial Olympus BLAC40TS cantilever was used for AFM imaging in liquid.

Commercially-available microelectrodes were obtained from Microarray Ltd., UK. These microelectrodes each comprised of a working electrode, a carbon counter electrode and a saturated Ag/AgCl strip serving as the reference electrode. The working electrode contained microcavities which exposed the underlying carbon substrates. These microcavities were formed sonochemically on the insulating polydiaminobenzene layer polymerized on the carbon substrate. Microelectrodes were used as-received.

All DNA sequences were bought either from 1st BASE Pte Ltd, Integrated DNA Technologies (IDT), Inc. or AIT Biotech Pte. Ltd.

Sequence of linear probe DNA for Aniline Microelectrode:

5'-ATG ATT ATG GCT CAG GTA CTG CTA TCC ACC-3'

Sequence of disulphide probe-T9 DNA:

ATG ATT ATG GCT CAG GTA CTG CTA TCC ACC –TTT TTT TTT –O-(CH₂)₃-S-S-(CH₂)₃-OH

Sequence of disulphide T9 spacer groups:

TTT TTT TTT –O-(CH₂)₃-S-S-(CH₂)₃-OH

Sequence of disulphide probe-T6 DNA:

ATG ATT ATG GCT CAG GTA CTG CTA TCC ACC –TTT TTT –O-(CH₂)₃-S-S-(CH₂)₃-OH

Sequence of disulphide T6 spacer groups:

TTT TTT –O-(CH₂)₃-S-S-(CH₂)₃-OH

Sequence of reduced probe-T9 DNA:

5'-ATG ATT ATG GCT CAG GTA CTG CTA TCC ACC-TTT TTT TTT-(CH₂)₆-SH-3'

Sequence of reduced T9 spacer groups:

5'-TTT TTT TTT-(CH₂)₆-SH-3'

Sequence of reduced probe-T6 DNA:

5'- GCT CAG GTA CTG CTA TCC ACC – TTT TTT - (CH₂)₆-SH-3'

Sequence of reduced T6 spacer groups:

5'-TTT TTT - (CH₂)₆-SH-3'

Sequence of complementary DNA: (cDNA)

5'-GGT GGA TAG CAG TAC CTG AGC CAT AAT CAT-3'

Sequence of complementary DNA for reduced T6 probe:

5' – GGT GGA TAG CAG TAC CTG AGC – 3'

Sequence of non-complementary DNA: (NcDNA)

5'-CAA CCT CAA ACA GAC ACC ACG G-3'

Sequence of single-mismatched non-complementary DNA: (N3)

5' – CGT GGA TAG CAG TAC CTG AGC CAT AAT CAT – 3'

Sequence of single-mismatched non-complementary DNA: (N4)

5' – GGT GGA TAG CAG TAG CTG AGC CAT AAT CAT – 3'

All PNA sequences were obtained from Panagene, Korea.

Sequence of reduced probe-T6 DNA:

(C) 3' – CCA CCT ATC GTC ATG GAC TCG TTT TTT - (CH₂)₆ – SH - 5' (N)

Sequence of reduced T6 spacer groups:

(C) 3' -TTT TTT - (CH₂)₆ – SH - 5' (N)

3.2 Solution Preparation

All solutions were prepared using deionized water (18MΩ.cm resistivity) from a Millipore Milli-Q system.

Pure aniline was first obtained by vacuum distillation. Aniline buffer (pH 1-2), containing 0.2 M purified aniline, 0.3 M HCl and 0.5 M KCl, was prepared.

Gold solution used for deposition was made up of 1mM HAuCl₄.

Incubation Buffer (IB) comprised of 1 M NaCl.

Hybridization Buffer (HB, pH 8.0) comprised of 4X SSC buffer.

Buffer (pH 7.3) comprised of 0.01M PBS and 0.01M MgCl₂.

0.01M Phosphate Buffer (1XPBS, pH 7.4) contained 137 mM NaCl and 2.7 mM KCl, diluted from 10XPBS

Electrolyte used for all electrochemical measurements comprised of 5 mM [Fe(CN)₆]^{3-/4-} in 1X PBS.

3.3 Electrode Surface Cleaning/Modifications

3.3.1 Cleaning of planar gold surface

Planar gold electrodes were first cleaned twice in RCA solution comprised of DI water, ammonia solution and hydrogen peroxide solution, in the ratio 5:1:1 at 80 °C for 5 min. After rinsing thoroughly with DI water, the electrodes were polished sequentially with 1 µm, 0.3 µm and 0.05 µm Al₂O₃ suspensions and sonicated in DI water for 10 min. Finally the electrodes undergo electrochemical cleaning with potential cycling from 0 V to 1.57 V (vs. Ag/AgCl) for 10 cycles at a scan rate of 0.06 V/s in 1 M H₂SO₄. After sonicating in DI water for about 2 min, the electrodes were dried in N₂ gas and used immediately for DNA immobilization.

3.3.2 Cleaning of quartz crystal / SPR gold surface

Quartz crystals and SPR gold surfaces were first cleaned twice in RCA solution comprised of DI water, ammonia solution and hydrogen peroxide solution, in the ratio 5:1:1 at 80 °C for 5 min. The cleaned surfaces were then washed thoroughly in DI water, dried in nitrogen gas and used immediately.

3.3.3 Aniline deposition on microelectrode

Electropolymerization of aniline was performed in aniline buffer solution from -0.2 V to 0.8 V (vs. Ag/AgCl) for 21 half cycles at a scan rate of 5 mV/s by cyclic voltammetry. The final linear sweep from -0.2 V to 0.8 V (vs. Ag/AgCl) was performed to leave the polyaniline in its protonated emeraldine salt form.

3.3.4 Gold deposition on microelectrode

Gold deposition on the microelectrodes was performed at -0.9 V for 300 s in gold solution. The depositing parameters have been optimized to form stable gold protrusions with the greatest active surface area for DNA immobilization.

3.4 DNA immobilization and hybridization

Solutions for immobilization for both probe and spacer groups were diluted in IB into the required concentration from a stock concentration of 1 mM. Complementary and non-complementary DNA solutions used for hybridization were diluted in HB into the required concentration from a stock concentration of 100 μ M. Concentration for probe was fixed at 5 μ M and subsequent concentration for spacers (x μ M) used were calculated according to their relative ratio to probe concentration (probe:spacers = [5]: [5x]) for immobilization, as specified in Chapter 4.

3.4.1 Electrochemistry

At each step, 7 μ l of the probe or spacer group solution was dropped onto the working electrode using a micropipette and covered with an inverted eppendorf tube to prevent drying of solution. Probe DNA was allowed to assemble overnight, while spacer group backfilling was incubated for 1 h. Target incubation was performed for 2 h. Incubation at each step was done in dark at the respective temperature.

After each incubation step, electrodes were rinsed in 1XPBS before cyclic voltammetry and impedance measurements. Cyclic voltammetry scans were performed from 0.7 V to -0.6 V at a scan rate of 0.06 V/s. AC impedance measurements were scanned from 10^5 Hz to 0.01 Hz at an amplitude of 0.01 V. The applied potential is fixed at 0.20 V.

3.4.2 QCM-D

A stable baseline was first established in buffer before the injection of DNA solution into the QCM-D chambers. 300 μ l of probe or spacer group solution was then added and was allowed to assemble overnight at room temperature in the chambers. Spacer group backfilling with the respective concentration was incubated for 1 h. ncDNA solution was first circulated for about 10 min or until the frequency change was stable. After rinsing with buffer for about 2-3 min, cDNA solution was added and circulated for about 10 min or until the frequency change was stable, followed by buffer rinsing. Solutions were allowed to circulate at a flow rate of 50 μ L/min at all incubation steps.

3.4.3 SPR

40 μ l of probe or spacer group solution was dropped onto the SPR gold chip using a micropipette and allowed to assemble overnight at room temperature in a self-built humidifying chamber to prevent drying of solution. Spacer group backfilling with the respective concentration was incubated for 1 h. After rinsing with 1xPBS, the SPR chip was loaded into the SPR setup and a stable baseline was first established in buffer. The SPR setup was programmed to allow successive flow of buffer, NcDNA and cDNA solutions to the sensing chip, with adequate rinsing time with buffer between each flow. Generally, 5 μ l of buffer will be injected first, followed by 50 μ l of NcDNA solution and finally 50 μ l of cDNA solution. Solution was circulated at a flow rate of 5 μ L/min and a dissociation time of 120 s was set at the end of cDNA hybridization.

3.4.4 AFM

Three ratios (1:0, 1:1 and 0:1) were prepared for AFM imaging. Cleaned SPR gold chips were first mount onto microscopic glass slides, individually, using nail polish. 40 μ l of probe or spacer group solution was then dropped onto the SPR gold chip using a micropipette and allowed to assemble overnight at room temperature in a self-built humidifying chamber to prevent drying of solution. For ratio 1:1, additional step of spacer group for backfilling was incubated for 1 h. The sensing chips were used directly for AFM imaging in 1XPBS after rinsing. Height images were recorded at a scan rate of 0.5 Hz, with a drive frequency of 29-33 kHz.

3.5 Instrumentations

All AC impedance measurements and cyclic voltammetry were performed using Autolab PGSTAT 302N electrochemical analyzer or CHI600C workstation (CH Instruments, Inc.).

QCM-D measurements were done using Q-Sense E4 (Q-Sense, Sweden) with 4-channels IPC pump (Ismatec SA, Switzerland).

SPR measurements were done using Biacore 3000 (GE Healthcare, Uppsak, Sweden).

AFM images were captured using Asylum MFP3D, operated in tapping mode.

SEM micrographs and EDX spectrums were obtained using SEM JEOL JSM-6360 and FESEM JEOL JSM-7600f equipped with Oxford Instruments INCA software, respectively.

4 Results & Discussions

In this chapter, there will be 3 sections which discuss: 1) DNA biosensors optimized using disulphide 9-Thymine bases DNA; 2) DNA biosensors optimized using reduced 9-Thymine bases DNA and 3) application of the 9-Thymine spacers using sonochemically-fabricated microelectrodes. Within each section, the discussion is sub-divided according to the different characterization tools used, such as SPR, QCM-D, AFM and electrochemistry.

Tables 1 to 3 show a summary of the different types of MRSA probes and spacer groups used, as well as the various sensing surfaces which are formed and will be discussed in the subsequent sections.

(i) DNA oligonucleotides and DNA-based spacer groups

Table 1: Different types and lengths of DNA probes and spacer groups used to form the sensing surfaces.

Type / Length of DNA molecules	MRSA oligonucleotides (30mers)		DNA-based Spacer group	
	T9	T6	T9	T6
Disulphide				
Reduced				

The various combination of molecules form surfaces with different properties, as shown ion Table 2:

Table 2: Schemes of the various sensing surfaces formed.

Type of sensing surfaces	T9	T6
Disulphide
	DNA probes on ternary sensing surface are sufficiently spaced apart by the presence of thymine spacers and mercaptopropanol, which is attached to the disulphide DNA probes and spacer groups.	
Reduced
	Binary sensing surface formed on planar gold using reduced DNA probes and spacer groups result in a dense surface which reduces target recognition. Such steric hindrance of the DNA probes to target can be reduced using gold-deposited microelectrodes.	Sensing ability of reduced T6 DNA and PNA strands immobilized on gold-deposited microelectrodes are compared, with PNA strands showing higher selectivity than its DNA form.

(ii) Commonly-used organic spacer groups

Table 3: Molecular structures of organic spacer groups and schemes of the sensing surfaces formed with disulphide DNA probes.

Organic Spacer group		
2-mercaptoethanol (ME)	3-mercapto-1-propanol (MP)	6-mercapto-1-hexanol (MCH)

Types of sensing surface formed with disulphide DNA probes

Sensing surfaces backfilled with organic spacer groups are generally densely-packed, with compactness in the order of MCH>MP>ME.		

The uncertainty shown for all subsequent experiments is the standard deviation calculated

according to the equation, $\sigma = \sqrt{\frac{1}{N} \sum_{i=1}^N (x_i - \mu)^2}$ where N is the total number of sample x and μ is

the average value for the data set.

4.1 DNA Biosensors optimized using strands of disulphide 9-Thymine bases DNA

4.1.1 SPR analysis

Figure 1: SPR sensorgrams with successive flow of buffer (green arrows), NcDNA (black arrows) and cDNA (red arrows) for a sensing Au surface immobilized with pure disulphide probe DNA (1:0) (Top) and pure disulphide T9 DNA spacer groups (0:1) (Bottom).

SPR reveals excellent selectivity and reproducibility for the sensing surfaces with varying ratios of probe DNA and T9. Typical sensorgrams are observed upon cDNA hybridization, but negligible response with buffer or NcDNA injections. The sensorgrams for measurements on Au surface immobilized with the extreme ratios, pure probe (1:0) and pure T9 (0:1), are shown in Figure 1. Full sensorgrams for all probe:T9 ratios are shown in Appendix (A).

Figure 2: SPR binding curve fitted with 1:1 Langmuir model (Purple) and Bivalent Ligand model (Black) for Au chip immobilized with pure disulphide probe DNA (1:0 ratio).

The kinetics of DNA hybridization is commonly assumed to follow the 1:1 Langmuir isotherm. The assumptions made include a neutral binding surface which will not affect the hybridization process, a homogenous binding surface and a non-competitive binding environment.

The binding curve from the cDNA hybridization for the current sensing surface is fitted to the 1:1 Langmuir model using the BIAevaluation program, as shown in Figure 2 (Purple curve). As shown, the binding curve using the 1:1 Langmuir model does not fit that well with the sensorgram, indicating a hybridization kinetics that does not comply with the assumptions for the ideal 1:1 Langmuir model. This also apply to all the other sensing surfaces immobilized with the various probe:T9 ratios.

Firstly, since negatively-charged DNA probes are being immobilized, one would expect that the SAM formed is not neutral but a highly-charged one. This surface is even more charged for other sensing ratios which involve further incubation with the thymine-based spacer groups, eg. ratio 1:0.5 to 1:2.5, which adds to the total negative charges on the surface.

Secondly, since the spacer groups are also DNA bases (eg. thymine), there is a higher possibility of interaction between the target and the thymine spacer groups before the actual hybridization between the target and its complementary DNA probe takes place. While such interactions between the thymine bases from the spacer group and the target DNA strand may only be transient, they can still influence the actual hybridization process, and this may include the reduction of available target DNA strands that can hybridize with the probe DNA and the additional hindrance of the probe DNA for actual hybridization due to the unintended interaction of the target DNA with T9.

Thirdly, there is also the possibility of more than one target strand attaching to the DNA probe before one of them becomes fully hybridized with the whole strand of DNA probe. This becomes likely at a high concentration of target DNA (1 μM) used in this study.

The highly-charged surface, the short-term interactions between the target DNA and T9 spacer groups, as well as the possible initial multiple target DNA attaching to one probe DNA which result in a competitive and heterogeneous binding surface, definitely challenge the ideal assumptions of kinetics calculations using 1:1 Langmuir model. Furthermore, it has also been shown that the 1:1 Langmuir model is accurate only for limited target concentrations up to 400 nM, which is much lower than the current concentration of 1 μM used herein.⁽⁷³⁾

From the BIAevaluation programme, an alternative model, Bivalent Ligand, is used to fit the sensorgrams (See Figure 2 (Black)). As shown, this model fits more accurately to the sensorgram than the 1:1 Langmuir model. Similar good fitting to the other sensorgrams can be seen in Appendix (B) using the same model. According to this Bivalent Ligand model, the proposed interactions are shown to be as follows:

, where A and B can be expressed as the target DNA and probe DNA respectively. AB1 and AB2 can be expressed as either the actual complementary hybridized double-stranded DNA strand or the short term binding between the target DNA and the thymine bases spacer group. Note that at any one time, there is a possibility of a cDNA to partially hybridize with the bases of the actual sequences of the probe DNA, as well as partial binding with the thymine bases spacer group.

Figure 3: Change in SPR response (RU) after hybridization with 1 μ M cDNA and rinsing the Au chip immobilized with varying ratios of disulphide probe DNA and disulphide T9 DNA spacer groups (t=100s).

Figure 3 shows the highest response unit (RU) after hybridization at the same ratio. This maximum response unit for the different sensing surfaces was taken during the dissociation phase at t = 100 s, about 40 s of buffer rinsing after hybridization with 1 μ M of cDNA. The number of RU corresponds to the relative amount of additional cDNA that remains on the surface (likely hybridized with the probe DNA) after rinsing.

An observation of utmost importance from Figure 3 is the large increase in the maximum RU obtained when even a small concentration of T9 spacer groups is added (eg. at ratio 1:0.25). A jump

of more than 10 RU is obtained as the ratio of T9 spacer groups increased from 1:0 to 1:1. This suggests the impact of the addition of T9 spacer groups on the surface density of the immobilized probe DNA. The increase in cDNA uptake (increase in RU) for ratios involving T9 spacer groups suggests that the steric hindrance observed for the closely-packed probe DNA is reduced upon the addition of T9 spacer groups. The spacers undoubtedly improve the accessibility of the cDNA to the hybridizing sequence of the probe DNA. Further increase in the ratio of T9 spacer groups added (eg. above ratio 1:1.5) reduces the response to a level that is still significantly higher than that of ratio 1:0, where no T9 spacer group strands are added. This suggests a possible reduction in the amount of probe DNA present for hybridization, which can be due to a replacement of the probe DNA by the shorter T9 spacer group strands during incubation.

From the SPR analysis, T9 spacer groups have successfully shown to be able to improve the amount of hybridization, likely by removing non-specific interaction between the DNA bases and Au surface, as well as by aligning and spacing the probe DNA strands apart. Though a higher amount of T9 spacer groups may help to distribute the probe DNA on the surface to reduce the steric hindrances, results from the maximum SPR response obtained here show that too high a T9 spacer group concentration can cause an adverse effect on the final number of probe DNA available for hybridization, thus reducing the number of hybridized DNA. The ratio 1:1 is hence concluded to be the optimum ratio required to get the highest hybridization response from SPR analysis.

4.1.2 QCM-D analysis

4.1.2.1 Monolayer Characterization

Figure 4: Average change in frequency from 3rd Harmonics evaluated from QCM-D measurements on gold-coated quartz crystal immobilized with varying ratios of disulphide probe DNA and disulphide T9 DNA spacer groups.

QCM-D measurements were carried out across the entire range of ratio of disulphide probe DNA and disulphide T9 DNA spacer group immobilized on Au-coated quartz crystal. The change in frequency from the 3rd harmonics is plotted in Figure 4.

As shown in Figure 4, the change in frequency is the lowest at ratio 0:1, where only disulphide T9 DNA spacer groups are immobilized. The other ratios which involve the immobilization of disulphide probe DNA (eg. ratio 1:0 to 1:2.5), obtained high change in frequencies of above 30Hz. Some variation in frequency change can be observed with the incubation of different concentration of disulphide T9 DNA spacer group. More will be discussed in the following section.

Figure 5: Mass change on gold-coated quartz crystal immobilized with varying ratios of disulphide probe DNA and disulphide T9 DNA spacer groups.

The change in frequency (Δf) observed from QCM-D measurements is directly related to the change in mass (Δm) on the quartz crystal with molecular immobilization, according to the Sauerbrey equation

$$\Delta f = - \frac{2f_o^2}{A\sqrt{\rho_q\mu_q}} \Delta m , \quad (1)$$

where f_o is the resonant frequency ($f_o = 14.88$ MHz for 3rd Harmonics Frequency), A is the gold-coated area on the quartz crystal, ρ_q is the density of quartz ($\rho_q = 2.648$ g/cm³) and μ_q is the shear modulus of AT-cut quartz crystal ($\mu_q = 2.947 \times 10^{11}$ g/cms²).

Figure 5 shows a plot of the mass changes on the quartz crystal after immobilization of the various ratios of disulphide probe DNA and disulphide T9 DNA spacer group according to Equation 1. As shown, the largest mass change occurs at ratio 1:0, where only disulphide probe DNA is immobilized. With the addition of disulphide T9 spacer groups, non-specific interactions between the DNA bases

and the Au surface is reduced because of the electrostatic repulsion by the disulphide T9 DNA spacer group. The lowered mass change at ratio 1:1 and ratio 1:1.5 may suggest the removal of loosely-bound probe by the shorter T9 spacer groups. Since the T9 spacer group ($M_w = 2872$ g/mol) has a lower molecular weight than that of probe ($M_w = 12139$ g/mol), it is possible for the mass change to decrease with the addition of a small concentration of T9 spacer group. As the concentration of disulphide T9 DNA spacer group further increase, the mass change starts to increase as shown in Figure 5. This increase may be due to immobilization of more T9 spacer group onto the sensing surface, which becomes more significant than the reduction of mass change due to the removal of probe DNA from the surface.

As expected, the lowest mass change is obtained from ratio 0:1, where the short disulphide T9 DNA spacer groups of the lower molecular weight are immobilized.

4.1.2.2 Hybridization

Figure 6: Average change in frequency from 3rd Harmonics evaluated from QCM-D measurements with 1 μ M cDNA hybridization on gold-coated quartz crystal immobilized with varying ratios of disulphide probe DNA and disulphide T9 DNA spacer groups.

QCM-D measurements were carried out across the entire range of ratio of disulphide probe DNA and disulphide T9 DNA spacer group immobilized on Au-coated quartz crystal. The change in frequency is calculated after subsequent injections of NcDNA and cDNA, the same procedure as used for SPR measurements.

From Figure 6, very little or no change in frequency is obtained after NcDNA injections, which suggest little or no binding of NcDNA for the sensing surfaces. A significantly higher average change in frequency is seen after cDNA injection for ratios involving disulphide probe DNA, mainly due to the additional mass of hybridized cDNA on the surface. For ratio 0:1, where only disulphide T9 DNA

spacer groups are present, the frequency changes after both NcDNA and cDNA injections remains small. Once again, the sensing surfaces show great selectivity in discriminating between NcDNA binding and cDNA hybridization.

Figure 7: Mass change with 1 μ M cDNA hybridization on gold-coated quartz crystal immobilized with varying ratios of disulphide probe DNA and disulphide T9 DNA spacer groups.

Mass changes with 1 μ M of cDNA injection for each sensing surfaces of different ratios are calculated according to Equation 1 and plotted in Figure 7.

Compared to the mass change due to cDNA hybridization on sensing surface immobilized with pure probe DNA (ratio 1:0), the mass change increases slightly when disulphide T9 DNA spacer groups are added into the sensing surface at ratio 1:0.25 and ratio 1:0.5, as can be seen in Figure 7. This shows that the cDNA strands can hybridize more easily to the probe DNA, likely because of the removal of non-specific interactions between the DNA bases and the Au surface. Even though the probe DNA may not be completely aligned with this small concentration of T9 DNA spacer group, the probe

DNA strands are likely to get lifted up by the T9 spacer group such that they are more exposed to the cDNA strands for hybridization.

With a further increase in disulphide T9 spacer groups, the mass change due to cDNA hybridization decreases in a small extent. This decrease in mass, which may also denote a decrease in the number of hybridized cDNA strands on the sensing surfaces, can suggest either that the probe DNA strands are unexposed for hybridization, or a lower number of probe DNA strands are immobilized on the sensing surface. Taking into account the mass change with immobilization in Figure 5, where mass change decrease from ratio 1:0.5 to ratio 1:1, it is unlikely that the same number of probe DNA remains on the sensing surface after incubation with a higher concentration of T9 spacer groups. Furthermore, the increase in mass change from ratio 1:1 to 1:2.5 in Figure 5 also show the possibility of having more T9 spacer groups immobilized on the sensing surface, which helps to align and expose the probe DNA sequences for hybridization. Hence, the slight decrease in mass change after cDNA hybridization starting from ratio 1:1 in Figure 7 is likely due to the lower number of probe DNA that remained on the sensing surface after the addition of higher concentration of disulphide T9 DNA spacer group.

A point to note is that large mass changes are not expected since it takes a large number of molecules, in the 10^{13} molecules range, to make a difference of about $1\text{ }\mu\text{g}^{(74)}$. In this case, the removal or addition of a large number of probe DNA or T9 spacer groups from the surface are not expected and hence only slight changes in mass are detected.

Figure 8: Average change in dissipation from 3rd Harmonics evaluated from QCM-D measurements with 1 μ M cDNA hybridization on gold-coated quartz crystal immobilized with varying ratios of disulphide probe DNA and disulphide T9 DNA spacer groups.

The change in dissipation of the various sensing surfaces is also recorded simultaneously with frequency change during successive injections of NcDNA and cDNA during the QCM-D measurements. The change in dissipation at the 3rd harmonics for the entire range of immobilizing ratios is plotted in Figure 8.

Besides ratio 0:1, it can be observed that the other sensing surfaces involving disulphide probe DNA can generally be separated into two regions. From ratio 1:0 to ratio 1:1, it can be seen that changes in dissipation due to both NcDNA and cDNA hybridization are significantly lower than those from ratio 1:1.5 to ratio 1:2.5. This may suggest two regions of different organisation/alignment of the probe DNA on the sensing surface.

In the region of ratio 1:0 to ratio 1:1 (Region 1), the low dissipation change close to zero suggest that the sensing surfaces remain rigid after the injection of NcDNA. This shows that little or no NcDNA sticks to the surface and no actual hybridization occurs, which is also evident from the low frequency change with NcDNA injection in Figure 6. With cDNA injection and hybridization, the dissipation change becomes higher due to the greater viscoelastic drag. The rigid double helix is likely to extend out into the solution and trapping liquid between the helices, causing greater viscoelasticity of the overall hydrated SAM.

The same explanation can be used for the higher dissipation change for cDNA hybridization than that due to NcDNA injection in the region with ratio 1:1.5 to ratio 1:2.5 (Region 2). However, it can be noted that the change in dissipation due to NcDNA is much higher in Region 2 than in Region 1. This suggests that the sensing surface in Region 2 may have probe DNA strands which are much more “lifted” (or aligned) than those in Region 1, due to the higher concentration of immobilized disulphide T9 spacer group in Region 2. Some NcDNA strands do not bind to the probe DNA but may be trapped between the aligned probe DNA strands, resulting in greater dissipation change even after rinsing. This effect may be undesirable since it leads to non-specific responses in the measurements. Nevertheless, the change in frequency and dissipation from QCM-D measurements provide useful insights, especially on the possible alignment of probe DNA strands, on the various SAMs formed by the immobilization of different ratio of disulphide probe DNA and disulphide T9 DNA spacer group.

Real-time experimental graphs of frequency and dissipation changes for each probe:T9 disulphide ratios can be found in Appendix (C).

4.1.3 AFM

AFM imaging was conducted on three ratios, namely 0:1, 1:1 and 1:0, with bare Au surface as a control. Ratio 1:1 was selected for comparison with the extreme ratios since it has been concluded to result in the greatest change in response upon cDNA hybridization using SPR. From QCM-D, ratio 1:1 is also one of the ratios which fall within Region 1, where probe DNA strands are likely to be sufficiently “lifted” and with insignificant frequency and dissipation changes after NcDNA flow.

4.1.3.1 Monolayer Characterization

Figure 9: AFM height images of Au surface immobilized with disulphide probe DNA and/or disulphide T9 DNA spacer group at ratio (A) 1:0, (B) 1:1 and (C) 0:1. Bare Au surface (D) is shown as a control.

Images captured were analyzed using Igor Pro programme and were applied with Cold/Warm colours to better present the observations. Areas with warm colours show greater height than those with cold colours, which depicts uncovered or partially covered areas (termed as pores for subsequent explanation) on the surfaces.

From the AFM images shown in Figure 9, differences in porosity between the various sensing surfaces can be seen. Density of pores was generally lower at ratio 1:0 and 1:1 (Figure 9(A) and 9(B)) than at ratio 0:1 (Figure 9(C)). As observed, images for ratio 1:0 and 1:1 are also more “blurry” than that of ratio 0:1. Bare Au surface expectedly show a surface with high density of uncovered areas, with observed height differences throughout the surface due possibly to external artefact contaminants sticking to the surface (Figure 9(D)).

The blurry images for ratio 1:0 and 1:1 are likely to be caused by the movement of the longer probe DNA strands while the surfaces are being probed by the cantilever tip in liquid. Such movement effects have shown to be insignificant for shorter T9 spacer groups, as clear images are being captured for ratio 0:1 as well as for bare Au surface.

Comparing the images between ratio 1:0 and ratio 1:1 of similar pore density, the pore size for ratio 1:1 seems to be larger than that of ratio 1:0. As defined earlier, these “pores” may either be uncovered or partially covered areas. For ratio 1:1, since the surface is made up of both the longer probe DNA strands and the shorter T9 spacer group strands, the pores may also represent areas filled with the shorter T9 spacer group strands.

Figure 10: Histogram of counts of event for range of height for bare Au (Red), ratio 1:0 (Green), 1:1 (Blue) and 0:1 (Yellow).

Using the same Igor Pro programme, histograms which show the number of counts of events for the range of height captured in the whole image can be plotted, as shown in Figure 10. Bare Au surface expectedly gave the broadest peak with arms extending to the extreme left (lowest height range). The slight asymmetric peak also shows more count of events towards low height range on the left of the plot. This signifies a surface with the highest exposed surface (low height) compared to the other surfaces.

A comparison between the individual sensing surfaces immobilized using the three different ratios, the surface exposure level may be induced as $0:1 > 1:0 \geq 1:1$. Both the peak maximum count at height 0nm and peak symmetry/asymmetry are used in the attempt of evaluating exposed level of the different surfaces. For ratio 0:1, even though the peak maximum count at height 0 nm is lower than that of 1:0 and 1:1, the peak is asymmetric and displays additional counts at the left of the plot. The relatively smaller right arm at higher height range is likely due to the presence of immobilized T9 spacer groups. The ratios 1:0 and 1:1 generally show similar surface exposure. From Figure 10,

even though ratio 1:0 may seem to have a higher maximum count at height 0 nm (green), the number of counts at height greater than 0 nm is also higher, as compared to that of ratio 1:1 (blue). The asymmetric peak which tends towards higher height range for ratio 1:1 is also apparent. Overall, the exposure surface may be similar between the surfaces of ratio 1:0 and 1:1.

Figure 11: Cross-sectional height analysis of sensing surface immobilized at ratios 1:0 (Black), 1:1 (Red) and 0:1 (Blue) at image position of 0.5 μm .

A comparison of the cross-sectional height analysis between the images also show ratio 0:1 having generally a lower height with pores of greater depth (Figure 11 Blue line). Once again, ratio 1:0 and 1:1 show similar pore depth from the random cross-sectional height comparison. The presence of greater height across the surface for ratio 1:0 is also observed. Such occurrences are attributed to the higher number of counts at heights greater than 0 nm for ratio 1:0 shown in Figure 10.

From the cross-sectional height analysis in Figure 11, an obvious observation which distinguishes the two ratios is that the surface with ratio 1:0 generally has a higher roughness than that of ratio 1:1,

which shows a more regular change in height across the surface. This may suggest a more homogenous surface due to the presence of T9 spacer groups for ratio 1:1. More cross-sectional height analysis for the individual sensing surfaces can be found in Appendix (D).

Table 4: Statistics for roughness for sensing surface of different ratios.

Ratio	Sample size	Mean	SD
Au	4	1016.93	94.9
1:0	4	1061.16	62.0
1:1	8	836.11	80.6
0:1	4	943.95	94.5

To further characterize the different surfaces, surface roughness of the various sensing surfaces was determined from the AFM experiments. The calculated statistics are presented in Table 4.

Generally, ratio 1:0 shows a surface with higher roughness than that of ratio 1:1 and 0:1. This may be due to the multiple interactions between the DNA bases and the Au surface, hence resulting in varying height changes across the surface. On the other hand, the smaller roughness mean for ratio 1:1 and 0:1 suggests more uniform surfaces, which can also be seen in the cross-sectional height comparison in Figure 11. The high roughness for Au surface may be attributed to possible surface artifacts, as seen in the AFM image in Figure 9(D).

Table 5: Results of 1-way ANOVA for roughness of sensing surfaces of different ratios.

Source of Variance	df	Sums of Square	Mean Square	F Statistics	P
Sample	3	168800	56260	6.471	0.0045 (P < 0.05)
Roughness	16	139100	8694		
Total	19	307900			

In order to show the significance of the difference in surface roughness for the various sensing surfaces, the roughness data is evaluated using 1-way ANOVA and presented in Table 5. Individual calculations can be found in Appendix (D).

According to the results, the ratio of mean squares is statistically significant ($F = 6.471$, $P = 0.0045$ ($P < 0.05$)). Even though sample size is small ($N = 20$), such calculation further validates the differences of the SAM formed on the surface using the various ratios.

Surface characterization using AFM has possibly brought further insights on the type of SAM formed on the surface immobilized with ratio 1:0, 1:1 and 0:1. The difference in roughness between the surfaces has shown to be statistically significant, which shows a change in sensing surface with the use of T9 spacer groups for backfilling. Most importantly, from the cross-sectional height analysis in Figure 11, sensing surface of ratio 1:1 has shown to form a SAM which seems more uniform than that of ratio 1:0. This shows the likelihood of the removal of non-specific interaction between the DNA bases and the Au surface (eg. higher roughness for ratio 1:0) and backfilling by T9 spacer group on the Au surface, which eventually makes the overall surface more uniform in height.

4.1.4 Electrochemistry

All experimental graphs for this section can be found in Appendix (E).

4.1.4.1 Optimization of Probe Surface Density by disulphide T9 DNA spacer group

4.1.4.1.1 Monolayer Characterization

Figure 12: Fractional coverage (Red line) and average current change (Black line) of overall immobilized ratios of disulphide probe DNA and disulphide T9 DNA spacer groups on planar Au surface.

Impedance spectroscopy and cyclic voltammetry scans were performed on the different sensing surfaces incubated with varying ratio of disulphide probe DNA and disulphide T9 spacer groups.

Fractional coverage (Ψ_{FC}) was calculated using the equation

$$\Psi_{FC} = 1 - \left(\frac{R_{ct}^{Au}}{R_{ct}^{SAM}} \right) , \quad (2)$$

where R_{ct}^{Au} is the charge-transfer resistance for bare gold surface and R_{ct}^{SAM} is the charge-transfer resistance for the self-assembled monolayer (SAM) formed on the sensing surface incubated with the respective ratios of probe and T9 spacer group.

Average current change was calculated from the anodic peak current change obtained from the cyclic voltammetry scans for the sensing surface before and after incubation with the respective ratio of probe and T9 spacer group. A high magnitude of the average current change signifies greater reduction in electron transfer across the Au surface, eg. lowered accessibility of redox probes to Au surface.

Electrochemistry is based on the flow of electrons across the conducting surface. This can be achieved with the addition of redox probes in the electrolyte, for example $[\text{Fe}(\text{CN})_6]^{3-/4-}$, which exchange electrons across the surface with every cycle of oxidation and reduction reaction due to an applied potential. (See Figure 13)

Figure 13: Exchange of electrons due to redox reaction of $[\text{Fe}(\text{CN})_6]^{3-/4-}$.

With additional immobilization of either or both of the probe and T9 spacer groups, a barrier is formed on the gold surface which reduces the number of redox probes close to the surface. The effect of this reduction of redox probes near to the surface will be shown as either an increase in the charge-transfer resistance or a decrease in the peak current. The extent of increase/decrease is largely dependent on the compactness and vertical length of the SAM formed on the gold surface. A

highly compact and vertically long SAM, which causes less channeling of the redox probes to the surface, will result in a low electron transfers across the surface. A large increase in charge transfer resistance and a large decrease in peak current will therefore be observed. The vice versa is true, where a loosely packed and relatively shorter SAM will result in increase channeling of the redox probes and thus a higher electron transfer across the surface (See Figure 14).

Figure 14: Schemes of (a) low electron transfer due to compact and long SAM on surface and (b) high electron transfer due to loosely-packed and short SAM.

According to Equation 2, after the formation of a SAM on the gold surface, if charge-transfer resistance increases relative to that of bare gold, fractional coverage should have a value less than 1. Evidently from Figure 12, calculated value for fractional coverage is less than 1 for all the different ratio of probe and T9 spacer group incubated on the gold surface. This shows that gold surface has successfully been coated with immobilized probe and/or T9 spacer group.

Figure 12 also shows varying fractional coverage value for the different ratio of probe and T9 spacer group, the lowest fractional coverage from the ratio 0:1 (incubated with pure T9 spacer group) and one of the highest from ratio 1:0 (incubated with pure probe). With a difference of 21 bases ($\approx 7.14\text{nm}$) between the probe (30 bases) and T9 spacer group (9 bases), the T9 spacer group is expected to form a more compact and vertically shorter SAM on the gold surface, as compared to that of probe SAM (See Figure 15). As mentioned in the above paragraph, a more compact SAM will

result in less electron transfer across the surface as compared to a loosely-packed one. However, at the same time, a shorter SAM should allow more electron transfer across the surface. Since the lowest fractional coverage comes from the surface immobilized with ratio 0:1 (pure T9 spacer group), it shows that the effects of high electron transfer due to the short SAM is more pronounced than a lowered electron transfer due to the more compact T9 spacer group SAM. This is also evident from the low peak current change obtained from cyclic voltammetry scans, which signifies a high accessibility of redox probes to the Au surface resulting in a high electron transfer (Figure 12 black line).

Figure 15: Schemes of (a) longer probe but less compact SAM and (b) shorter T9 but more compact SAM.

Sensing surface immobilized with probe, with or without the addition of T9 spacer group, generally results in a high fractional coverage, as seen in Figure 12. Probe, which has a length of 30 bases, is expected to form a loosely-packed SAM on the gold surface and hence a low fractional coverage (See Figure 16(a)). However, the high fractional coverage for the entire range of ratios involving probe, shows that the effects brought by the longer length of probe relative to T9 spacer group has a more dominating effect than that brought by the compactness of the SAM formed.

Traditionally, organic spacer groups have been added to probe SAM to improve the compactness of the layer, due to the removal of non-specific interaction between the nucleotide bases and the gold surface. In this case, when DNA-based spacer groups are added, the fractional coverage for the

different ratios of probe:T9 generally remains unchanged (See Figure 12). The fractional coverage reduces about 3% of the fractional coverage for pure probe immobilization (1:0) when a small concentration of T9 spacer groups are added (1:0.25), but gradually increases back to the initial fractional coverage when a higher concentration of T9 spacer group is added.

Figure 16: Schemes of immobilized (a) pure disulphide probe DNA on Au with non-specific interactions between DNA bases and Au, (b) probe with addition of low concentration of disulphide T9 spacer groups and (c) probe with high concentration of disulphide T9 spacer groups.

At 1:0, the immobilized probes are expected to form a loosely-packed SAM, and likely with a high degree of non-specific interaction between the bases and the gold surface. As shown in Figure 16(a), the probes are not aligned and some of the probes can lie on the gold surface. Since the probes are longer and hence more highly charged than the T9 spacer group, assuming one unit of negative charge per base, this spread of probes on the gold surface forms a pseudo-compact layer on the surface, which blocks the flow of electrons between the redox probes and the gold surface. A relatively high fractional coverage and low peak current (high average current change) are hence obtained.

With the addition of T9 spacer group at small concentration (eg, at 1:0.25), some of the non-specific interaction between the bases and the gold surface will be replaced by the formation of stronger

Au-S bonds from the thiolated T9 spacer group. This makes the probes more aligned which increases the length of the SAM (See Figure 16(b)). At the same time, however, regions of shorter T9 spacer group newly immobilized on the gold surface may improve the electron transfers across the gold surface. Thus, an increase in peak current (decrease in average current change) and a small reduction of about 3% in fractional coverage are seen in Figure 12.

With a further increase in T9 spacer group concentration, the probes become more aligned since more non-specific interactions are being removed. The formed layer become more compact and at the same time, increase in the vertical length of the SAM (See Figure 16(c)). The fractional coverage and peak current gradually reached similar values to that of 1:0, noticeably when ratio is above 1:1.

A point to note is that even though the fractional coverage and peak current for the different ratio are similar to that of 1:0, the formed SAMs are of different configurations and alignment as that of 1:0. This will be further discussed in the following sections.

4.1.4.1.2 Hybridization

Figure 17: Average current change after incubation with 1 μM of NcDNA (Grey) and cDNA (Red) in 4XSSC buffer at room temperature (22°C) on varying ratios of disulphide probe DNA and disulphide T9 DNA spacer groups.

Cyclic voltammetry scans were recorded on the entire range of planar Au surfaces immobilized with different ratios of disulphide probe DNA and disulphide T9 spacer group, before and after incubation with 1 μM non-complementary DNA (NcDNA) and complementary DNA (cDNA) at room temperature (22°C) for 2 h. NcDNA and cDNA incubation were done on separate electrodes for each ratio, with N=3 for each set of experiments.

Figure 17 shows the average current change for the various ratios according to the equation

$$\text{Average Current Change} = I_{\text{After Target}}^p - I_{\text{Before Target}}^p, \quad (3)$$

where $I_{After\ Target}^p$ is the peak current after target (NcDNA/cDNA) incubation and $I_{Before\ Target}^p$ is the peak current before target incubation.

The extent of current change, which is affected by the efficiency of electron transfer between the negatively-charged redox probes ($[Fe(CN)_6]^{3-/4-}$) and the gold surface after cDNA hybridization, are typically controlled by two main factors.

With the formation of rod-like double helix after cDNA hybridization, channeling of the redox probes to the surface is expected to increase and result in an increase in current change. However, with the increase in the overall negative charges on the sensing surface after hybridization, repulsion of the negatively-charged redox probes from the surface is also possible. This will result in less electron transfers across the surface and thus a decrease in the current change.

In Figure 17, the average measureable current change is positive for all ratios. This shows that current increases after target binding, which is due to the increase in the channeling of the redox probes through the SAM. Unexpectedly, the current change due to NcDNA binding is also significant for all the ratios. This may suggest non-specific binding of the shorter NcDNA with the probe DNA and/or T9 spacer groups, likely via base-stacking interaction rather than Watson-Crick base pairing. This observation is unlike in the case of SPR and QCM-D studies presented in the previous sections, where the introduction of NcDNA strands using dynamic flow result in insignificant signal changes. The static hybridization method used in electrochemistry results in additional non-specific interaction which may interfere with the actual signal change due to hybridization. The additional “brushes” formed by the attached NcDNA on the immobilized DNA (probe/T9) on the surface may also “sweep” and trap redox probes within the layers closer to the electrode surface, which facilitates electron transfer (See Figure 18). The current change observed after NcDNA is therefore not an indication of hybridization, but rather, an additional factor of non-specific binding to the surface probes.

Figure 18: Schemes of sensing surface (a) without NcDNA binding and (b) with NcDNA binding which result in more redox probes being trapped within probe layers closer to the electrode surface.

Considering that neither NcDNA nor cDNA should interact with the surface compactly immobilized with pure T9 spacer group (0:1), the current change after target binding at 0:1 shows the degree of non-specific interaction that can occur on the sensing surface or with the T9 spacer groups. In order to show the current change due solely to hybridization, the current change for all the ratios from 1:0 to 1:2.5 will be corrected with the “ultimate control”, which is the current change at 0:1 where only non-specific interactions can possibly occur. The corrected current change is shown in Figure 19.

Figure 19: Average current change due to hybridization (corrected with non-specific interaction from “ultimate control”) for both NcDNA (Grey) and cDNA (Red) on varying ratios of disulphide probe DNA and disulphide T9 DNA spacer groups.

With the current change corrected with the “ultimate control” 0:1, Figure 19 shows the average current change due solely to hybridization for all the ratios of disulphide probe DNA and disulphide T9 spacer group. Significantly seen is the large jump in current change after cDNA hybridization from 1:0.5 to 1:1 (See Figure 19 Red). This marks a possible change in alignment of the immobilized probes on the surface, which affects the accessibility of the probes to the cDNA for hybridization. With the addition of T9 spacer group, non-specific interactions between the probe bases and the gold surface can be reduced by the formation of Au-S bonds between the thiolated T9 spacer group and the gold surface. This results in the “lifting” of the probe bases and better alignment of the probes on the surface. With adequate amount of T9 spacer group and spontaneous rearrangement of the probes and T9 spacer group on the surface, the probes can be spaced well apart to reduce the steric hindrance for efficient hybridization.

While the increase in current changes after cDNA incubation is due to hybridization, current changes after NcDNA incubation are due to the additional factor of non-specific binding between the NcDNA and the surface probes. As mentioned in the previous section, the shorter NcDNA is likely to bind with the probe DNA and/or T9 spacer groups via base-stacking interaction. The NcDNA attached to the immobilized DNA on the surface introduces additional “brushes” which may trap redox probes within the DNA layers to facilitate electron transfer. With more aligned probes, such non-specific interactions may increase since more bases are relatively more exposed for interaction as compared to unaligned ones. This is apparent from the higher current changes observed after NcDNA binding for ratio 1.5 and above in Figure 19.

Figure 20: Signal-to-noise ratio with 1 μ M cDNA in 4XSSC for varying ratios of disulphide probe DNA and disulphide T9 DNA spacer groups.

In order to show the ability of each ratios of probe and T9 spacer group to distinguish between the cDNA hybridization and NcDNA interaction, the signal-to-noise ratio is being calculated according to the equation

$$\text{Signal-to-noise } (S/N) = I_{\text{Corr.cDNA}}^p / I_{\text{Corr.NcDNA}}^p, \quad (4)$$

where $I_{\text{Corr.cDNA}}^p$ is the corrected average peak current change after cDNA hybridization and $I_{\text{Corr.NcDNA}}^p$ is the corrected average current change after NcDNA interaction. Uncertainty is

calculated according to $\partial(S/N) = \pm (S/N) \sqrt{\left(\frac{\partial A}{I_{\text{Corr.cDNA}}^p}\right)^2 + \left(\frac{\partial B}{I_{\text{Corr.NcDNA}}^p}\right)^2}$, where ∂A is the

standard deviation of the corrected average peak current change after cDNA hybridization and ∂B is the standard deviation of the corrected average current change after NcDNA interaction.

From Figure 20, the S/N ratio increases from ratio 1:0 to a maximum value at ratio 1:1 ($S/N = 5.91$) and drops significantly to a plateau S/N value eventually, with further increase in backfilled T9 spacer group (eg. ratio 1:1.5 to 1:2.5). Such a trend has also been observed by Boozer *et al.*, where a maximum SPR response is observed at a specific probe/OEG (oligo(ethylene glycol) ratio of 0.02⁽⁷⁵⁾. A higher or lower concentration of OEG backfiller has shown to decrease SPR response, which the authors attribute such observations to be due to the high surface coverage by the backfillers at high OEG concentration and a poor orientation of the probe DNA, that restrict hybridization, at a low OEG concentration.

From the trend observed, there is a switch from negative S/N values at 1:0 and 1:0.25, to a positive one at 1:0.5. This particular ratio between 1:0.25 and 1:0.5 where the switch occurs is critical as it shows the minimum concentration of T9 spacer group required to cause a change in the alignment of the probe on the sensing surface. This alignment of probe on the sensing surface not only affects the binding of the probe with the cDNA, but also that with the NcDNA.

For the case of the sensing surfaces incubated with ratios 1:0.5 and above, there is sufficient T9 spacer group to “lift” the probes upright by removing non-specific interaction and to fill up the exposed Au surface. The probes are not only more aligned than those formed from lower ratio (eg. 1:0 and 1:0.25), the probes are also likely to be separated further apart from the neighboring probe by the backfilled T9 spacer group. This reduction in steric hindrances results in an increased in accessibility of the target DNA to hybridize (for cDNA)⁽⁷⁶⁻⁷⁸⁾, as well as to bind (for NcDNA), to the aligned probes.

The negative S/N values as seen in ratio 1:0 and 1:0.25 is due to the negative current change of NcDNA due solely to hybridization after deduction from the “ultimate control”, shown in Figure 19. This shows that NcDNA has very little or no direct interaction with the unaligned probes, which is indicative of excellent selectivity. However, since the probes are unaligned with possibility higher steric hindrance, actual hybridization with cDNA is also reduced.

Even though a decrease in current change for NcDNA binding is more desirable than a positive one, the S/N values in Figure 20 has shown that the best sensing ratio to discriminate between the NcDNA binding and cDNA hybridization occurs at 1:1.

4.1.4.2 Optimization of Hybridization conditions

Having optimized the ratio of the sensing surface to be 1:1, hybridization was carried out at different conditions to find their effects on hybridization ability. Three different hybridization time (2 hours, 1 hour and 15 minutes) and two different hybridization temperatures (22°C and 55°C) were used in study.

4.1.4.2.1 Room Temperature

Figure 21: Average current change after incubation with 1 μ M of NcDNA (Grey) and cDNA (Red) in 4XSSC buffer at room temperature (22°C) for varying hybridization time at 1:1 disulphide probe DNA: T9 sensing surface.

Hybridization time were varied from 2 hours to 15 minutes to observe the effects of hybridization time on the ability of the optimized sensing surface (ratio 1:1) to discriminate between the NcDNA binding and the cDNA hybridization. Cyclic voltammetry scans were performed and the average current change was calculated according to Equation 3.

From Figure 21, varying the hybridization time shows significant effects on cDNA hybridization. NcDNA binding remains almost the same for hybridization time of 2 hours and 1 hour, but increased significantly at short hybridization time (15 minutes).

Figure 22: Average current change due to hybridization (corrected with non-specific interaction from “ultimate control”) for both NcDNA (Grey) and cDNA (Red) incubated at room temperature (22°C) for varying hybridization time at 1:1 disulphide probe DNA: T9 sensing surface.

Figure 22 shows the average current change due solely to hybridization for varying hybridization time on 1:1 sensing surface. This current change was obtained after the current change was corrected from the “ultimate control” 0:1.

As mentioned in the above section, varying the hybridization time affects cDNA hybridization significantly. Evidently from Figure 22, the average current change due solely to hybridization is greatest at long hybridization duration (2 hours). The current change drops significantly when hybridization time is reduced to 1 hour, but discrimination between the cDNA hybridization and its NcDNA binding is still possible at this hybridization time. However, when hybridization is only

allowed to occur within 15 minutes, discrimination between cDNA hybridization and NcDNA binding is no longer possible. This can be seen from the similar change in current at hybridization time of 15 minutes in Figure 22. This will be further discussed in the subsequent section.

4.1.4.2.2 Elevated Temperature

Hybridization was repeated for all three hybridization times at an elevated temperature of 55°C. This is the calculated optimum hybridization temperature (T_{hyb}), which is 25°C lower than the melting temperature (T_m) of the hybridized double helix. As its name suggest, this is the temperature where hybridization is expected to occur the most efficiently. At T_{hyb} , the temperature is high enough to improve the mobility of the target DNA strands for higher possibility of hybridization, but at the same time still low enough so that the hydrogen bonds formed between the complementary bases are maintained and the the secondary structure of the formed double helix is retained.

T_m is first obtained from an online T_m calculator⁽⁷⁹⁾ from IDT Oligo Analyzer using 5uM as the probe concentration, 780mM as the sodium ion concentration during hybridization and 1uM as the target concentration. T_{hyb} is then calculated to be about 55°C according to the equation

$$T_{hyb} = T_m - 25^{\circ}\text{C} \quad (5)$$

At this temperature, hybridization has also shown to occur from the drop in absorbance in the UV-vis spectrum after hybridization is allowed to occur at 55°C for 1 hour. (Appendix (F))

This temperature is similar to the value ($T_{hyb} \approx 57^{\circ}\text{C}$) calculated according to the equation⁽⁸⁰⁾

$$T_{hyb} (^{\circ}\text{C}) = 81.5 - 16.6 (\log[Na^{+}]) + 0.41 (\% GC) - 0.63 (\% \text{Formamide}) - \frac{600}{n} - 25, \quad (6)$$

where $[Na^{+}]$ is the concentration of sodium ions present during hybridization, % GC is the percentage of guanine and cytosine base pairs involved in the hybridization process per strand of

probe, % *Formamide* is the percentage of formamide present during hybridization and n is the number of bases per strand of probe.

Figure 23: Average current change after incubation with 1 μ M of NcDNA (Grey) and cDNA (Red) in 4XSSC buffer at elevated temperature (55°C) for varying hybridization time at 1:1 disulphide probe DNA: T9 sensing surface.

Hybridization time were varied from 2 hours to 15 minutes to observe the effects of hybridization time on the ability of the optimized sensing surface (ratio 1:1) to discriminate between the NcDNA binding and the cDNA hybridization at 55°C. Cyclic voltammetry scans were performed and the average current change was calculated according to Equation 3.

Once again, varying hybridization time affects cDNA hybridization but to a lesser extent for NcDNA binding, as shown in Figure 23.

Figure 24: Average current change due to hybridization (corrected with non-specific interaction from “ultimate control”) for both NcDNA (Grey) and cDNA (Red) incubated at elevated temperature (55°C) for varying hybridization time at 1:1 disulphide probe DNA: T9 sensing surface.

Figure 24 shows the average current change due solely to hybridization for varying hybridization time on 1:1 sensing surface at 55°C. This current change was obtained after the current change was corrected from the “ultimate control” 0:1. A point to note is that this “ultimate control” 0:1 used for correction is the current change obtained when cDNA and NcDNA are incubated at 55°C for 2 hours, which is different from what has been used so far. This is necessary since temperature may cause different interactions between the sensing surface and the target molecules, which in turn will cause a different current change. From the data obtained, an increase in hybridization temperature from 22°C to 55°C generally causes an increase of about 7-8% of the average measureable current change after cDNA and NcDNA incubation on 0:1 sensing surface.

The maximum current change due solely to hybridization occurs when cDNA is incubated for 2 hours, as shown in Figure 24. The current change was shown to be negative when hybridization time is 1 hour, which shows that the current change is less than that of the “ultimate control” 0:1. This

suggests either no hybridization or incomplete hybridization occurring on the sensing surface, which in the case of incomplete hybridization, the “lingering” ends of the cDNA may lie across the surface, restricting the accessibility of the redox probes to the electrode surface for electron transfer. A decrease in current change will thus result, as oppose to the expected increase channeling of the redox probe through the probe/T9 SAM on the electrode surface when complete hybridization takes place.

An interesting observation when comparing between the current change after NcDNA binding in Figure 22 and Figure 24, is that an increase in hybridization temperature to 55°C result in smaller current change after NcDNA incubation for all three hybridization time. This shows that the elevated temperature not only allows more efficient hybridization, it also helps in the hybridization process more effectively, such that complementary bases binds while non-complementary ones will dissociate even if there is some interaction between the bases.

4.1.4.2.3 Optimized Hybridization Conditions Determination

Figure 25: Signal-to-noise ratio with 1 μM cDNA in 4XSSC incubated at room temperature (22°C) (Dark blue) and elevated temperature (55°C) (Cyan) for varying hybridization time at 1:1 disulphide probe DNA: T9 sensing surface.

To show the overall effects of hybridization temperature and time on the sensing ability of the optimized probe-to-T9 ratio at 1:1, the S/N ratios for all the six combination of hybridization conditions are calculated according to Equation 4 and plotted in Figure 25.

Firstly, a decrease in hybridization time generally results in a decrease in S/N ratio, regardless whether hybridization occurs at 22°C (Set A- dark blue) or 55°C (Set B -cyan). This shows that the ability of the sensing surface to discriminate between NcDNA binding and actual cDNA hybridization is reduced as hybridization time reduces.

Assuming that all external factors (such as temperature and pressure) remained constant for each set of experiments (Set A or Set B), the rate-determining factor that can affect the efficiency of

hybridization is the diffusion of the target molecules to the immobilized probes on the surface, which is also a time-dependent process. When hybridization is forced to stop at the respective hybridization time, especially at short hybridization time of 15 minutes, the amount of target molecules that can arrive and bind at the site of hybridization may be lower than that when hybridization is allowed to occur at a longer duration. It is therefore expected that, given a shorter time for target molecule incubation, the degree of hybridization will be lesser. Hence, when the hybridization time is too short, in the case of 15 minutes, the possibility of complete hybridization is lower. This results in a similar channeling effect of the redox probes to the electrode surface after either ncDNA or cDNA incubation, hence a very small S/N ratio is observed.

Secondly, increasing the hybridization temperature to the optimum hybridization temperature at 55°C also results in a generally higher S/N ratio. At long hybridization time of 2 hours and 1 hour, the S/N ratio is almost double when the hybridization temperature is elevated.

According to the First law of Thermodynamics, where energy can neither be created nor destroyed, an increase in the temperature of the system will increase the kinetic energy of the molecules present in the system according to the equation

$$E = \frac{3}{2} k_B T \quad , \quad (7)$$

where k_B is the Boltzmann constant (1.38×10^{-23} J/K) and T is the temperature (K).

This increase in kinetic energy will result in, not only higher rate of diffusion, but also higher mobility of the target molecules. The rate of collision between the complementary bases between the probe and cDNA is hence expected to increase and hybridization can take place more efficiently within the same hybridization time.

Finally, it can be observed that when hybridization is allowed to take place at 55°C for 15 minutes, the S/N ratio is almost zero, implying very little or no cDNA hybridization. This phenomenon is due to the combined factors mentioned in the above paragraphs. At the elevated temperature, with the

increase in mobility of the cDNA, the rate of collision between the cDNA molecules is possibly greater than the rate of hybridization (eg. rate of hydrogen formation). Together with the short hybridization time, where some hybridization process is forced to stop before it is fully completed, the overall combination of elevated temperature (55°C) and short hybridization time (15 minute) has shown to be undesirable for efficient hybridization.

From Figure 25, the maximum S/N ratio (≈ 12) occurs when hybridization is allowed to occur at 55°C for 2 hours. These were concluded to be the optimum hybridization conditions, where the best discrimination between the NcDNA binding and cDNA hybridization is observed within the six hybridization conditions in study.

4.1.4.3 Determination of Sensitivity and Selectivity

4.1.4.3.1 Sensitivity

Figure 26: Signal-to-noise ratio with varying target concentration in 4XSSC buffer incubated at optimized hybridization conditions (55°C for 2h) on 1:1 disulphide probe DNA: T9 sensing surface.

Using the optimized ratio (1:1) and optimized hybridization conditions (55°C for 2 hours) concluded in the above sections, the concentration of the cDNA is varied to show the sensitivity of the sensing surface and determine the limit of detection.

From Figure 26, the maximum S/N ratio occurs when target concentration is 1 μM and reduce gradually when target concentration increases or decreases beyond this specific concentration.

Ideally, as target concentration increases, S/N ratio should increase gradually before reaching a plateau where further hybridization does not cause a difference in electrochemical signal. In this

case, however, there is an inverse trend of signal, where further increase in target concentration beyond 1 μM result in a reduction in the S/N ratio.

A possible explanation for this is the increase in negative charges on the surface brought about by the larger number of DNA strands on the surface. When higher concentration of cDNA is introduced, number of hybridized probe increases, which result in more negatively-charged DNA strands on the surface. This increase the overall negative charges on the surface, which causes more repulsion of the negatively-charged redox probes responsible for the electron exchange across the Au surface. The increased repulsion leads to a reduced electron transfer across the electrode, resulting in lower S/N ratio.

As the target concentration gradually decreases, at least down to 1 μM , the formation of rigid double-helix which allows more efficient channeling of the redox probes through the SAM layer is reduced. Since there is lesser number of DNA strands on the surface as compared to the case where more hybridization can take place at higher target concentration, the number of negative charges on the surface is smaller and thus caused relatively lower repulsion with the redox probes. Both of these factors result in higher electron transfer as compared to when the target concentration is higher, thus higher S/N ratio.

As the target concentration continue to decrease to a very low concentration, the S/N ratio reaches a low value, since the number of double helix can no longer improve the channeling of the redox probe great enough to cause a significant change in the electrochemical signal. The limit of detection for this sensing surface is hence determined to be 0.1 μM , after which further reduction in target concentration no longer shows smaller S/N ratio value.

The S/N ratio has shown to peak at a target concentration of 1 μM , after which the S/N ratio starts to decrease again. This can suggest an optimum concentration, where the effects of increased channeling of the redox probes through of the SAM due to the double-helix formation is more

dominant than the charge repulsion between the surface and redox probes which cause a decrease in signal. A point to note is that these two factors are constantly competing with each other and one factor may be more dominant than the other at different situations, in this case, the target concentration. This competition is likely to be higher for this work, since both the probe and the spacer groups are negatively charged.

4.1.4.3.2 Selectivity

The optimized 1:1 sensing surface was used to test its ability for single-mismatch (SMM) detection. Two SMM DNA strands were used, N3 having the single-mismatch at the 1st position and N4 having the single-match at the 15th position from the attachment site of immobilized probe DNA (3').

Figure 27: Average current change after incubation with 0.1 μM or 1 μM of N3 (Green), N4 (Black), cDNA (Red) and NcDNA (Grey) in 4XSSC buffer at optimized hybridization conditions (55°C for 2h) on 1:1 disulphide probe DNA: T9 sensing surface.

Cyclic voltammetry scans were performed and the average current change was calculated according to Equation 3. Single-mismatch detection is possible using the optimized 1:1 sensing surface. As seen in Figure 27, average current change for both N3 and N4 is smaller than that for cDNA detection for both target concentrations.

Figure 28: Average current change due to hybridization (corrected with non-specific interaction from “ultimate control”) after incubation with 0.1 μM or 1 μM of N3 (Green), N4 (Black), cDNA (Red) and NcDNA (Grey) in 4XSSC buffer at optimized hybridization conditions (55°C for 2h) on 1:1 disulphide probe DNA: T9 sensing surface.

Figure 28 shows the average current change due solely to hybridization after incubation with the single-mismatch strands (SMM - N3 and N4), cDNA and NcDNA on 1:1 sensing surface at 55°C. This current change was obtained after the current change was corrected from the “ultimate control” 0:1.

Selectivity of the optimized 1:1 sensing surface is once again evident from Figure 28, where the change in current due solely to hybridization is smaller when incubated with SMM strands as compared to that after incubation with cDNA. From the results, it can be deduced that hybridization still takes place between the probe and the SMM strands, though the resulting effect caused by hybridization with the SMM strands are smaller than that with the cDNA strands. Another observation is that only at the optimum concentration of 1 μM can the SMM detections be distinguished from the NcDNA binding. This may be due to the low concentration of the target molecules at 0.1 μM, where the effects caused by small degree of hybridization with the SMM

strands are not significant enough to result in a higher current change than the NcDNA binding. Furthermore, since the NcDNA strands are shorter than the SMM strands, more non-specific interaction between the NcDNA and the probes may occur as compared to the partial hybridization between the longer SMM and probes. The trapping of redox probes close to the surface by the attached NcDNA to the immobilized DNA on the surface are thus more pronounced than that caused by the hybridized SMM, resulting in higher current changes after NcDNA binding than that for the SMM strands at the concentration of 0.1 μM .

Figure 29: Signal-to-noise ratio after incubation with 0.1 μM or 1 μM of N3 (Green), N4 (Black) and cDNA (Red) in 4XSSC buffer at optimized hybridization conditions (55°C for 2h) on 1:1 disulphide probe DNA: T9 sensing surface.

The signal-to-noise magnitudes for the different detections are calculated according to Equation 4 and plotted in Figure 29. Besides showing the ability to differentiate between SMM DNA hybridization and cDNA hybridization, the S/N magnitudes give a clearer picture of how

hybridization with SMM strands are possibly causing different conformational changes which result in different signal changes.

At low target concentration of 0.1 μ M, the resultant S/N values for N3 and N4 are similar but lower than that for cDNA. This shows that selectivity between SMM DNA and cDNA is distinguishable, though the type of SMM strands used cannot be determined.

For the case of higher concentration at 1 μ M, all the S/N values are much higher than those detected with 0.1 μ M, with increasing S/N values from N3, to N4 and finally to cDNA detections. Mismatch at the 1st position near the probe attachment site (N3) result in a 38% decrease in S/N value while central mismatch (N4) cause a 22% decrease in S/N value as compared to when fully complementary strands hybridize (See Figure 29).

Double-stranded DNA formed from hybridization with single-mismatch strands (DNA-SMM) is known to be less stable than those formed with cDNA, though the actual mechanism that affects the signal output has not been fully addressed. Factors affecting the thermostability of the formed duplex with SMM DNA include base mismatch defect type, nearest-neighbour effects and defect position.^(81, 82)

Depending on the defect type and nearest-neighbour base pairings present, the stability of the base mismatch pairing differs due to the formation of different numbers of hydrogen bonds between SMM pairs. Base pairing involving GC bases are generally more stable than that of AT bases, even for like-bases pairing such as A-A, T-T, G-G and C-C, due to the different stacking ability of the purine and pyrimidine rings. While the relative stability of A-A and T-T like-mismatch pairings are generally similar in all reports, vast differences have been reported for G-G and C-C pairings.⁽⁸²⁻⁸⁶⁾ For similar DNA-DNA mismatch hybridization on microarrays, Wick et al. showed a higher stability of G-G base pair than C-C base pair⁽⁸³⁾, while Naiser et al. showed the opposite, with C-C pairing being more stable than G-G pairing⁽⁸⁶⁾. However, based on the influence of nearest-neighbour base pairing,

Peyret et al summarizes G-G mismatches as commonly most stable due to the presence of two hydrogen bonds and C-C mismatches as the least stable due to presence of at most one hydrogen bond.⁽⁸⁵⁾

In this case, a C-C mismatch binding is present in N3, while a G-G mismatch binding is involved in N4. According to the stability of the defect type as explained in the above paragraph, duplex formation with N4 is likely to have a similar conformation as that of the complementary duplex formed with cDNA. C-C mismatch in N3 may result in a duplex with loosely-attached ends, which can restrict the accessibility of the redox probes to the surface (See Figure 31(c)). Hence, a lower S/N ratio is observed for N3 than that for N4.

Besides the type of SMM base at the defect site between N3 and N4, the defect position is also another major factor that can cause a difference in the electron transfer.

A recent study of ferrocene-tagged PNA-DNA duplex shows a decrease in the electron transfer rate constant with increasing defect position from the attachment site of the strand.⁽⁸⁷⁾ The defect site, which acts as the anchoring site, increases the flexibility and thermal motion of the duplex segment above the defect site. Such flexibility of the duplex segment above the defect site, where the ferrocene is tagged, is likely to improve the electron transfer between the ferrocene and the electrode surface. With increasing defect position from the attachment site of the strand, the duplex segment above the defect site is shorter, which makes such effects from the thermal motion less significant, resulting in a decrease in electron transfer between the ferrocene and electrode surface. This decrease rate of electron transfer of increasing defect position from the attachment site is likely to be applicable only for tagged DNA probes, since the distance between the tagged ferrocene and the electrode surface is the factor that affects the electron transfer between them.

When the DNA probe is unlabeled and the redox probes responsible for the electron transfer are available throughout the bulk solution, accessibility rather than distance is the factor that affects the

electron transfer. In this case, when the duplex segment above the defect site is longer, the possibility of the neighbouring duplex segments tilting and overlapping each other is larger. This tilted structure is likely to restrict the accessibility of the redox probes to the electrode surface for electron transfer, reducing the rate of electron transfer (See Figure 30). Hence, for unlabelled DNA-SMM hybrid, the shorter duplex segment above the defect site may well improve electron transfer, rather than decreasing it like in the case reported by Hüsken, N., et al ⁽⁸⁷⁾.

Figure 30: Schemes of possible tilted structure with duplex formed when (a) single-mismatch is at 1st position and (b) at central position (15th) from the attachment site of probe. Pink ring represents mismatch position which acts as anchoring site for flexibility and thermal motion of duplex.

From Figure 29, N3, which has the single-mismatch at the 1st position from the DNA probe attachment site, exhibits a smaller S/N ratio than N4, which has the single-mismatch at the 15th position (central) from the attachment site. Since the duplex segment above the defect site is shorter in N4 than in N3, the electron transfer after hybridization with N4 is expected to be better than with N3, as explained in the above paragraph. In addition, it has also been shown that end mismatches promote “unzipping” of the duplex⁽⁸⁸⁾. The flanking ends of the duplex formed by N3 can further reduce the accessibility of the redox probes to the surface for electron transfer. “Unzipping” of the duplex due to the mismatch base at the central position (N4) is less likely to occur due to the stabilization by neighbouring complementary base pairs at both ends of the defect

site. The duplex formed by N4 is relatively similar to that formed with cDNA, which allows greater channelling of the redox probes to the surface for electron transfer (See Figure 31).

Figure 31: Schemes of redox probes channeling through duplex formed with (a) cDNA, (b) N4 (central single-mismatch) and (c) N3 (single-mismatch at 1st position with neighbouring “unzipped” bases).

The current sensing surface using thymine bases as spacer group enables us to discriminate, not only between fully complementary DNA and shorter non-complementary DNA, but also with single-mismatch strands at two different defect positions and defect types. Even though more validation is needed to further prove the selectivity of the sensing surfaces, the possibility to detect different single mismatch strands at different defect position using such simple, label-free platform is commendatory.

4.1.4.4 Comparison with other spacer groups

In order to show the advantage of DNA-based spacer group over the commonly used organic spacer groups, the same hybridization experiments were conducted on the sensing surface first immobilized with probe DNA and backfilled with 1mM of 2-mercaptoethanol (ME), 3-mercapto-1-propanol (MP) or 6-mercapto-1-hexanol (MCH). Besides the organic spacer groups, probe DNA concentrations and all experimental procedures carried out in this section remained unchanged and can be found in Chapter 3. Comparison was done with previous results obtained using the optimized ratio of 1:1 disulphide probe DNA:T9 sensing surface on planar gold surface.

4.1.4.4.1 Organic Spacer Group

4.1.4.4.1.1 Monolayer Characterization

Figure 32: Average current of overall immobilized disulphide probe DNA and various types of spacer group incubated at 22°C in dark on planar Au surface.

Results from cyclic voltammetry scans show varying average current change after the Au surfaces are immobilized with the DNA probe and the respective spacer groups, as seen in Figure 32. The average current change was calculated from the anodic peak current change obtained from the cyclic voltammetry scans for the sensing surface before and after incubation with the probe and respective spacer groups. As mentioned in Section 4.1.3.1.1, a high magnitude of average current change signifies greater reduction in electron transfer across the Au surface, eg. lowered accessibility of redox probes to Au surface. The DNA surface that is incubated with T9 spacer groups results in the lowest average current change. This signifies a sensing surface with the highest electron transfer across the Au surface after immobilization, as compared to those formed using the other organic spacer groups. MCH, being the longest chain among the three organic spacer groups, have shown to form a sensing surface which blocks the accessibility of the redox probes to the Au surface most efficiently, resulting in the highest average current change.

From Figure 32, the addition of organic spacer groups to the DNA probe surface generally shows a higher average current change than that with the addition of T9 spacer groups. This higher surface coverage after addition of the organic spacer groups is likely due to their –OH head groups, which most likely are responsible for the removal of non-specific interactions between the DNA bases and Au surface by repelling the negatively-charged DNA bases from the surface.^(68, 89) As mentioned in the above paragraph, MCH is the longest chain, having 6-carbon chains, while MP and ME have 3 and 2 carbons in the chain. Short carbon chains, such as ME, are known to promote the direct diffusion of the redox probes through the SAM layer for electron transfer across the surface^(43, 65, 66), as compared to longer carbon chains. It is therefore reasonable for the obtained decreasing average current change, which signifies an increasing ease of electron transfer across the Au surface, when the length of spacer group decreases from MCH to MP and ME. This is also in agreement to the difference in E_{pp} (peak-to-peak potential) for the different sensing surface, where the largest E_{pp} , which signifies a highly compact layer with minimal access of redox probes to the Au surface for

electron transfer, is obtained after MCH incubation (See Table 6). The peak-to-peak potential (E_{pp}) is the difference in the redox peak potentials due to the oxidation (anodic) and reduction (cathodic) of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ during a cyclic voltammetry scan. An increase in E_{pp} signifies a more compact barrier on the electrode surface which impedes the transfer of electrons across the surface ^(2, 68, 70), in this case due to the immobilization of additional spacer groups on the DNA probe-immobilized gold surface.

Table 6: Average peak-to-peak potential (E_{pp}) obtained from cyclic voltammetry scans after incubation with various spacer groups on DNA probe-immobilized Au surface.

Type of Spacer group	Average E_{pp} (mV)
ME	312
MP	394
MCH	467
T9	331

With the use of T9 spacer groups, both the average current change (Figure 32) and E_{pp} (Table 6) shows a less compact sensing surface possessing high electron transfer. This may be due to the difference in the charge and hydrophobicity of the T9 spacer groups with the other organic spacer groups. A report by Dharuman et al. showed that the more highly charged and hydrophilic $-\text{COOH}$ head groups in 3-mercaptopropionic acid (MPA) are likely to repel the negatively-charged DNA more effectively than the more hydrophobic MCH, thus creating more exposed Au surface for electron transfer with $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox probes ⁽⁶⁸⁾. This results in a much smaller peak current change and E_{pp} than that obtained by MCH after spacer group immobilization. Similarly, when T9 spacer group is used, a smaller average current change and E_{pp} are obtained. Negatively-charged T9 spacer groups, which are highly hydrophilic, are likely to repel the DNA bases from the surface more effectively than the organic spacer groups, removing the non-specific interactions between the DNA bases and Au, while creating larger surface defect sites (exposed Au) for electron transfer. Moreover, since both the DNA probe and T9 spacer groups are now hydrophilic, multiphase separation is less likely to occur than with organic spacer groups, which is advantageous in forming a more homogenous sensing surface.

Even though a more compact surface is commonly more desirable for an electrochemical biosensor, it will be shown in the following section that the use of T9 spacer groups, though formation of a less compact sensing surface exhibits great capability to form a highly selective biosensor.

4.1.4.4.1.2 Hybridization Detection

Figure 33: Signal-to-noise ratio with 1 μ M cDNA in 4XSSC incubated at optimized hybridization conditions (55°C for 2h) on sensing surface immobilized with disulphide probe DNA and backfilled with various spacer groups.

Signal-to-noise (S/N) ratio is calculated according to Equation 4 using the corrected peak current changes after cDNA hybridization and NcDNA interaction using the various sensing surface backfilled by the respective spacer groups.

As shown in Figure 33, the use of T9 as the backfilled spacer groups results in about 10 times increase in the S/N ratio as compared to those that are backfilled with commonly used organic spacer groups. This signifies a more selective sensing surface when T9 spacer groups are used as the backfillers. As mentioned in the previous section, T9 spacer groups result in a less compact and highly-charged surface. Since the redox probes are also negatively-charged, electrostatic repulsion between the redox probes and the highly-charged surface may think to be undesirable for the detection. This is, however, shown to be otherwise from the obtained results.

Consider the case when the surface is compactly filled with organic spacer group and exposed defect sites are small. Assuming all the probes on the surface are hybridized by the cDNA targets, the formation of rigid double helix will increase the channelling and accessibility of the redox probes through the defect sites and increase the electron transfers. With NcDNA interaction, some strands may also be lifted and increase the electron transfer, though to a smaller extent. However, at a more charged surface where defect sites are expectedly larger, as also deduced experimentally when using T9 spacer group as backfillers, the accessibility of the redox probes are greatly improved through the larger exposed Au surface during cDNA hybridization. Once again, NcDNA interaction may lift some of the strands, but this time, the increase in electron transfers will relatively be lower than that caused by the cDNA hybridization. Overall, the calculated S/N ratio for a charged surface will be much higher than that of the uncharged surface.

Despite the general idea that highly charged surface can deter electron transfer and reduce the selectivity of the sensing surface, the use of T9 spacer group at the optimized probe:T9 ratio of 1:1 has shown to have a better capability in discriminating between NcDNA binding and cDNA hybridization, making DNA-based spacer group a better backfiller than the organic spacer groups.

4.1.4.4.2 Shorter Thymine Spacer group (T6)

Figure 34: Signal-to-noise ratio with 1 μ M cDNA in 4XSSC incubated at optimized hybridization conditions (55°C for 2h) on 1:1 sensing surfaces immobilized with either disulphide T6 DNA or disulphide T9 DNA, each backfilled with their respective length of DNA spacer group.

Signal-to-noise (S/N) ratio is calculated according to Equation 4 using the corrected peak current changes after cDNA hybridization and NcDNA interaction using the various sensing surfaces backfilled with the respective length of thymine spacer groups.

A point to note is that the DNA probes contain the same number of thymine spacer group at the immobilization end as the length of thymine spacer groups used for backfilling in each type of sensing surfaces respectively. This ensures, either that the DNA bases specific for hybridization are not being blocked by the longer backfillers, or that additional exposed thymine bases from the DNA probes are present for hybridization due to the shorter backfillers.

As seen in Figure 34, the use of shorter six thymine spacer group (T6) as backfillers results in an almost equivalent S/N ratio (difference ≈ 1 S/N) as compared to that using T9 to backfill the sensing

surface, though the use of longer T9 spacers results in better reproducibility than using T6 spacers.

This shows that the inherent properties of thymine bases (eg. negatively-charged and hydrophilic)

can serve their function as spacers and are not affected by the number of thymine bases used.

4.2 DNA Biosensors optimized using strands of reduced 9-Thymine bases DNA

Having shown the capabilities of thymine-based DNA spacer groups in the previous section, similar experiments were conducted using the reduced form of probe DNA and T9 spacer groups. Using this reduced form, binary sensing surfaces are formed instead of ternary ones. With one less type of molecule on a binary surface, complications due to the DNA interactions may be reduced as compared to a ternary surface. Though binary surfaces are usually used in a biosensor rather than ternary surfaces, experimental results in subsequent sections show that a binary surface on planar electrode using the current DNA strands may not perform as well as a ternary one.

4.2.1 SPR analysis

SPR measurements were conducted on binary surfaces made up of the various ratios of reduced probe DNA and reduced T9 spacer groups.

Figure 35: SPR sensorgrams with successive flow of buffer (green arrows), NcDNA (black arrows) and cDNA (red arrows) on sensing Au surfaces immobilized with (a) pure reduced probe DNA (1:0) and (b) pure reduced T9 DNA spacer groups (0:1).

The SPR sensorgrams obtained from measurements on Au planar surface immobilized with the extreme ratio, pure reduced probe DNA (1:0) and pure reduced T9 spacer group (0:1), are shown in Figure 35. Full sensorgrams for all reduced probe:T9 ratios are shown in Appendix (G).

Excellent sensitivity for all the sensing surfaces immobilized with varying ratio of reduced probe DNA and reduced T9 spacer groups can be observed, with obvious binding curve only with cDNA hybridization but negligible response with buffer or NcDNA injections.

Four simultaneous measurements were taken from each SPR chip for each sensing ratio. As compared to ternary sensing surface using pure disulphide probe DNA (See Figure 1), the binary sensing surface using pure reduced probe DNA (See Figure 35) seemed to give a lower reproducibility in terms of response signal upon cDNA hybridization. While all four measurements from the ternary sensing surfaces in Figure 1 gave similar response, only two out of the four measurements gave similar response when a binary sensing surface was used (Figure 35 1:0 G3 and

1:0 G4). This indicates that the sensing surface formed from pure reduced DNA strands may not be as homogenous as that using pure disulphide DNA strands, which forms the ternary sensing surface.

For the 0:1 sensing surface, no binding curves can be observed since probe DNA is not present for hybridization. However, it can be noticed that NcDNA interactions (Black arrow) seemed to cause a greater change in response than with cDNA interactions (Red arrow), even though both result in no permanent bindings (eg. response returns to baseline during buffer rinse). This is not being observed for disulphide 0:1 sensing surface, where similar changes in response are shown. The greater interactions from NcDNA for reduced 0:1 may pose problems when a static hybridization method in electrochemistry is used. More will be discussed in the following electrochemistry section.

Figure 36: Association (ka-Red) and dissociation (kd-Black) rate constants evaluated from SPR measurements with 1 μ M cDNA hybridization on varying ratios of reduced probe DNA and reduced T9 DNA spacer groups.

The sensorgrams were fitted using the Bivalent Ligand Model, similar to that used for the ternary sensing surfaces. (Appendix (H)) The evaluated average corresponding association and dissociation rate constants for all the sensing surfaces are plotted in Figure 36.

As shown, the rate of association of cDNA binding generally occurs in the $10^4 \text{ M}^{-1}\text{s}^{-1}$ range while rate of dissociation occurs at a low 10^{-3} s^{-1} range, showing strong association of the hybridized double helix DNA. However, as compared to the ternary sensing surfaces where rate of association occurs at a high $10^5 \text{ M}^{-1}\text{s}^{-1}$ range (See plot in Appendix (B)), the binary sensing surfaces have shown their inferiority in hybridization kinetics shown by the lower association range by an order of magnitude, possibly due to greater steric hindrances which lowers target recognition, as also suggested from electrochemistry in the following section. This is unlike for the ternary sensing surfaces where probe DNA strands are spaced more apart due to the presence of mercaptopropanol, thus improving target recognition and hybridization kinetics.

4.2.2 Electrochemistry

All experimental graphs for this section can be found in Appendix (I).

4.2.2.1 Optimization of Probe Surface Density by reduced T9 DNA spacer group

4.2.2.1.1 Monolayer Characterization

Figure 37: Fractional coverage (Red line) and average current change (Black line) of overall immobilized reduced probe DNA and reduced T9 DNA spacer groups on planar Au surface.

Impedance spectroscopy and cyclic voltammetry scans were performed on the different sensing surfaces incubated with varying ratios of reduced probe DNA and reduced T9 spacer groups.

Fractional coverage (Ψ_{FC}) was calculated using Equation 2 and the average current change was calculated from peak current change obtained from the cyclic voltammetry scans for the sensing surface before and after incubation with the respective ratio of reduced probe DNA and reduced T9 spacer group. A high magnitude of average current change signifies greater reduction in electron transfer across the Au surface, eg. caused by lowering of the accessibility of redox probes to Au surface.

As explained in the previous section, with immobilization of either or both of the probe DNA and T9 spacer groups, the changes in charge-transfer resistance and peak current are largely dependent on the compactness and vertical length of the SAM formed on the gold surface. Generally a compact or long SAM will reduce the accessibility of the redox probes and result in a low electron transfer across the Au surface, depicted by a high fractional coverage and high average current change.

From Figure 37, the highest fractional coverage and average current change are observed for pure reduced T9 spacer group immobilization (0:1). As mentioned, this is due to the highly compact SAM that is formed by the reduced T9 spacer groups, which lowers the accessibility of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ ions for electron transfer across the Au surface.

With the addition of more reduced T9 spacer groups to the immobilized reduced probe DNA on Au, the overall fractional coverage and average current change generally decreases (See Figure 37). This is likely due to the increase in electron transfer as a result of improved accessibility of the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ ions via the larger regions of the immobilized short reduced T9 spacer group on the surface.

As compared to the use of disulphide DNA strands, shown in Figure 12, immobilization of the reduced form generally results in higher fractional coverage and lower average current change. For all the different ratios, immobilizing the reduced form will have one less molecule type, the mercaptopropanol, on the surface as compared to the immobilization of the disulphide form. Initially, the presence of the additional mercaptopropanol was thought to be non-beneficial to the sensing surface since complications may increase with a more hydrophobic molecule. However, experimental results comparing the presence of this smaller spacer group show that this third molecule is actually required to form a more sensitive and selective sensing surface.

When immobilized with the reduced form, the higher fractional coverage suggests a more compact SAM on the Au surface. This is possible, since the lack of the small hydrophobic mercaptopropanol molecule can prevent multiphase separation with the hydrophilic DNA strands, which may have

caused the lower fractional coverage for the ternary surfaces when using disulphides. However, the average current change with the immobilization of the reduced DNA strands occur in the 10^{-6} A range, which is one order of magnitude lower than that obtained from the immobilization of the disulphide DNA strand (10^{-5} A range). This lower average current change after immobilizing the reduced form of DNA shows the possibility of exposed defect spots which could have been filled up by the smaller mercaptopropanol. The use of disulphide DNA, which is already attached to the mercaptopropanol by the disulphide bond, further ensures that such defect spots are less likely to occur since both strands will be immobilized together.

4.2.2.1.2 Hybridization

Figure 38: Average current change after incubation with 1 μ M of NcDNA (Grey) and cDNA (Red) in at room temperature (22°C) on varying ratios of reduced probe DNA and reduced T9 DNA spacer groups.

Cyclic voltammetry scans were performed on the entire range of sensing Au planar surface immobilized with different ratios of reduced probe DNA and reduced T9 spacer groups, before and after incubation with 1 μ M non-complementary DNA (NcDNA) and complementary DNA (cDNA) at room temperature (22°C).

The average current changes for all ratios are calculated according to Equation 3 and plotted in Figure 38. Similar to the ternary sensing surface immobilized with the disulphide DNAs (Figure 19), current changes due to NcDNA binding are high for all ratios. The difference between the current changes due to NcDNA binding and cDNA hybridization is especially large for the extreme ratios of 1:0 and 0:1.

As mentioned in the above SPR section, NcDNA binding results in higher temporary response than cDNA binding for 0:1 even during dynamic flow. For static incubation in the case of electrochemistry, this non-specific binding seemed to have amplified, since more time is given for the NcDNA strands to rearrange and bind more permanently into the reduced T9 spacer group SAM in static incubation as compared to that in dynamic flow. The smaller response from cDNA binding on reduced 0:1 surface may be due to two factors. Firstly, the cDNA strands are longer (30 mers) than NcDNA strands (22 mers), so the shorter NcDNA may be able to integrate into the SAM more easily than the cDNA. Secondly, even though both strands possess the same total number of adenine bases, there is only one pair of consecutive adenine bases in cDNA while there are more adenine bases in close proximity in the NcDNA strand. This makes interaction between the thymine bases in reduced T9 spacer group and NcDNA strand stronger than that with cDNA.

Figure 39: Average current change due to hybridization (corrected with non-specific interaction from “ultimate control”) for both NcDNA (Grey) and cDNA (Red) on varying ratios of reduced probe DNA and reduced T9 DNA spacer groups.

With the current change corrected with the “ultimate control” 0:1, Figure 39 shows the average current change due solely to hybridization for all the ratios of reduced probe DNA and reduced T9 spacer groups.

A significant observation is the higher average current change due to hybridization with cDNA than NcDNA binding, for all the ratios involving backfilling by reduced T9 spacer groups (eg. 1:0.25 onwards). Without the T9 spacer group in the 1:0 surface, the average current change due to cDNA hybridization is much lower than that due to NcDNA binding, making the changes due to cDNA hybridization inaccurate. This again shows the importance of the presence of T9 spacer group on the sensing surface.

Figure 40: Signal-to-noise ratio with 1 μ M cDNA for varying ratios of reduced probe DNA and reduced T9 DNA spacer groups.

The signal-to-noise ratio (S/N) for all the different ratio of sensing surfaces is calculated according to Equation 4 and plotted in Figure 40.

As compared to the ternary sensing surfaces (Figure 20), the S/N ratios for the binary sensing surfaces in Figure 40 do not give a clear trend of the possible switch in alignment of the DNA probes due to the addition of different concentration of reduced T9 spacer group. Nevertheless, the advantage brought about by the addition of T9 spacer group is still seen from the increase in S/N values with just a small concentration of reduced T9 spacer groups added to the sensing surface immobilized with pure reduced probe DNA (ratio 1:0).

An interesting observation is that even with the use of the reduced form of DNA strands and having a conclusion that the ternary sensing surface possibility performs better than their reduced form, the optimized ratio for binary sensing surface that best discriminate between the NcDNA binding and cDNA hybridization also occurs at 1:1.

4.3 Applications

With the possibility of creating miniaturized and portable biosensors as one of the objectives of using the electrochemical technique, similar sensing surfaces optimized by DNA-based spacer groups are applied on 3-in-1 sonochemically-fabricated microelectrodes which can be purchased commercially. As mentioned in Chapter 2, microelectrodes have the capability of an improved sensitivity of the biosensors as compared to planar electrodes. This is however not part of the scope of this thesis and thus will not be further investigated.

Pre-surface modifications to these as-received carbon-based microelectrodes are required before the actual probe DNA/ spacer group immobilization (See Figure 41). Besides the commonly done aniline deposition for this type of microelectrodes ^(27, 28), Au deposition was also performed for the thiolated probe DNA immobilization. Experimental conditions used for aniline and gold deposition on these microelectrodes can be found in Section 3.3.3 and Section 3.3.4, respectively.

Note that since electrostatic attraction between the positively-charged aniline and the negative-charged DNA is the main contribution to binding, the DNA and spacer group strands used for aniline-deposited microelectrodes are non-functionalized at both ends in order to reduce any complications which may arise with unused functional groups. For gold-deposited microelectrodes, thiolated probe DNA is used for immobilization on the gold surfaces via the Au-S bonds.

The experimental procedures used for subsequent electrochemistry measurements in this section remain unchanged and they can be found in Chapter 3.

Figure 41: Schematics of as-received surface of the 3-in-1 sonochemically fabricated microelectrode and pre-surface modification step before probe immobilization.

4.3.1 Detection using aniline-deposited microelectrode

4.3.1.1 Electropolymerization of aniline

Figure 42: Cyclic voltammetry of polyaniline electrodeposition on microelectrode (a) 1st cycle, (b) 5th cycle, (c) 8th cycle, (d) 10th cycle, (e) Last segment

Aniline was deposited on sonchemically-fabricated microelectrodes by sequential cycling to form polyaniline protrusions. Each full cycle of the electropolymerization process was terminated at +0.8 V since irreversible aniline oxidation has shown to occur at around 0.85 V, regardless of the type of substrate used for deposition⁽⁹⁰⁾. Electropolymerization at potentials greater than 0.8 V was not performed to prevent possible side reactions and by-products formation, such as the benzoquinone, which can remain on the surface of the electrode and affect polyaniline growth⁽⁹¹⁾. A reversible redox couple is observed at around 0.2 V, as shown in Figure 42. The anodic peak, A, corresponds to the oxidation of the aniline monomer from its fully reduced state, known as the leucoemeraldine, to the emeraldine base⁽⁹⁰⁾. Upon doping, in this case in the presence of HCl, the emeraldine base converts into an emeraldine salt, which is highly conductive^(92, 93). Hence, to ensure that the

polyaniline protrusions are conductive for further use, a final single sweep from -0.2V to 0.8V was performed, as indicated by the curve (e) in Figure 42.

With successive sequential scans, oxidative peak A shifts anodically while reductive peak C shifts cathodically (See Figure 42). Peak current at A has also shown to increase with the number of deposition cycles. This indicates that the deposited polyaniline film is conductive and that the growth of the polyaniline is expected to occur at the polyaniline-electrolyte interface on the microelectrode⁽⁶⁸⁾.

4.3.1.2 SEM Micrographs

Figure 43: SEM micrographs of microelectrode surface before (A) and after (B) electropolymerization.

Before electropolymerization, randomly-distributed microcavities of various sizes can be observed on the microelectrode surface, as seen in Figure 43 (A). After electropolymerization, polyaniline layers build up from within the microcavities and eventually form protrusions as seen in Figure 43(B). The presence of microcavities seen even after electropolymerization, shows that not all of the sonochemically-formed microcavities are actually ablated deep enough to expose the underlying carbon layer (See Figure 43(B)). Nevertheless, the presence of protrusions formed after

electropolymerization show that the parameters used for electropolymerization are sufficient to form polyaniline protrusions, and not a polyaniline film, on the microelectrode surface.

4.3.1.3 Hybridization

Figure 44: Average change in Z' at 10Hz after hybridization with different concentration of NcDNA (Grey) and cDNA (Red) on reduced probe DNA immobilized on aniline-deposited microelectrode.

Figure 44 shows a comparison of the change in the real component of impedance (dZ') with the concentration of target DNA for hybridization. Only the real component of impedance (Z') was used for comparison since the increase in Z' dominates the overall increase in impedance. A positive dZ' indicates an increase in impedance after hybridization.

As shown in Figure 44, dZ' after cDNA hybridization is larger than that after NcDNA hybridization, in particular for 500 fM and 1 μ M of target DNA. A point to note for these sets of experiments is that the probe DNA immobilization on the electrode surfaces were not optimized before target DNA hybridization. Assuming that the electrode surfaces were fully covered with probe DNA before target DNA hybridization, such that there is no room for the formation of a twisted double helix, the target DNA could only form base-pairs at the surfaces of the immobilized probe DNA. The increase

in the number of DNA strands on the electrode surface after hybridization therefore increases the number of negative charges on the electrode surface. This causes greater repulsion of the negatively-charged redox probes, the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ ions, to the electrode surface, resulting in an effective increase in impedance after hybridization.

Hybridization with a lower concentration of 50 fM cDNA resulted in an unexpected decrease in impedance (eg. negative dZ'). This observation could be a result of the electrode surface not fully covered with the probe DNA before hybridization, giving space for the twisting and rolling during formation of double helix between the probe DNA and the target cDNA. Electrostatic attraction between the positively-charged polyaniline surface and the double helix weakens, since the negatively-charged phosphate groups are likely to be embedded within the DNA helix. At the same time, electrostatic repulsion between these DNA helices and the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ ions is also reduced, as compared to that before hybridization, where the negatively-charged phosphate groups from the immobilized probe DNA are exposed. Another possibility is the removal of these weakly bounded double helices to the polyaniline surface during rinsing, hence improving the accessibility of the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ ions to the electrode surface for electron transfers. Both cases lead to the decrease in impedance (eg. negative dZ').

4.3.2 Detection using gold-deposited microelectrode

4.3.2.1 SEM Micrographs & EDX Spectrums

Figure 45: SEM micrograph of microelectrode surface before (A) and after (B) electrodeposition of gold.

Figure 46: EDX spectrum before (left) and after (right) electrodeposition of gold.

As seen in Figure 45, randomly-distributed protrusions are formed after electrodeposition. EDX spectrums in Figure 46 show presence of gold peaks after electrodeposition, indicating the formation of gold protrusions. The gold protrusions formed are of various sizes, similar to that shown in Figure 43(B) after electropolymerization of aniline. However, the size range of protrusions formed after gold deposition ($< 1 \mu\text{m}$) is much smaller than that after aniline electropolymerization ($< 15 \mu\text{m}$). Even though smaller protrusions represent smaller surface area for DNA immobilization,

the deposition parameters have been optimized to form stable gold protrusions with the highest possible surface area.

4.3.2.2 Reduced 9-Thymine bases DNA

4.3.2.2.1 Monolayer Characterization

Figure 47: Average current change of overall immobilized reduced probe DNA and reduced T9 DNA spacer group on gold-deposited microelectrode.

Cyclic voltammetry (C-V) plots were recorded to study the electron transfer kinetics upon probe DNA immobilization. Average current change was calculated from the peak current change obtained from the cyclic voltammetry scans for the sensing surface before and after incubation with the respective ratio of reduced probe DNA and reduced T9 DNA spacer group. As mentioned in the previous section, a high magnitude of average current change signifies greater reduction in electron transfer across the Au surface, eg. due to the lowering of the accessibility of redox probes to Au surface.

The assembly of the DNA layer on the gold surface blocks the accessibility of the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ ions to the electrode surface, thus reducing electron transfer across the surface, as denoted by the negative change in current (See Figure 47). The efficiency of electron blockage, due to self-assembled monolayer formation on the gold electrode surface, increases to a maximum for a 1:2 ratio of probe DNA and T9 spacer groups. Once again, the addition of T9 spacer groups have shown to improve the formation of a closely-packed layer of DNA comprising of both the probe DNA and T9 spacer groups, as compared to a loosely-packed layer formed using pure probe DNA. The efficiency in the blocking of the surface for electron transfer by the assembled layer on the Au-deposited microelectrodes improves with the addition of T9 spacer groups, which has shown to be optimized at a ratio of 1:2.

As the ratio increases to 1:4, the average current change decreases. This can be due to the lower number of strands of randomly-oriented probe DNA sequence on top of the T9 spacer group regions, which blocks the movement of the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ ions to the electrode surface. Thus, the resultant blocking effect is lower than that at 1:2, where the presence of higher concentration of probe DNA can further reduce the movement of the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ ions to the electrode surface.

When the ratio further increase to 1:10, the average current change is similar to that when pure T9 spacer groups are being immobilized on the surface (eg: ratio 0:1). At a ratio 1:10, the minimum concentration of the probe DNA results in a similar blocking effect as that when T9 spacer groups are used for assembly.

4.3.2.2.2 Hybridization

Figure 48: Average current change after incubation with 1 μ M of NcDNA (Grey) and cDNA (Red) at room temperature (22°C) on gold-deposited microelectrode immobilized with varying ratios of reduced probe DNA and reduced T9 DNA spacer groups.

Cyclic voltammetry scans were performed on the entire range of sensing surface immobilized with different ratios of reduced probe DNA and reduced T9 spacer group immobilized on gold-deposited microelectrodes, before and after incubation with 1 μ M NcDNA and cDNA at room temperature (22°C).

The average current changes for all ratios are calculated according to Equation 3 and plotted in Figure 48. As compared to the current changes when reduced probe DNA and reduced T9 spacer groups are immobilized on planar Au (Figure 38), current changes after NcDNA incubation is generally much lower when gold-deposited microelectrodes are used as the substrate, relative to the current changes after cDNA hybridization. This may be attributed to the hemispherical shape of the Au protrusions on the microelectrode which helps to “spread out” the immobilized probe DNA

as compared to those immobilized on the planar Au substrate. The spreading out of the immobilized probe DNA reduces the steric hindrance of the cDNA to the immobilized probe DNA, leading to a more effective hybridization process. At the same time, NcDNA can also be washed away more easily from the sensing layer on the microelectrode surface than that on the planar gold surface, since the “spread-out” immobilized probe DNA on the Au protrusions will not hinder the removal of the weakly binded NcDNA. Therefore, as compared to using planar Au electrodes (Figure 38), current changes caused by the interaction of the sensing surface with NcDNA may be relatively lower when gold-deposited microelectrodes were used.

Figure 49: Average current change due to hybridization (corrected with non-specific interaction from “ultimate control”) for both NcDNA (Grey) and cDNA (Red) on gold-deposited microelectrode immobilized with varying ratios of reduced probe DNA and reduced T9 DNA spacer groups.

With the current change corrected with the “ultimate control” 0:1, Figure 49 shows the average current change due solely to hybridization for all the ratios of reduced probe DNA and reduced T9 spacer groups immobilized on the Au-deposited microelectrodes.

A gradual increase in cDNA hybridization is seen when ratio increases from 1:0 to 1:2, after which current changes starts to reduce when more reduced T9 spacer groups are added. Note that the current change due to NcDNA binding at ratio 1:10 is higher than that of cDNA hybridization, making 1:10 an undesirable ratio to use. Current changes for NcDNA binding remains relatively low for all other ratios immobilized on the gold-deposited microelectrodes.

Figure 50: Signal-to-noise ratio with 1 μ M cDNA for varying ratios of reduced probe DNA and reduced T9 DNA spacer groups immobilized on gold-deposited microelectrode.

The signal-to-noise ratio is calculated according to Equation 4 and plotted in Figure 50. As observed, the largest change in S/N value occurs at a ratio of 1:2. A lower or higher ratio than 1:2 has shown to reduce the S/N value significantly.

While a ratio of 1:1 is optimum for planar Au surface (Figure 41), a ratio of 1:2 is required for gold-deposited microelectrodes (Figure 47). For gold-deposited microelectrodes, where the surface area-to-volume ratio of the gold protrusions is expected to be higher than that of the planar Au surface, a

higher ratio of probe to spacer group seems to be required to form a sensing surface optimum for the best sensitivity.

The exceptionally high S/N ratio obtained by using gold-deposited microelectrodes, optimized with 1:2 ratio, indicates the superiority in the sensitivity of microelectrodes as compared to planar electrodes. As discussed in Chapter 4.1 and 4.2 which use planar electrodes, the best S/N ratio obtained is about 12 when used with disulphide DNA strands. This S/N value is, however, about 8 units lower than that obtained by microelectrodes (S/N ratio ≈ 20). The greater sensitivity from the microelectrodes is likely due to the enhanced mass transport of the target strands to the immobilized probe DNA, a result of the hemispherical solute diffusional profile inherent to microelectrodes. The extent of sensitivity and selectivity of microelectrodes will not be further investigated since they are not within the scope of this thesis.

4.3.2.3 Reduced 6-Thymine bases DNA

4.3.2.3.1 Monolayer Characterization

Figure 51: Average current change of overall immobilized reduced probe DNA and reduced T6 DNA spacer groups immobilized on gold-deposited microelectrode.

The average current change was calculated from the peak current change obtained from the cyclic voltammetry scans for the sensing surface before and after incubation with the respective ratios of reduced probe DNA and reduced T6 DNA spacer group.

Similar blocking efficiency by 6-thymine spacer groups (T6) is obtained even though the spacer groups are now shorter (6 thymine DNA bases rather than previously used 9 thymine DNA bases). Electron transfers between the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ ions and the electrode surface are reduced with the addition of the reduced T6 DNA spacer groups, denoted by the gradual increase in average current change of ratio 1:1 onwards when compared to 1:0 (pure probe DNA) (See Figure 51). The average

current changes reaches a plateau at a ratio of 1:4, where further increase in T6 DNA spacer groups do not result in higher current changes.

With an increase in the concentration of spacer groups, a lower number of probe DNA is expected to be immobilized since the spacer groups are much shorter than the probe DNA and will be able to replace some of the already immobilized probe DNA. The lower number of strands of probe DNA sequences on top of the T6 DNA spacer group regions (eg. ratio 1:10) should not block the accessibility of the $\text{Fe}(\text{CN})_6]^{3-/4-}$ ions from the electrode surface for electron transfer as much as those with a higher number of probe DNA strands (eg, ratio 1:4). Even though similar average current changes are seen between 1:4 and 1:10, the effect of electron blockage by the DNA sequences on top of the spacer group regions may be deduced from the much larger standard deviations. Note that such electron blockage effect is random and may result in smaller reproducibility of the sensing surface, thus larger standard deviations.

Immobilization of pure T6 DNA spacer groups on gold-deposited microelectrode form a highly compact SAM, indicated by the largest average current change in Figure 51. Even though a shorter vertical length of SAM is likely to allow higher electron transfer across the electrode surface, a more compact SAM is also expected to be form due to the smaller size of the shorter DNA strand, thus reducing the electron transfer.

4.3.2.3.2 Hybridization

Figure 52: Average current change after incubation with 1 μ M of NcDNA (Grey) and cDNA (Red) at room temperature (22°C) on gold-deposited microelectrodes immobilized with varying ratios of reduced probe DNA and reduced T6 DNA spacer groups.

Cyclic voltammetry scans were performed and the average current change was calculated according to Equation 3 and plotted in Figure 52.

Average current changes due to cDNA hybridization are generally lower than that due to NcDNA binding, indicating sensing surfaces which are non-selective. Selective surfaces only occur on sensing surface immobilized with ratio 1:0 and 1:10.

Greater channeling of the redox probes are expected with cDNA hybridization since rigid double helix is formed. However from Figure 52, negative current changes are seen from ratio 1:2 and 1:4, which indicates a reduction in electron flow after cDNA hybridization. Assuming that some of the probe DNA strands are not aligned upright on the surface, the double helix formed after cDNA

hybridization may not have the space to truly extend outwards into the solution. There is also the possibility of having a cDNA strand hybridizing with more than one probe DNA strand which is in close proximity. This makes it impossible for the hybridized ends to extend outwards to allow greater channeling of the redox probes. These additional negative charges on the surface from the hybridized cDNA strands will instead repel the negatively-charged redox probes from the surface, resulting in a lower current after hybridization and a negative calculated current change. Such explanations, though possible, may not be truly conclusive to the actual sensing surface, since the trend for such occurrence is not consistent.

Figure 53: Average current change due to hybridization (corrected with non-specific interaction from “ultimate control”) for both NcDNA (Grey) and cDNA (Red) on gold-deposited microelectrodes immobilized with varying ratios of reduced probe DNA and reduced T6 DNA spacer groups.

With the average current change corrected with the “ultimate control” 0:1, Figure 53 shows the average current change due solely to hybridization for all the ratios of reduced probe DNA and reduced T6 spacer groups immobilized on the Au-deposited microelectrodes.

Current changes due to NcDNA binding are generally negative, which show a reduction in electron transfer after NcDNA incubation. As concluded in the above section, the selective sensing surfaces remain at ratio 1:0 and 1:10, where current changes due to cDNA hybridization can be easily discriminated from NcDNA binding.

Figure 54: Signal-to-noise ratio with 1 μ M cDNA for varying ratios of reduced probe DNA and reduced T6 DNA spacer groups immobilized on gold-deposited microelectrodes.

Signal-to-noise ratios (S/N) are calculated according to Equation 4 and plotted in Figure 54. As observed, the largest S/N ratio occurs at ratio 1:10 for reduced probe DNA and reduced T6 DNA spacer group immobilized on gold-deposited microelectrodes. The negative S/N value is due to the negative current change after NcDNA binding.

Comparing T6 DNA with T9 DNA immobilized on gold-deposited microelectrodes, it can be observed that T9 DNA generally gives a better trend of current changes with cDNA and NcDNA incubations. While no concrete evidence can be used for explaining such observation, this is likely due to the

shorter spacer group for T6 DNA. With a shorter spacer between the actual DNA sequences and the Au surface, interactions between the DNA bases and the Au surface are higher. Such interactions may interfere with the actual interactions with the cDNA sequences. Even though monolayer characterization shows a more compact SAM for immobilized T6 DNA (Figure 51) than that for T9 DNA (Figure 47), it does not equate such compact layer as having better sensitivity or selectivity. Evidently from the experimental results, the longer T9 DNA, though overall obtain less compact sensing surfaces, produce more sensitive and selective surfaces.

4.3.2.4 Reduced 6-Thymine bases PNA

Peptide nucleic acid (PNA) is an artificially synthesized analogue of DNA, which can also selectively hybridize with its complementary bases via the Watson-Crick base pairing. The backbone of PNA is, however, made up of repeating units of N-(2-aminoethyl)-glycine linked by peptide bonds, making it a neutral strand. When immobilized together, they form a neutral SAM which should not repel the negatively-charged cDNA for hybridization, unlike the case for negatively-charged DNA SAM.

In the following section, similar experiments were conducted on Au-deposited microelectrodes, but this time, using reduced probe PNA and reduced T6 PNA spacer group. The cDNA and NcDNA used for detection remains unchanged. A comparison between DNA and PNA SAM will be the main focus for this section and how the difference in the SAM charge can possibly affect the hybridization events will be shown.

4.3.2.4.1 Monolayer Characterization

Figure 55: Average current change of overall immobilized reduced probe PNA and reduced T6 PNA spacer groups on gold-deposited microelectrodes.

The average current change was calculated from the peak current change obtained from the cyclic voltammetry scans for the sensing surfaces before and after incubation with the respective ratios of reduced probe PNA and reduced T6 PNA spacer groups.

As can be observed from Figure 55, there is a general increase in average current change with an increase in the concentration of T6 PNA spacer group. This suggests an improvement in the compactness of the SAM when more T6 PNA spacer groups are being immobilized on the Au surface.

An interesting observation is the similar average current changes for the different ratios formed by the T6 PNA SAM (Figure 55) and that by T6 DNA SAM (Figure 51) on the Au-deposited microelectrodes. Neutral PNA is commonly thought to be able to pack better than DNA strands since repulsion between the PNA strands during immobilization should not exist. However, the experimental results suggest a similar packing ability of the PNA and DNA during the formation of the SAM, even though PNA is neutral and DNA is negatively-charged.

For the case of 0:1 where only spacer groups are immobilized, T6 PNA spacer group SAM results in a smaller current change as compared to that of T6 DNA spacer group SAM. From the similar current changes obtained for the other ratios SAM, the large difference for that of 0:1 PNA and DNA SAM is likely not due to their difference in packing ability, but rather due to the charges of the SAM formed by the two different type of molecules. Since 0:1 PNA SMA is neutral, repulsion with the negatively-charged redox probes will be reduced significantly. Electron transfer can thus occur more easily across the short T6 PNA spacer groups, resulting in a small current change with T6 PNA spacer group SAM. In the case of T6 DNA spacer group SAM, repulsion between the negatively-charged redox probes and the negatively-charged SAM will increase the distance between the Au surface and the redox probes, resulting in much lesser electron transfer across the surface. Even though such repulsion should also exist for the other ratios using the probe DNA, such that a higher current

changes should be obtained when compared to probe PNA, the much longer length of the probe as compared to the spacer group may have made such effects due to repulsion less significant.

4.3.2.4.2 Hybridization

Figure 56: Average current change after incubation with 1 μ M of NcDNA (Grey) and cDNA (Red) at room temperature (22°C) on gold-deposited microelectrodes immobilized with varying ratios of reduced probe PNA and reduced T6 PNA spacer groups.

Cyclic voltammetry scans were performed and the average current change was calculated according to Equation 3 and plotted in Figure 56.

As observed from Figure 56, average current change due to NcDNA binding relatively remains similarly high for all ratios immobilized on the Au-deposited microelectrodes. A significant increase in current change for cDNA hybridization is seen when T6 PNA spacer group is added to the immobilized probe PNA (ratio 1:0), which continue to increase with increasing concentration of T6 PNA spacer group. This signifies the importance of the addition of the T6 spacer groups to the

sensing surface for improved current response. However, it can also be observed that both the current changes for NcDNA and cDNA binding at 0:1 sensing surface is very high as well. This shows high degree of non-specific interaction between the target strands and the T6 PNA spacer groups. In fact, only the current change at ratio 1:10 shows true response due to cDNA hybridization, since only at this ratio is the current change for cDNA hybridization higher than that obtained by cDNA binding at 0:1, where only non-specific interactions can occur.

Figure 57: Average current change due to hybridization (corrected with non-specific interaction from “ultimate control”) for both NcDNA (Grey) and cDNA (Red) on gold-deposited microelectrodes immobilized with varying ratios of reduced probe PNA and reduced T6 PNA spacer groups.

With the average current change corrected with the “ultimate control” 0:1, Figure 57 shows the average current change due solely to hybridization for all the ratios of reduced probe PNA and reduced T6 PNA spacer groups immobilized on the Au-deposited microelectrodes.

As mentioned in the above section, true current change due to cDNA hybridization only occurs at ratio 1:10, as can be seen from the only positive current change due to cDNA hybridization in Figure 57. The current change due to NcDNA binding is also very low at this ratio.

PNA, besides having a neutral charge, is likely to differ from DNA in many ways which may not have been discovered yet. While DNA-based spacer group has shown to perform better for DNA SAM in the previous sections than organic spacer groups, it is possible that PNA-based spacer group may not be as beneficial to PNA SAM as the organic spacer group. From the results in Figure 57, positive current change due to cDNA hybridization only occurs at ratio 1:10, which suggest that more PNA-based spacer groups may be required to get an even more selective sensing surface (eg. 1:20 or higher). However, since PNA studies are not within the scope of this thesis, in-depth investigations on PNA strands will be not carried out.

Figure 58: Signal-to-noise ratio with 1 μ M cDNA for ratio 1:10 immobilized on gold-deposited microelectrode using T6 DNA strands or T6 PNA strands

As concluded in the above section that only at ratio 1:10 is the current change truly due to cDNA hybridization, Figure 58 shows a comparison between the signal-to-noise ratio (S/N) calculated for both 1:10 sensing surfaces formed using T6 DNA strands and T6 PNA strands. Note that for T6 DNA sensing surface, ratio 1:10 is also concluded as being the best ratio (eg. highest S/N value) that can discriminate between the NcDNA binding and cDNA hybridization.

From Figure 58, it can be observed that using PNA strands result in a larger S/N value as compared to that using DNA strands. This can be due to the neutral SAM formed by the PNA, which reduces repulsion of the redox probes from the SAM layer. While both sensing surfaces improve the channeling of redox probes to the Au surface due to the formation of rigid double helix after hybridization, the reduction in electrostatic repulsion between the redox probes and PNA SAM will allow the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ ions to diffuse closer to the Au surface for electron transfer, thus improving the current changes due to hybridization relative to that due to NcDNA binding. DNA SAM, on the other hand, will have lesser channeling $[\text{Fe}(\text{CN})_6]^{3-/4-}$ ions since repulsion between the same charged ions and DNA SAM will reduce the number of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ ions near the surface.

5 Conclusions

In conclusion, DNA-based spacer group has shown to be a possible alternative to commonly-used organic spacer group to form an assembled layer of high selectivity. Both SPR and QCM-D measurements showed distinct binding curves with 1 μM of fully complementary DNA (cDNA) strands, while little or no response was seen with non-complementary DNA (NcDNA) strands. From electrochemistry, blocking of the planar gold electrode surface for electron transfer by the assembled layer improves with the addition of DNA-based spacer groups, in this case with the use of T9 spacer groups.

Evaluated using the Bivalent Ligand Model, the ratio of 1:1 (probe: T9) results in the greatest response from cDNA hybridization in SPR measurements. QCM-D analysis showed varying frequency and dissipation changes with the different SAM made up of various ratios of probe DNA and T9 spacer groups. Using the Sauerbrey equation, the calculated mass changes, after immobilization of the probe DNA and/or T9 spacer groups, increased with the addition of T9 spacer groups. Dissipation changes showed possible greater alignment of the probe DNA at ratios higher than 1:1.5. Such alignment of probe DNA may, however, allows the trapping of NcDNA strands within the layers, resulting in a lower selectivity of the sensing surface.

The blocking efficiency by the ternary mixed layer is shown to be optimized at a ratio of 1:1 (probe: T9) from electrochemistry. At this 1:1 ratio, when probe DNA was hybridized with 1 μM of cDNA strands, the increase in current measured was significantly larger than that measured when hybridization was carried out using NcDNA. Upon cDNA hybridization, the formation of double helices helped to enhance the accessibility of the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ ions to the electrode surface, thereby increasing electron transfer and thus current flow. The hybridization conditions were optimized at 55°C for 2 hours, where the high temperature and long hybridization duration resulted in the greatest discrimination between cDNA hybridization and NcDNA binding.

With the optimized ratio and hybridization conditions, the sensitivity was largest at 1 μM and the detection limit was estimated to 0.1 μM . Single-mismatch discrimination was possible at a concentration of 0.1 μM , though the mismatch position cannot be determined. Through the distinct difference in S/N ratio from electrochemistry, mismatch position with single-mismatch hybridization at the optimized ratio and conditions, could be determined at a concentration of 1 μM . Such capability in distinguishing the different mismatch positions using label-free platform was highly commentary, though more can be done to improve the sensitivity of such sensing surface using DNA-based spacer groups.

Compared with sensing surfaces backfilled with commonly-used organic spacer groups, namely 2-mercaptoethanol (ME), 3-mercapto-1-propanol (MP) or 6-mercapto-1-hexanol (MCH), T9 spacer groups formed a sensing surface which was about 10 times more selective as those before. Once again, this showed great capabilities of DNA-based spacer groups as backfiller molecules.

Binary sensing surfaces formed using reduced probe T9 DNA and reduced T9 spacer groups were also conducted. SPR sensorgrams showed the formed SAM surfaces being less homogenous than that of ternary sensing surfaces, though excellent selectivity between cDNA hybridization and NcDNA binding could still be observed. Even though the optimized ratio using binary sensing surface was found to be 1:1 by electrochemistry (similar to that for ternary sensing surface), a clear conclusion regarding the alignment of the probe DNA with T9 spacer groups could not be drawn. The overall S/N ratio to discriminate between the cDNA hybridization and NcDNA binding at ratio 1:1 for the binary surface was found to be essentially the same as for the 1:1 ternary surface, both using target concentration of 1 μM .

In the attempt to improve the sensitivity of such sensing surfaces using DNA-based spacer groups, commercially available sonochemically fabricated microelectrodes were used. These microelectrodes were first surface modified to form gold protrusions ($< 1 \mu\text{m}$) by electrochemical

deposition. Using such gold-deposited microelectrodes, 8 units of improvement in S/N ratio is achieved as compared to that obtained using planar gold electrode. The hemispherical solute diffusional profile inherent to microelectrodes is likely to have a positive effect on the S/N of the biosensor.

Experiments involving shorter DNA-based spacer group and PNA-based spacer groups, both with 6 thymine strands (T6), were also conducted on the gold-deposited microelectrodes.

Compared to using T9 DNA strands, SAM formed from T6 DNA strands result in a higher current change than that formed by T9 DNA strands, indicating a more compact SAM on the surface by the T6 DNA strands. However, the trend of S/N across the range of ratios using shorter T6 DNA strands is not as consistent as that when using longer T9 DNA strands. When using shorter T6 DNA strands, there may be more non-specific interactions between the probe DNA sequences and the Au surface, which can interfere with the actual hybridization and hence result in an inconsistent trend of S/N. This makes longer T9 DNA strands more desirable in forming a sensor with better robustness and consistency.

Comparing between negatively-charged T6 DNA strands and neutral T6 PNA strands of the same length, the SAM formed generally results in similar average current changes, except for ratio 0:1. At ratio 0:1 (pure spacer groups), the negatively-charged SAM formed by T6 DNA spacer groups will result in more repulsion of the negatively-charged redox probes from the surface than that formed from the neutral PNA SAM, thus reducing the electron exchange and current flow. Current flow after immobilization of either the DNA or PNA strands for other ratios remained generally in the same range, likely due to the longer probe strands which can also significantly block the surface from the redox probes. Interestingly, both T6 DNA and PNA strands showed ratio 1:10 as the optimized ratio to obtain a sensing surface with the highest S/N value. Having formed a SAM of slightly higher average current change (eg. better compactness), the PNA sensing surface result in about 3 units

improvement of S/N ratio at the optimized 1:10 ratio as compared to that using DNA strands. This is likely due to the neutral backbone of PNA which allows the redox probes to channel closer to the Au surface for more efficient electron transfer after cDNA hybridization, as compared to that of negatively-charged DNA.

Using the microelectrodes, aniline protrusions were also formed from within the randomly-arranged microcavities by electropolymerization. Non-functionalized DNA probes of 30 mers were attached to the positively-charged aniline protrusions via electrostatic interactions. Detections at 500 fM of target DNA strands were shown to be possible, with significant difference in impedance change between cDNA hybridization and NcDNA binding. The higher sensitivity of such an approach is likely due to the use of microelectrodes with the impedimetric sensing method (Electrochemical Impedance Spectroscopy), both which is more sensitive to changes at the electrode-surface interface as compared to the use of planar electrodes and voltammetric sensing method.

6 Future Work

Due to the scope of project and time constraints, current investigation using DNA-based spacer groups are focused mainly on how their presence on sensing surface affect electrochemical signal change due to hybridization and their capabilities as backfillers when compared to commonly-used organic spacer groups.

The following section suggests possible extension to further the study on the use of thymine-based spacer group.

6.1 Vary length of thymine bases

In the current study on planar Au substrate, strands of nine thymine bases (T9) have been used to find the optimized ratio and conditions. While shorter six thymine bases strands (T6) have been shown to result in lesser sensitivity (eg. lower S/N), the probe:spacer group ratio used to prepare the sensing surface is based on that using T9 spacer groups for comparison. Effects of using strands longer than T9 have also not been explored. Since the length of organic spacer groups have shown to affect the sensing ability of the electrochemical biosensors, optimization work using the different length of thymine spacer groups can be further extended to investigate the effects of DNA-based spacer group on the sensing ability.

6.2 Vary end group of thymine bases

More work can also be done to find the effects of the thymine strands functionalized with an end-group on the overall sensing ability. As mentioned in Chapter 2, Dharuman *et al* has shown the function of organic spacer group functionalized with different end groups, on the packing and sensitivity of the sensing surface.⁽⁶⁷⁻⁶⁹⁾ Effects of using end group functionalized thymine spacer groups, such as –OH and –COOH, can also be investigated. The presence of hydroxyl group, for example, can increase the solubility of the thymine strands and possibility extends the strands out

into the electrolyte. This may help to improve the orientation of the DNA probe and increase the effective blocking of the gold substrate before target recognition.

6.3 Comparison between PNA and DNA strands with thymine-based spacer group

Peptide nucleic acid (PNA) has, in recent years, created great interest in the sensing research field as an alternative to DNA, due to its neutral-charged backbone and the higher thermal stability of a hybridized PNA-DNA strand as compared to a hybridized DNA-DNA strand. More research can be explored, both to improve the sensitivity of the sensor using neutral PNA strands and to the use of PNA-based spacer groups for modulating the density of the receptors on the sensing surfaces.

6.4 Effect of spacer groups for other type of receptors

Most work on receptor density optimization has been focused on DNA-based sensing surface using organic spacer groups. For sensing surfaces based on antibody-antigen recognition, research is largely focused on orientating the active sites of antibodies for maximum antigen recognition using different linkers and immobilization methods.^(62, 94-96) By far, little has been done to study the effects of the addition of spacer groups in modulating the density of immobilized antibodies on the sensing surface. Similar to that of DNA-based sensing surface, optimizing the density of oriented antibodies on the sensing surface can have an improvement in the sensitivity of the sensor due to the reduction in steric hindrances to the antigen targets, making such study important.

6.5 Use of thymine-based spacer groups as capturing agents

Calorimetric biosensors based on conjugated polythiophene (PT) polymer have been used to detect biomolecules such as nucleotides^(97, 98) and ATPase⁽⁹⁴⁾. The ability of the PT polymer to interact with single-stranded DNA allows immobilized DNA strands to serve as a capturing agent for PT. Thymine-based spacer groups, which has shown to form a passive layer on the Au surface, can be used to capture and stabilize PT polymer onto the surface. Such an assay allows the detection of small molecules, such as miRNA⁽⁹⁹⁾, using other surface characterization tools (eg. QCM-D).

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CONFERENCES ATTENDED

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APPENDIX (A)

SPR sensorgrams after subsequent injections of buffer, 1 μ M NcDNA and 1 μ M cDNA on Au chip each immobilized with the various probe:T9 ratios.

1) 1:0

2) 1:0.25

APPENDIX (A)

3) 1:0.5

4) 1:1

APPENDIX (A)

5) 1:1.5

6) 1:2

APPENDIX (A)

7) 1:2.5

8) 0:1

APPENDIX (B)

Three measurements were done simultaneously on the same Au chip each immobilized with the various probe:T9 ratios. Binding curves after injection of 1 uM cDNA were fitted with “Bivalent Ligand” model installed in the BIAevaluation programme, in which the association and dissociation rate constants are evaluated from the binding curve after cDNA injection (red arrow) and dissociating curve after rinsing (green arrow). A plot of the evaluated average corresponding rate constants for all sensing surfaces is shown at the end of the Appendix (B).

1) 1:0

APPENDIX (B)

2) 1:0.25

3) 1:0.5

APPENDIX (B)

4) 1:1

5) 1:1.5

6) 1:2

APPENDIX (B)

7) 1:2.5

APPENDIX (B)

Association (k_a -Red) and dissociation (k_d -Black) rate constants evaluated from SPR measurements with 1 μM cDNA hybridization on varying ratios of disulphide probe DNA and disulphide T9 DNA spacer groups.

APPENDIX (C)

QCM-D graphs after overnight incubation with the respective ratios of probe:T9 disulphide solutions and subsequent injections of 1 μ M NcDNA and 1 μ M cDNA on 5 MHz AT-cut piezoelectric quartz crystal coated with 100 nm Au.

1) 1:0

2) 1:0.25

APPENDIX (C)

3) 1:0.5

4) 1:1

APPENDIX (C)

5) 1:1.5

6) 1:2

APPENDIX (C)

7) 1:2.5

8) 0:1

APPENDIX (D)

Cross-sectional height analysis for sensing surface immobilized with ratio 1:0, 1:1 and 0:1, as well as Au surface as a control.

1) 1:0

APPENDIX (D)

2) 1:1

APPENDIX (D)

3) 0:1

APPENDIX (D)

4) Au

APPENDIX (D)

Roughness vales for each sample and calculations for 1-way ANOVA:

Sample (<i>i</i>)	Roughness (rms)			
	Au	1:0	1:1	0:1
	1170	992.624	768.104	882.816
	986.799	1010	1005	875.472
	909.937	1142	915.719	911.496
	1001	1100	775.92	1106
			844.518	
			822.277	
			747.055	
			810.296	
Mean ($\bar{x}$)	1016.93	1061.16	836.11	943.95
Standard Deviation	94.9	62.0	80.6	94.5

Total number of samples (*i*), $a = 4$.

Total number of observations (*j*), $N = 20$.

Source of Variance	df	Sum of Squares	Mean Square
Sample	$a-1$	SS_{sample}	MS_{sample}
Roughness	$N-a$	$SS_{\text{roughness}}$	$MS_{\text{roughness}}$
Total	$N-1$	SST	

df is the degree of freedom, which is the effective number of independent observations used in forming each sum of squares (SS).

APPENDIX (D)

Calculations are made as follows:

$$SS_{sample} = \sum_{i=1}^a n_i (\bar{x}_i - \bar{x})^2$$

$$SS_{roughness} = \sum_{i=1}^a \sum_{j=1}^{N_i} (x_{ij} - \bar{x}_i)^2$$

$$MS_{sample} = SS_{sample} / (a-1)$$

$$MS_{roughness} = SS_{roughness} / (N-a)$$

$$SST = SS_{sample} + SS_{roughness}$$

$$F \text{ statistics} = MS_{sample} / MS_{roughness}$$

APPENDIX (E)

I. Optimization of Probe surface density by disulphide T9 DNA spacer group

Average values of current changes on separate Au electrodes after immobilization with the various ratios of probe:T9 disulphide (N=6).

Triplicate values of current changes after incubation with 1uM cDNA on separate Au electrodes immobilized with the various ratios of probe:T9 disulphide.

APPENDIX (E)

Triplicate values of current changes after incubation with 1 μ M NcDNA on separate Au electrodes immobilized with the various ratios of probe:T9 disulphide.

II. Optimization of Hybridization Conditions

Triplicate values of current changes after incubation with 1 μ M NcDNA/cDNA at room temperature for varying hybridization time on separate Au electrodes immobilized with ratio 1:1 disulphide DNA.

APPENDIX (E)

Triplicate values of current changes after incubation with 1uM NcDNA/cDNA at 55°C for varying hybridization time on separate Au electrodes immobilized with ratio 1:1 disulphide DNA.

Triplicate values of current changes after incubation with 1uM NcDNA/cDNA at RT/55°C for 2h on separate Au electrodes immobilized with ratio 0:1 disulphide DNA ("ultimate control").

APPENDIX (E)

III. Determination of Sensitivity and Selectivity

Triplicate values of current changes after incubation with 1 μ M N3 at 55°C for 2h on separate Au electrodes immobilized with ratio 1:1 disulphide DNA.

Triplicate values of current changes after incubation with 1 μ M N4 at 55°C for 2h on separate Au electrodes immobilized with ratio 1:1 disulphide DNA.

APPENDIX (E)

IV. Comparison with other spacer group

Triplicate values of current changes after incubation with 1uM cDNA at 55°C for 2h on separate Au electrodes immobilized with disulphide probe DNA and the respective organic spacer groups.

Triplicate values of current changes after incubation with 1uM NcDNA at 55°C for 2h on separate Au electrodes immobilized with disulphide probe DNA and the respective organic spacer groups.

APPENDIX (E)

V. Shorter Thymine Spacer group (T6)

Triplicate values of current changes after incubation with 1uM cDNA/NcDNA at 55°C 2h on separate Au electrodes immobilized with ratio 1:1 disulphide DNA and T6 spacer group.

Triplicate values of current changes after incubation with 1uM cDNA/NcDNA at 55°C 2h on separate Au electrodes immobilized with ratio 0:1 T6 spacer group (“ultimate control”).

APPENDIX (F)

UV-vis spectrums of single-stranded probe T9 disulphide (probe), complementary DNA (cDNA) and non-complementary DNA (NcDNA) were measured before hybridization. After mixing the respective DNA solutions and heating at 55°C for 1h, where hybridization was allowed to occur, UV-vis measurements were taken again.

The drop in absorbance for the mixture of probe and cDNA solutions showed hybridization has occurred after heating at 55°C for 1h.

APPENDIX (G)

SPR sensorgrams after subsequent injections of buffer, 1 μ M NcDNA and 1 μ M cDNA on Au chip each immobilized with the various probe:T9 reduced ratios. Note some ratios experience an unexpected “jump” in response at the start and end of cDNA injection for unknown reasons.

1) 1:0

2) 1:0.25

APPENDIX (G)

3) 1:0.5

4) 1:1

APPENDIX (G)

5) 1:1.5

6) 1:2

APPENDIX (G)

7) 1:2.5

8) 0:1

APPENDIX (H)

Three measurements were done simultaneously on the same Au chip each immobilized with the various probe:T9 reduced ratios. Binding curves after injection of 1 uM cDNA were fitted with “Bivalent Ligand” model installed in the BIAevaluation programme. Note the “jump” in response at the start and end of cDNA injection in some ratios were removed for better representation.

1) 1:0

APPENDIX (H)

2) 1:0.25

APPENDIX (H)

3) 1:0.5

APPENDIX (H)

4) 1:1

APPENDIX (H)

5) 1:1.5

APPENDIX (H)

6) 1:2

APPENDIX (H)

7) 1:2.5

APPENDIX (I)

I. Optimization of Probe surface density by reduced T9 DNA spacer group

Average values of current changes on separate Au electrodes after immobilization with the various ratios of probe:T9 reduced (N=6).

Triplicate values of current changes after incubation with 1uM cDNA on separate Au electrodes immobilized with the various ratios of probe:T9 reduced.

APPENDIX (I)

Triplicate values of current changes after incubation with 1uM NcDNA on separate Au electrodes immobilized with the various ratios of probe:T9 reduced.

APPENDIX (J)

Current changes on separate Au-deposited microelectrodes after immobilization with the various ratios of probe:spacer groups.