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DNA Detection System of Homo-oligomers by Electrochemical Impedence Measurements and Quantification through Radiolabeling

By: Nicholas Deligiannis

A Thesis

In

The Department

Of

Chemistry and Biochemistry

Presented in Partial Fulfillment of the Requirements
For the Degree of Master of Science
At Concordia University
Montreal, Quebec, Canada

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Abstract

DNA detection system of homo-oligomers based on Electrical Impedance Measurements and Quantification through Radiolabeling

Nicholas Deligiannis

A system based on electrical impedance measurements using chemically modified Silicon/Silicon dioxide electrodes with covalently bound single stranded homo-oligonucleotides was developed for DNA detection. Silanating agents. (3-aminopropyltriethoxysilane, 3-glycodoxyethylpropylamine) were used to modify the electrodes and immobilize single stranded DNA. A method based on the electrochemical modification of Si substrates with bromobenzene diazonium was also used to immobilized single stranded DNA. The DNA detection system is able to recognize any electrical changes occurring at the electrode through in-phase and out-of-phase impedance measurements. Hybridization of the complementary strands causes a shift in the flat band potential due to variations in charge accumulating at the modified oxide/electrolyte interface. With these measurements, we were able to make reliable comparisons between the electrical measurements and the quantification of immobilized and hybridized single stranded DNA through radiolabeling.

ACKNOWLEDGMENT

I would like to express my earnest appreciation to Dr. Marcus Lawrence for his guidance and effervescent motivation throughout the three years I spent working with him. I would also like to thank Dr. Constantine Polychronakos on his guidance on his extensive knowledge on the DNA subject-matter.

I would like to thank Dr. Paul Joyce and Dr. R. Le Van Mao for serving on my research committee.

I would like to thank Dr. Isabelle Lawrence for her guidance in the first year of the project, the staff in the Department of Chemistry and Biochemistry and all of the undergraduate students I worked with Robert, Isabelle, Sebastien, Carine.

Finally, I would like to dedicate this thesis to my family: my father, my brother, my fiancee Astero and her family especially the constant push of my future mother-in-law.

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List of Symbols

OligodTBr	20 bases of T oligonucleotides with 5' Br
OligodTam	20 bases of T oligonucleotides with aminolinker
OligodTA	20 bases of A oligonucleotides
C	Capacitance
C_d	Capacitance of dielectric
R	Resistance
R_s	Resistance due to ohmic contacts
Z_{sc}	Impedence of semi-conductor
V	Potential
$\tilde{Z}$	Complex impedence
Z_p	in-phase impedence
Z_q	out-of-phase impedence
I	current
ω	angular frequency
ε	permittivity of semiconductor
ε_o	permittivity of free space
f	frequency
q	elementary charge
N	dopant density
V_{fb}	flat band potential

ssDNA	**single stranded deoxyribonucleic acid**
GPTS	**3-glycidoxyethylpropylamine**
APTS	**3-aminopropyltriethoxysilane**
DPTA	**diisopropylethylamine**
T_m	**melting temperature**
XPS	**X-ray photoelectron spectroscopy**

1 Background

Accurate measurements of biologically related substances have been a major goal in the late twentieth century. Detection of nucleic acids (deoxyribonucleic acid (DNA) and ribonucleic acid (RNA)) remains among the most challenging due to the variety of substance in the sample, the complexity of the nucleic acid structure and the time–dependent protocols.

An electrochemical DNA hybridization detection system based on impedance measurements[1,2] was devised to discriminate between simple repeat sequence alleles and to eventually distinguish differences in nucleic acid sequence polymorphisms.

1.1 Importance of Nucleic Acid Detection

Nucleic acids are the genetic makeup of any living organism. DNA, in particular, is the basic building block of genes which governs various functions of the organism. The Human Genome Project[3] was a project set out in 1990 to map the entire 3 billion base pairs that makeup human DNA and was set for completion in the year 2006. However, with new technologies, The Human Genome Project is now set for a summer 2000 completion (90%) along with a high-resolution mapping of every base pair by the year 2003. DNA sequence polymorphisms is responsible for a number of genetic or Mendelian disorders. Mendelian disorders account for the various inherited diseases caused by a DNA sequence mutation found in the gene. The gene is an

information-containing element that is past from one generation to the next and with over 100 000 genes that make up the human genome, the significance of detecting such indisposition is of high importance. Therefore, methods of detecting nucleic acids has recently become a major goal in biotechnology aiming at a variety of objectives.

- **1.2.1 Analysis of genetic disorders**

  Current detection of mutations inside a genetic marker depends solely on PCR-based methods utilizing size differences in polymorphic PCR products, usually simple sequence repeats, electrophoretically separated by size[4]. A method that is time consuming and not amenable to large-scale automation.

  Despite these limitations, genetic analysis by current methods has resulted in the successesful study of Mendelian disorders caused by mutation of a single gene; however, it accounts for a small portion of the genetically determined human diseases. Overall, diseases like diabetes, hypertension, and cancer involve more than one gene, which amplifies the logistics of linkage analysis[5].

- **1.2.2 Population Screening**

  Population screening is an important application in the identification of couples in which both partners are carriers of a recessive genetic disorder. Screening is used in order to detect Mendelian disorders such as cystic fibrosis, or breast cancer, or to

detect immunological[6] disorders such as insulin-dependent diabetes that is found in the family history of the patients. Other applications involve the population of various ethnic backgrounds that are at risk such as sickle cell anemia in blacks or Tay-Sachs disease in Jews. Programs to screen the population at large for complex disorders are currently being envisaged.

- **1.2.3 New DNA Detection Technologies**

  There is a consensus that to make DNA detection methods amenable to massive throughput, the electrophoresis step (i.e. Polymerase Chain Reaction method) needs to be annulled. Gel-less technologies have been under development and until recently it was demonstrated that genetic information in a DNA sample can be detected with the use of a biosensor[7,8].

1.3 What is a Biosensor?

The need of more detailed information about DNA and other biological molecules (proteins, RNA) led to the innovation and development of bioanalytical sensors, referred to simply as biosensors. Biosensors were forecasted originally as discrete analytical devises that will measure analytes selectively. The high degree of selectivity required for such measurements was to be based on biological recognition processes occuring via immobilized biocomponents on ordinary detectors. In addition, biosensors do more than provide selective detection, they provide new options for high selectivity in direct

measurements, they may respond in a continuous reversible manner, they can accomplish a measurement with little disturbance of the sample and can also be compatible with the environment in which the measurement is made. Biosensors are convenient for industrial applications, they can be economical and lend themselves to miniaturization, portability, low cost of mass production and easy to use.

Biosensors consist of two main components: (a) bioreceptor and (b) transducer.

- **1.3.1 (a) Bioreceptor**

  The bioreceptor consists of a chemically selective molecular recognition layer that is bound to the transducer. The bioreceptor acts as a molecular recognition sight. For example, antibodies fixed on transducers can act as receptors to the analye[10,11] (in this case the complementary antigens). Hence the bioreceptor is sight–specific and responds with high affinity.

- **1.3.2 (b) Transducer**

  Tranducer, from the Latin word *transucere*, means to lead across. The function of a transducer is to lead across chemical information about an analyte so it can be processed as an electrical signal for data acquisition and interpretation.

  Various transducers are used ranging from electrochemical transducers such as potentiometric[12] and amperometric analysis.

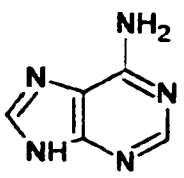
Optical sensors based on fluorescence[13] measurements and various acoustic wave sensors (quartz crystal microbalance[14]) based on surface acoustic wave measurements have also been investigated. Finally, chemical transduction which involves, for example, immunochemical binding or binding ofcomplementary oligonucleotides are also being explored.

1.4 DNA Biosensor

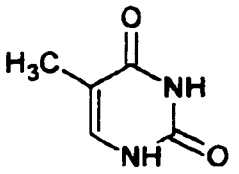
The Human Genome Project has created an explosive growth in sensor development for nucleic acid detection through hybridization. In the area of DNA sensors, high density oligonucleotide arrays have been produced by different technologies.

Fodor et al[15], at Affymetrix, designed a light directed oligonucleotide synthesis for constructing high density DNA probe arrays using photolithography. Mikkelsen et al[16] developed an electrochemical biosensor for DNA detection.

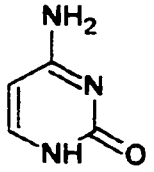
In more detail, how does a DNA biosensor works? A closer look at the structure of DNA provides more detail on how a DNA biosensor works. DNA, deoxyribonucleic acid[17], is a macromolecule made up of deoxyribonucleotides composed of a pentose sugar, a phosphate group and one of four nitrogenous bases namely: Adenine (A), Thymine (T), Cytosine (C), Guanine (G).



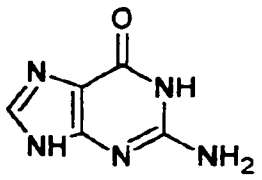
Adenine



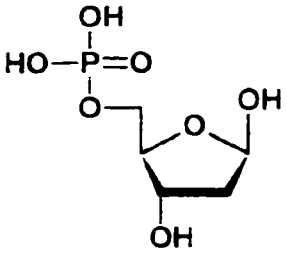
Thymine



Cytosine



Guanine



2-Deoxyriboso-5-phosphate

Figure 1.1: Four bases of DNA Adenine (A), Thymine (T), Cytosine (C), Guanine (G) and 5 Carbon ribose sugar with phophate group at 5' end

DNA is a long polymer of these deoxyribonucleotides that are linked together covalently via a phosphodiester bond that binds the 5' carbon of one deoxyribose (pentose sugar) group to the 3' carbon of the next.

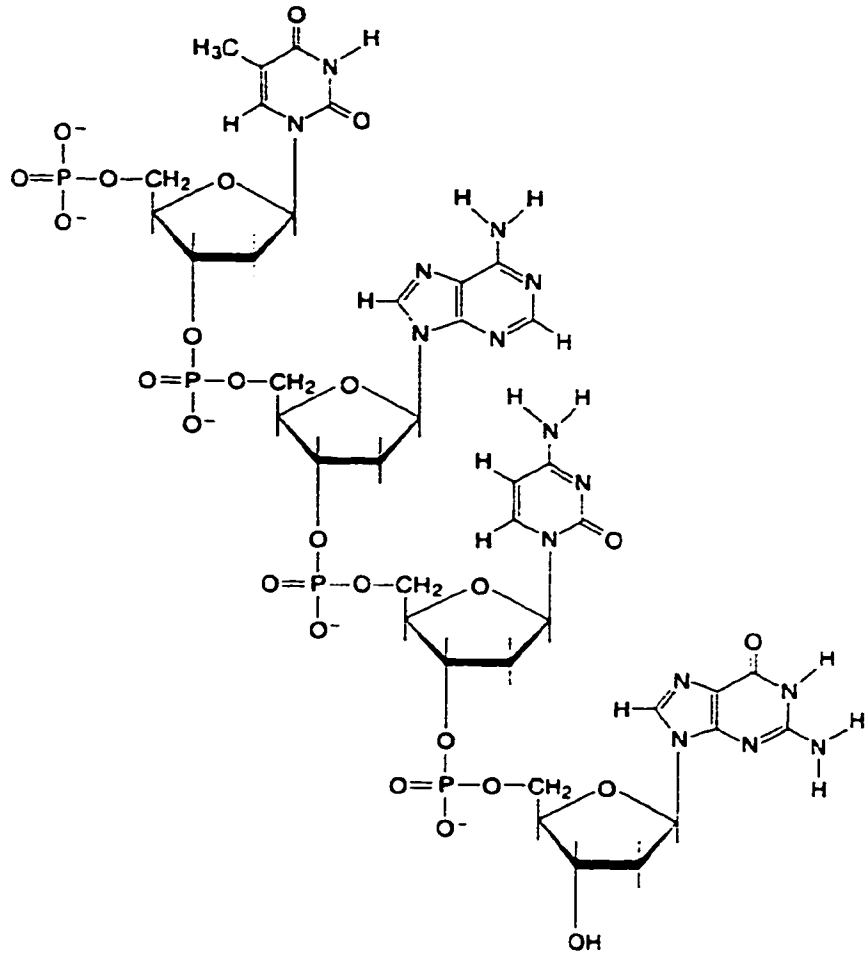


Figure 1.2: Four deoxyribonucleotides with phosphate at 5' end (top left) and 3' hydrohyl group (bottom)

The DNA molecule is a double helix that is composed of two complementary strands. This complementary base pairing deduced by Watson and Crick involves the specific interaction between adenine with thymine and guanine with cytosine. As illustrated, the base pairs are held together by hydrogen bonds. adenine and thymine base pairing are formed by two hydrogen bonds as compared to the three hydrogen bonds between cytosine and guanine.

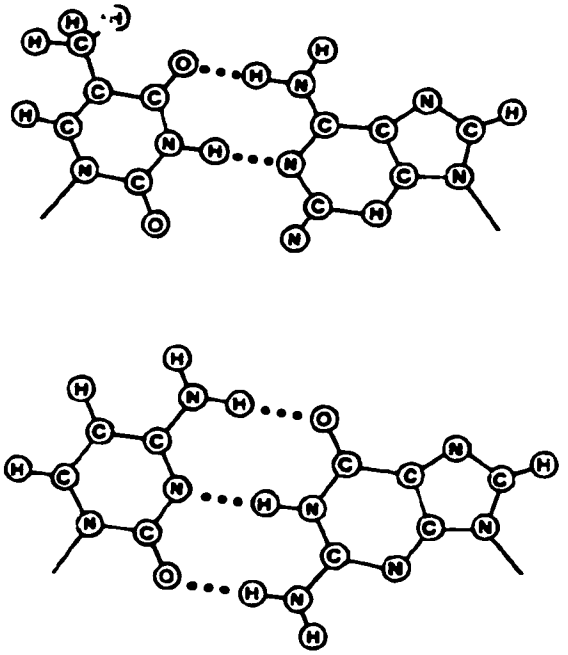


Figure 1.3: Top: Thymine and Adenine (two hydrogen bonding)
Bottom: Cytosine and Guanine (three hydrogen bonding)

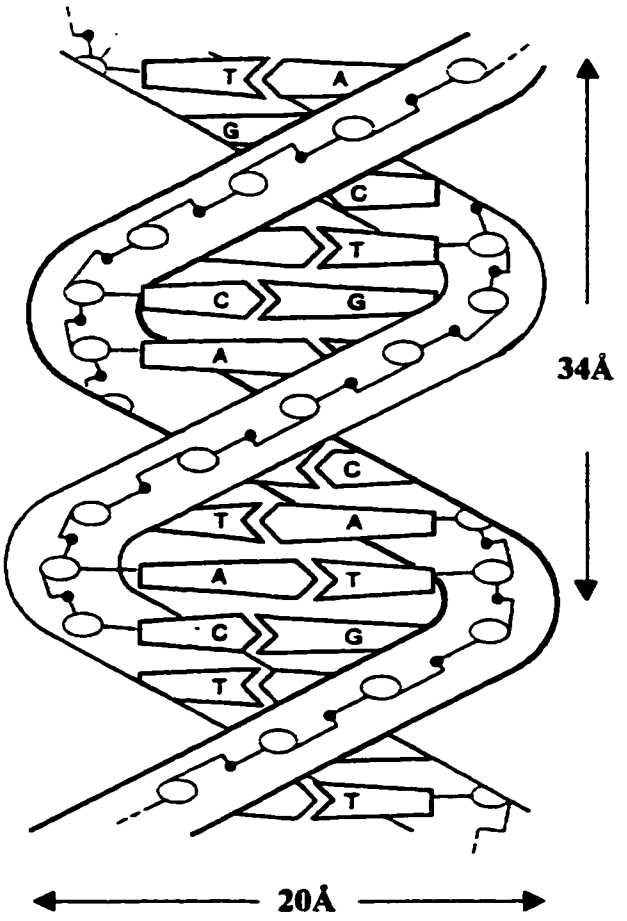


Figure 1.4: Double Helix of a DNA Strand
Diameter 20Å and 10 base-pair spacing represents the pitch length of 34Å

Single stranded DNA is sight specific and, under the proper environment, will bind with a complementary strand. This is known as hybridization.

Conversely, under elevated heat or at extreme pH, the two complementary strands will separate. This is known as denaturation. Hence, hybridization and denaturation are reversible.

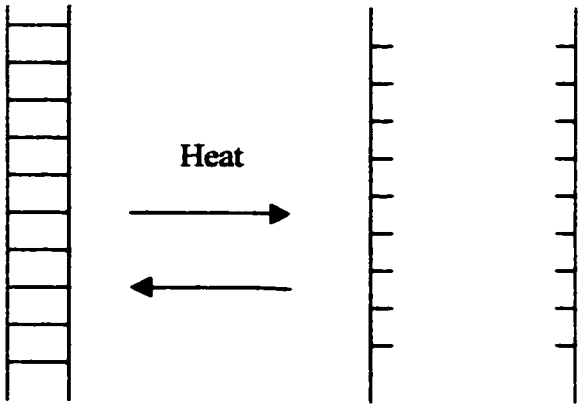


Figure 1.5: Schematic representation of Hybridized and Denatured DNA

To profit from the hybridization and denaturation events, a single stranded DNA, in short ssDNA, can be immobilized onto a transducer to acts as the bioreceptor probe layer. When complementary strands hybridize with the immobilized ssDNA, the transducer generates a signal indicative of the recognition event.

The probe layer may consist ssDNA of various length which may range from 15 to 20 base long ssDNA (oligonucleotides) to 1000 (polynucleotides) base pairs.

1.4.1 The Transducer

The several choices of transducers may vary from optical, amperometric to piezoelectric devices. However, pertinent to this thesis, an electrochemical transduction system will be used to directly detect hybridization of specific DNA sequences. At Concordia University[2], an approach has been developed based on a transducer/probe layer structure where molecular recognition (hybridization between complementary strands) is detected by measuring the variation it causes in the electrical impedance of the transducer/probe structure.

1.5 Thesis Overlay

The overall scheme of the thesis consists:

(a) a theoretical analysis of the transducer used, a semiconductor/oxide (Si/SiO_2) electrode.

(b) Theoretical analysis in the immobilization of ssDNA probe onto the transducer by first modifying the transducer with different silanization techniques namely: APTS/bromine, GPTS/aminolink and Aryldiazonium functionalization.

(c) The electrochemical impedance measurements performed when the semiconductor/oxide transducer is modified with the silanes, the immobilized oligonucleotide, followed by hybridization of complementary strand.

(d) Experimental results based on impedence measurements using two different silanization techniques namely GPTS/amino and with diazonium salt, followed by immobilization of oligonucleotides, and finally hybridization and denaturation of its complementary strands.

(e) Experimental results based on quantification of immobilized and hybridized DNA through the use of radiolabelled oligonucleotides. The quantification of oligonucleotides will include all three silanization methods by varying the concentration of the oligos (short for oligonucleotides) immobilized, and finally by quantifying the amount of potentially hybridized complementary strand.

Finally, a comparison between the electrochemical impedance measurements and the quantification results will be assessed.

2 Theory

2.1 The EIS Structure

The electrochemical system is comprised of a semiconductor/oxide electrode acting as the transducer along with the ssDNA probe layer immobilized on the oxide. This structure is placed in direct contact with an electrolyte solution.

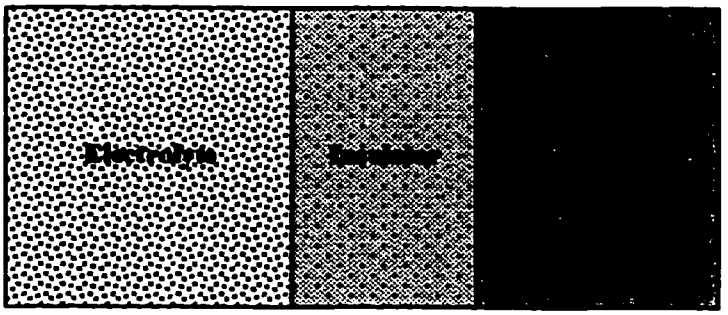
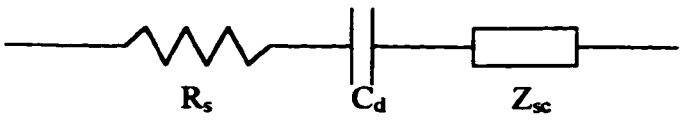


Figure 2.1: The EIS Structure

The electrolyte/insulator/semiconductor (EIS) structure[18,19,20] can be represented by the following equivalent electrical circuit.



Where R_s represents the resistance of the ohmic contacts to the electrode and of the electrolyte solution. C_d is the capacitance of the insulating oxide (dielectric) layer and Z_{sc} is the impedence of the semiconductor.

2.1.1 The Semiconductor

Semiconductors[21] can be depicted as two energy bands which may be occupied by electrons. The lower energy band is called the valence band which is where electrons are located. Removal of electrons from the valence band leaves behind holes (positive charges) that contribute to the semiconductor conductivity. The higher band is called the conduction band which is either vacant or contains conduction electrons. The difference in energy between the lowest point of the conduction band and the highest point of the valence band is called the band gap.

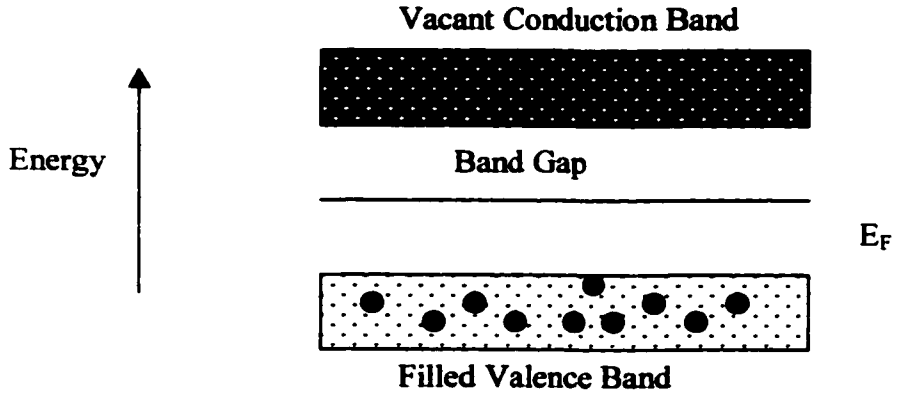


Figure 2.2: Band Scheme for intrinsic conductivity in a semiconductor

The Fermi level of semiconductors is the potential of the electrons inside the solid and is defined as the energy at which the probability of occupation by electrons is one-half. So for ideal situations, the Fermi level will dwell in the center of the band gap. Realistically, due to the presence of acceptor or donor chemical dopants, the Fermi level

is either shifted towards the valence band which makes the material a **p-type** semiconductor or towards the conduction band making it an **n-type** semiconductor.

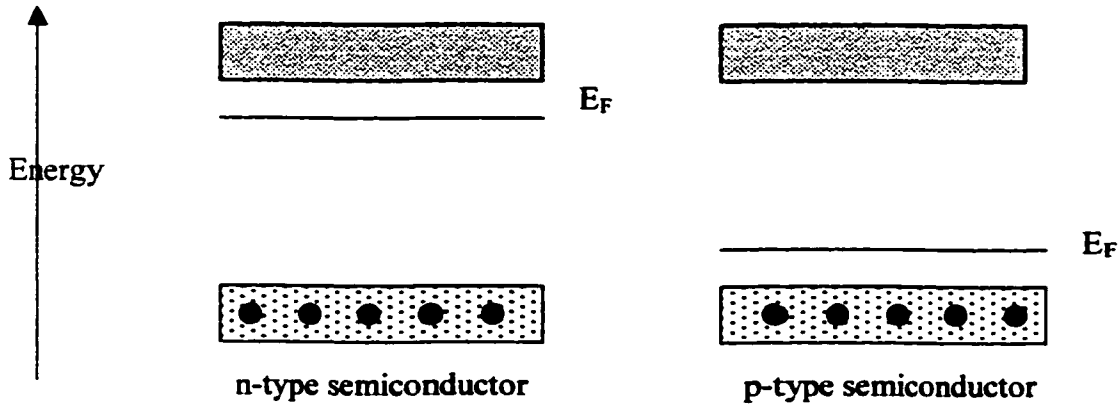


Figure 2.3: Band Scheme for p- and n-type semiconductors

2.1.2 The Insulator

The thin silicon dioxide, insulating layer acts as a blocking contact. This insulator is used to prevent the incident of faradaic phenomena between the semiconductor and the electrolyte solution. In addition, the oxide layer surface is where chemical grafting of the various silanization agents was performed.

2.1.3 The Electrolyte

The role of the electrolyte is to provide a constant potential throughout the bulk of the solution except for the region close to the oxide/electrolyte interface.

2.2 The Measurements

The electrochemical impedance measurements were performed by using a potentiostat and a three electrode set-up (working, counter and reference electrodes). The potentiostat superimposes an a.c. sinusoidal potential of 10mV amplitude at a frequency range of 100kHz to a d.c. potential ramp. The potentiostat monitors the d.c. potential

applied to the working electrode from –1.0 V to +1.0 V, at 100mV increments, all with respect to a Calomel reference electrode.

The equipment used to perform the potentiostatic measurements is a VoltaLab 40 (model: PGZ301 Dynamic EIS Voltammetry). The Voltalab 40 is an all in one Potentiostat/Galvanostat, programmable function generator, and electrochemical impedance meter. The data is analysed with a Voltamaster 4 window-based application software.

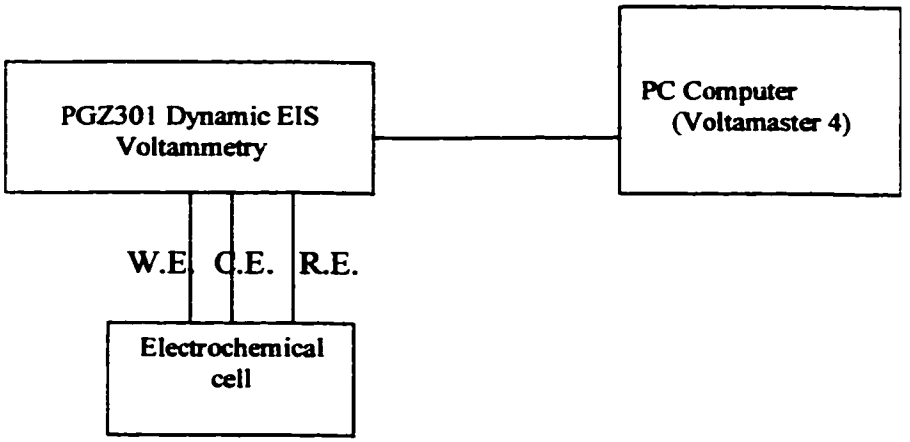


Figure 2.4: Block diagram of experimental set-up. W.E. (working electrode), C.E. (counter electrode), R.E. (reference electrode)

The impedance measurements are related to the complex form of Ohm's Law:

$$\tilde{V} = \tilde{Z}\tilde{I} = (Z_p + iZ_q)\tilde{I} \qquad (A)$$

where

$$\tilde{V} = V_p + iV_q \qquad \text{(B)}$$

and

$$\tilde{I} = I_p + iI_q \qquad \text{(C)}$$

V_p and I_p are the in-phase voltage and current, V_q and I_q are the out of phase values.

The in-phase, Z_p, and out-of-phase, Z_q, impedances are then calculated with the following expressions when incorporating equations (A), (B) and (C):

$$Z_p = \frac{V_p I_p + V_q I_q}{I_p^2 + I_q^2} \qquad \text{(D)}$$

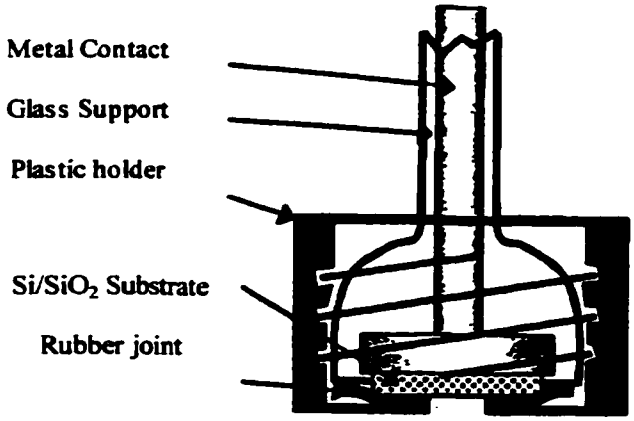
and

$$Z_q = \frac{V_q I_p - V_p I_q}{I_p^2 + I_q^2} \qquad \text{(E)}$$

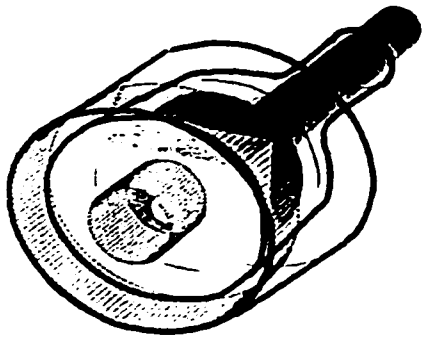
The Z_p and Z_q values are calculated for each continuous polarization value, for given modulation frequency.

The Si/SiO_2 substrate acts as the working electrode in a 20ml Pyrex beaker container half filled with the electrolyte. Included in the set-up is a saturated Calomel reference electrode (Fisher Scientific) and a platinum counter electrode (fabricated at Concordia University). As shown in the figure, the support for the working

electrode was fabricated at Concordia University to fit the Si/SiO_2 substrate. This apparatus is easy to use and very quick to assemble. A total run lasts for 10 minutes.



Cross Section of working electrode



Dimensional View of working electrode

Figure 2.5: The Working Electrode with supports

2.3 The Substrate

The Si/SiO_2 substrates used as electrodes are 1 X 1 cm^2 in area taken from a diced wafer 0.3mm thick purchased from Tronics Microsystems in Grenoble, France. The substrates are doped to a density of 10^{21} m^{-3} (P doped for n-type and Al doped for p-type) and a chromium/gold film was deposited on the back side to provide an ohmic contact.

The top surface is a thermally grown 100Å thick silica insulating layer which provides the active area of the substrate where chemical graftings are performed to produce the probe layer. During the impedance measurements, the 100Å also acts as a blocking layer preventing any passage of electrons through the substrate. Underneath the silica layer is the silicon semiconductor layer which is about 0.3μm in thickness.

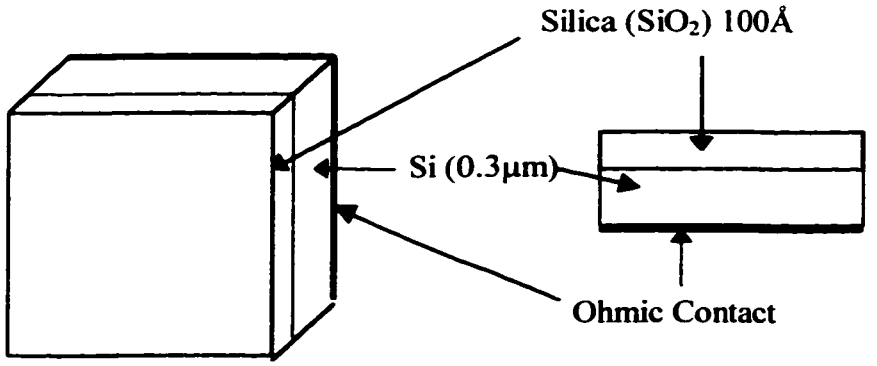


Figure 2.6: The Si/ SiO_2 Substrate

The electrolyte/insulator/semiconductor heterostructure can be considered to be made of two capacitors in series to give a total capacitance, C, where $C = C_1 + C_2$. The out-of-phase impedance, Z_q, can be related to the total capacitance given by

$$Z_q = \frac{1}{\omega C}$$

where ω is the angular frequency of the a.c. signal given by $\omega = 2\pi f$. In addition. The total capacitance of the heterostructure is inversely proportional to the thickness of the dielectric and is given by

$$C = \frac{\varepsilon_o \varepsilon A}{d}$$

where ε_o is the permittivity of free space, ε is the permittivity of the diectrectric material, d is the capacitance thickness and A is the surface area[23].

2.4 The Results

As mentioned, it is possible to represent the EIS structure as a simplified equivalent circuit. When a d.c. potential is applied to the working electrode, the silicon semiconductor layer continuously compensates any modification occuring at the electrolyte/oxide interface by modifying the charge distribution at the Si/SiO_2 interface (the space charge layer). This is illustrated by shifts of in-phase, Z_p, out-of-phase, Z_q, impedances as a function of the d.c. applied potential ramp.

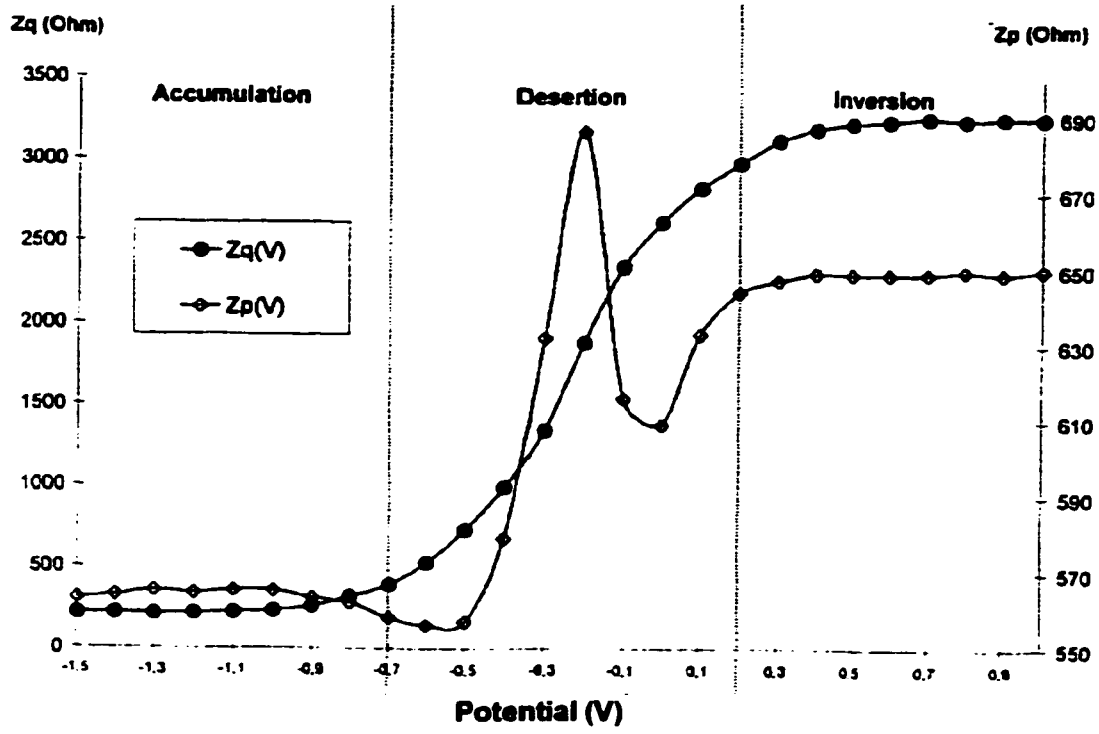


Figure 2.7: Impedence Curves as a function of applied potential

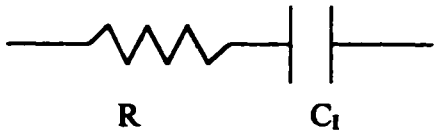
Figure 2.7 shows the general shape of $Z_p(V)$ and $Z_q(V)$ curves as a function of applied potential. The semiconductor in this case is a n-type (doped 10^{21} m^{-3}) and measurements were done at a modulating frequency of 100kHz. The impedances Z_p and Z_q are correlated with the resistance and capacitance of the heterostructure, respectively.

It is the out-of-phase impedance curve that is of interest to us in these studies. The Z_p versus potential curve reflects the presence of surface states in the band gap. The amplitude of the maximum in this curve corresponds to the density of surface states,

while its position corresponds to the energy of these surface states. The Z_q versus applied d.c. potential shows three distinct regions.

(a) 2.4 1 The Accumulation Region

It is in this region that the density of the majority charge carriers (electrons for n-type) is the greatest. The equivalent circuit is represented by as well as the energy diagram corresponding to this situation.



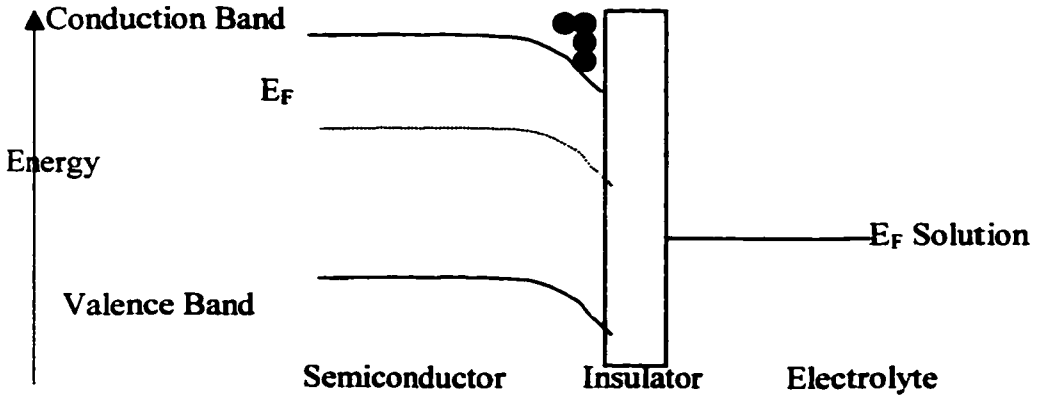


Figure 2.8: Band bending under Accumulation Condition

R represents the resistance due to the electrical contacts and the electrolyte solution. C_I is the capacitance of the oxide layer. The capacitance of the semiconductor, C_{SC} is insignificant.

(b) 2.4.2 The Depletion (Desertion) Region

This is where the density of majority charge carriers decreases in the conduction band. The equivalent circuit and energy diagram for this situation are illustrated below.

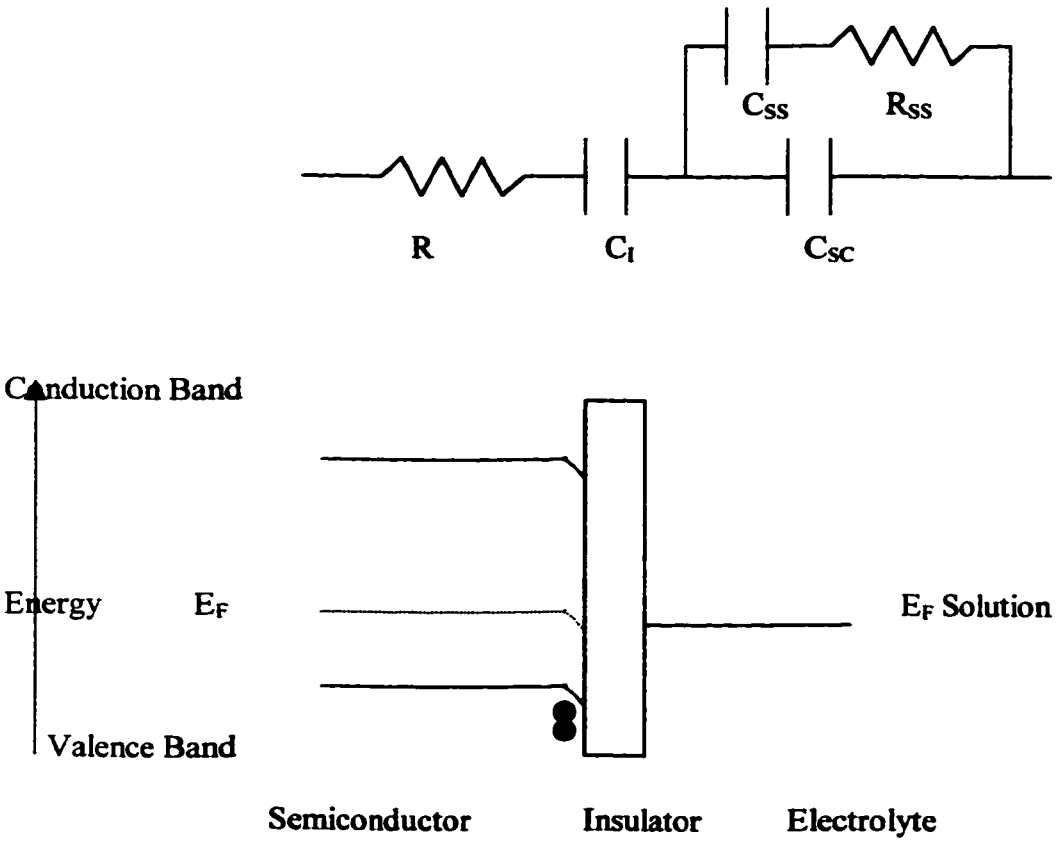


Figure 2.9: Depletion Condition

Here the capacitance is due to the semiconductor, C_{SC}, as well as the capacitance of the surface states C_{SS} . R_{SS} is the resistance due to the surface states.

(b) 2.4.3 The Inversion Region

In this region, the minority charge carriers (holes for n-type) are now in excess in the valence band. The resistance and capacitance of the surface states are not considered.

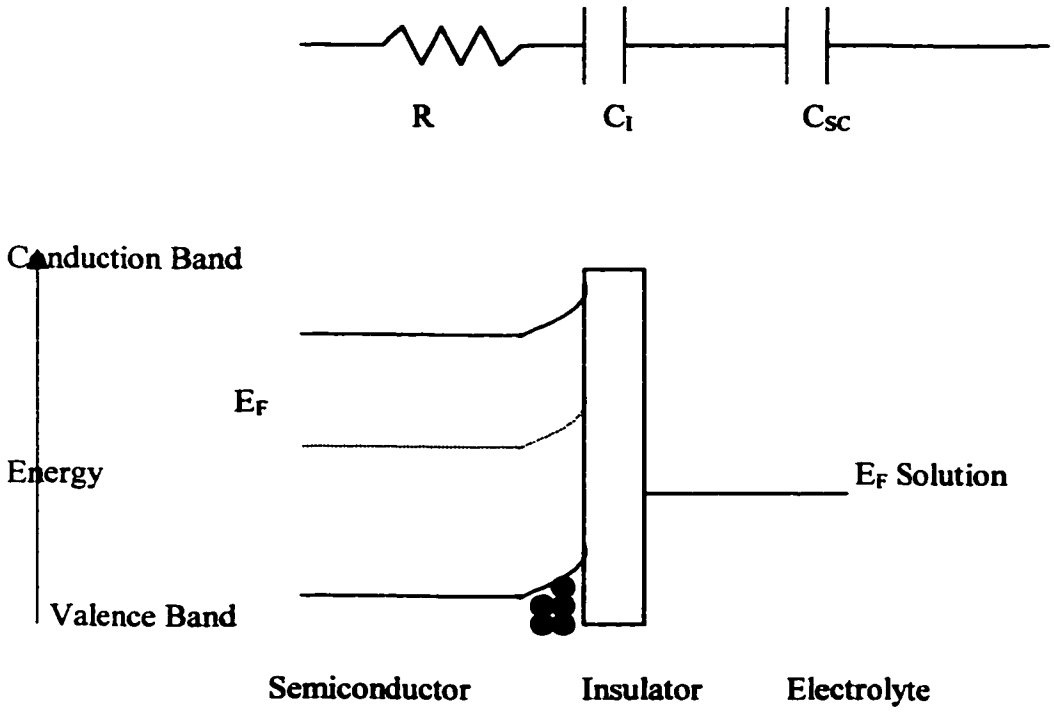


Figure 2.10: Inversion Condition

2.4.4 Flat-Band Potential

The importance of the Z_q versus V curve lies in the depletion region. When the semiconductor is exposed to an electrolyte solution, the fermi level of the semiconductor equilibrates with redox potential of the electrolyte. This is represented by band bending within the semiconductor. The flat band potential, V_{fb}, is defined as the potential that must be applied to the device (vs a reference electrode in solution) to cancel this band bending, and the value of V_{fb}, is determined by extrapolating the slope of the $Z_q(V)$ curve in the depletion regime to the applied potential axis. If the charge at the oxide/electrolyte interface is modified, the underlying semiconductor compensates for this modification by a new charge distribution inside its space charge layer to maintain electrical equilibrium. This introduces a change in the band bending and therefore yields a new value for the flat-band potential, which is reflected by a displacement of the impedance curves along the potential axis.

Variations of the flat-band potential may be observed when chemical binding or adsorption occurs at the SiO_2/electrolyte interface. The total capacitance of the EIS structure is related to the d.c. potential through the Mott-Schottky equation[24]:

$$V - V_{fb} = \frac{q N \varepsilon_o \varepsilon}{2 C^2}$$

where N is the dopant density per unit area, q is the elementary charge, ε_o is the permittivity in free space, ε is the relative permittivity of the semiconductor, V is the applied potential, V_{fb} is the flat-band potential.

A linear plot of $1/C^2$ versus V allows the extrapolation of the slope in the depletion region to the applied potential axis, the intercept of which yields the flat-band potential[25].

2.5 Functionalization of the Si/SiO_2 substrates

Chemically modifying the substrates is an essential process in order to immobilize any single-stranded DNA (ssDNA) onto the surface and eventually hybridizing it with its complementary strand. Preparing the surface for immobilization of the ssDNA involves the following steps;

(a) Cleaning of the substrates

(b) Hydroxylation of the oxide layer

(c) Silanization of the oxide layer prior to immobilization.

(a) 2.5.1 Cleaning

The surface of the silica contains many contaminants such as grease and other organic pollutants. Cleaning of the silica is accomplished by immersing it in organic solvants such as acetone and/or methanol[26]. In addition, elevating the temperature so that boiling acetone or methanol is obtained also aids in the removal of the contaminants on the surface.

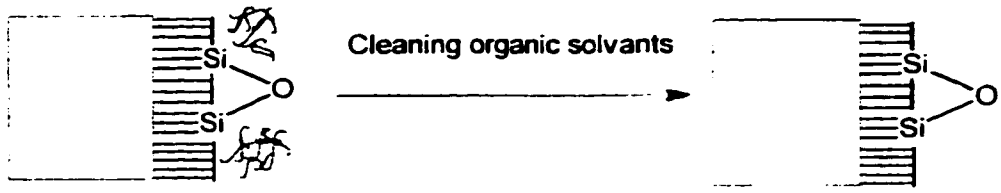


Figure 2.11: First stage, cleaning of substrates

(b) 2.5.2 Hydroxylation

The silica surface is composed of siloxane bonds as shown figure 2.11. Hydroxylation[27] of the surface, producing silanol groups, is essential for binding the silanization agents (GPTS, 3-glycidoxyethylpropylamine and APTS, 3-aminopropyltriethoxysilane) onto the surface. Hydroxylation is carried out by treating the surface with a concentrated sulfochromic acid solution. Furthermore, this treatment totally eliminates any unwanted organic pollutants on the surface. The figure below shows the hydroxylation of the silica surface in which siloxane bonds are hydrolysed to form silanol groups.

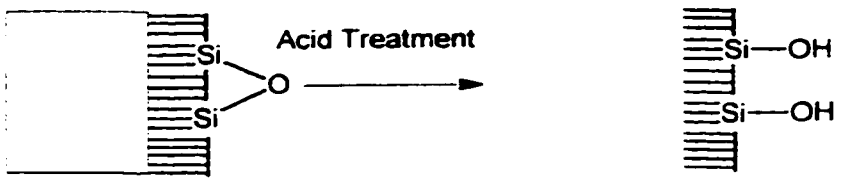


Figure 2.12: Stage Two, hydroxylation of substrates

Furthermore, under normal ambient conditions, the presence of water in the atmosphere may adsorb onto the surface of the silanol groups by hydrogen-bonding. Heating of the substrates is performed in order to evaporate the adsorbed water molecules. Iler et al[28] showed that a temperature between 120 – 150°C used to perform this task was ideal in removing adsorbed water while retaining most of the functional silanol groups in tact. Therefore, temperature treatment is essential after hydroxylation.

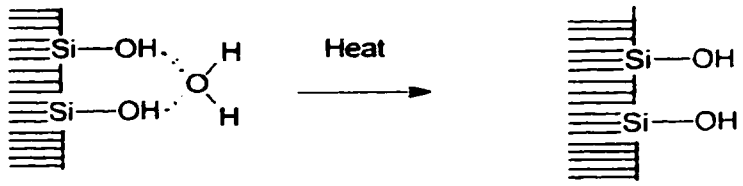


Figure 2.13: Stage Three: Thermal Treatment of substrates

(a) 2.5.3 Silanization/Immobilization

Silanization is accomplished by depositing an alkyl silane with a terminal functional group, such as a primary amine or epoxy group, allowing the attachment of biocomponents, i.e. single-stranded oligonucleotides.[29]

(i) APTS/Bromine Method

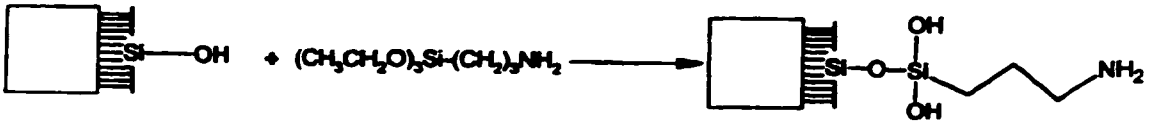


Figure 2.14: Modification of silanol surface with APTS

The first method that will be discussed is modifying the surface silanol groups with 3-aminopropyltriethoxysilane (APTS). Caravajal et al[30], and Keller et al[31] modified silica by preparing a 5% by volume aqueous solution of APTS and depositing for 10 minutes onto hydrolyzed silica at room temperature.

The APTS solution is soluble in water and the reaction can take place under ambient temperature. APTS is able to autocatalyse[32] with the silanol groups of the silica surface. Under a pH of 8.0 to 8.5, the stabilization of APTS in solution forms a 7 atom ring[33]. The terminal amino group of APTS forms hydrogen bonds with the hydroxyl groups as shown in figure 2.15.

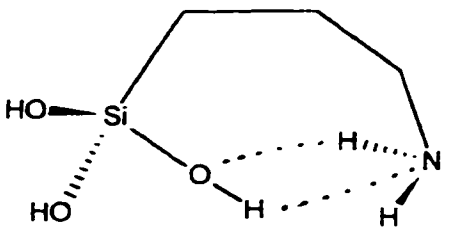


Figure 2.15: Stability of APTS under aqueous conditions

At a basic pH of 8.0, brominated ssDNA can bind onto the amino group of APTS. Figure 2.16 shows how brominated ssDNA or a ss-oligonucleotide in this case at C_6 carbon of the thymine base can react with the amino group of the APTS modified Si/SiO_2 substrate. On that basis, the conception of a DNA biosensor derived by using APTS as a functional monolayer group was justified.

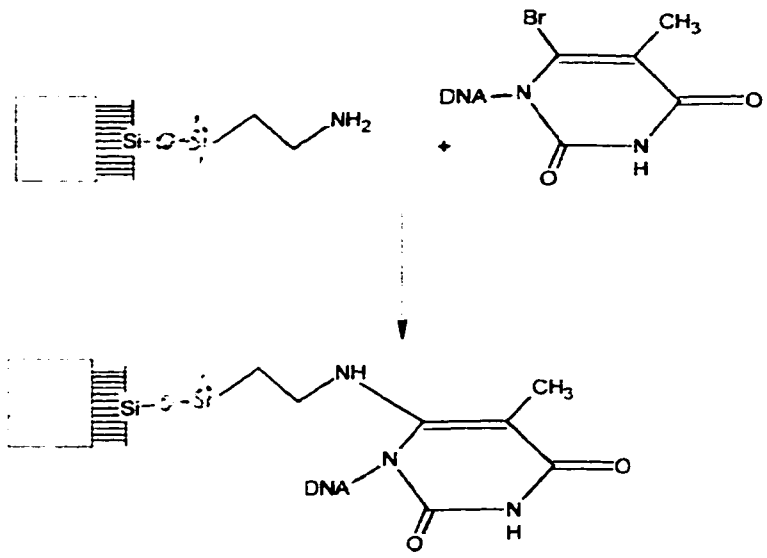


Figure 2.16: Immobilization of ssDNA strand through displacement of bromine by the amino group

(b) GPTS/aminolinker Method

The use of 3-glycidoxypropyltrimethoxysilane (GPTS) for functionalization purposes was first explored by the works of Southern et al[34,35,36] and Lamture et al [37,38] . They proposed to use GPTS as (1) an epoxy functional group for binding ssDNA and (2) binding the oligonucleotide onto the silane by using a 6 – carbon aminolinker (6 carbon chain with a primary amino group).

In this method, a more controlled environment is required to carry out the procedure. It requires using proper solvents, using a catalyst, and keeping the entire silanization process under anhydrous conditions.

GPTS lacks the autocatalyzing properties of APTS and binding onto the surface is accomplished with the aid of diidopropylethylamine as catalyst. Diisopropylethylamine or DPTA has a primary amino group that activates the silanol group of the silica and is able to chemically bind. For maximum binding, the reaction must be performed at a temperature of 80°C.

A mechanism for this silanization procedure was put forth by Blitz et al[39], and Tripp and Hair[40]. Blitz et al used ammonium as a catalyzing agent to bind trimethoxysilanes onto the silanol surface. Tripp and Hair also performed this silanization method under nitrogenous and anhydrous conditions. Hence, the formation of a silane monolayer onto the silica layer was achieved by employing an organic solvent (for instance O-xylene), and a catalyst (diisopropylethylamine), all under anhydrous conditions.

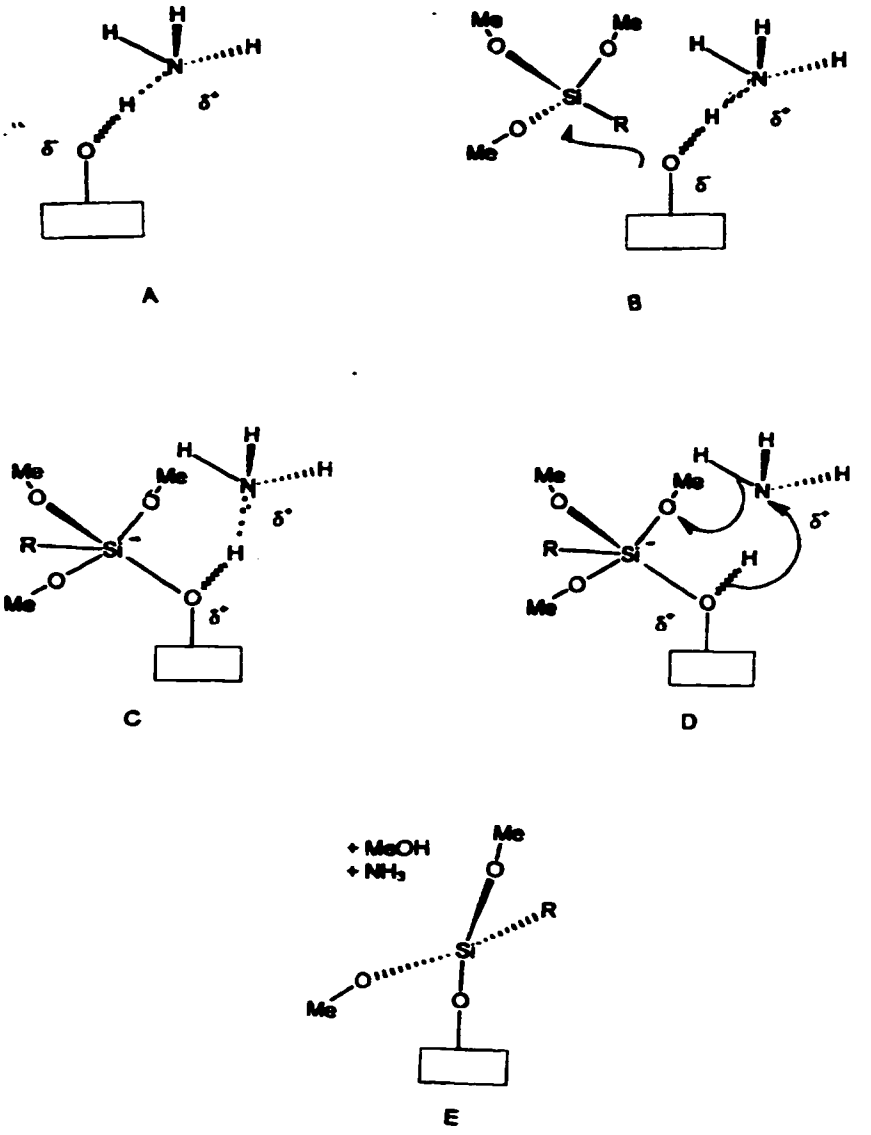


Figure 2.17: Catalysing Action of ammonia on Silanol surface (Blitz et al)[39] (A) Adsorption of base on silanol surface, (B) nucleophilic attack of SiO^- on trimethoxysilane, (C) resulting in intermediate form, (D) proton is displaced by methoxy group with the aid of electrophilic properties of ammonia and deprotonation of silanol, (E) Surface is silanated.

Southern et al proposed that the ssDNA is better able to attach onto the silane by either incorporating a spacer fixed on the monosilane layer or a linker fixed at one end of the DNA strand. This allows for an improved chance that there will be a covalent linkage between the silane and DNA. Secondly, there is an increase in the degrees of freedom in which the complementary ssDNA molecule may be able to bind. In this case a short, 6-carbon length, aminolinker is attached to the 5' end of the ss-oligonucleotide.

The coupling of the oligonucleotide with GPTS is executed under basic conditions (potassium hydroxide, KOH, solution). The KOH opens the epoxide group and the aminolinker is able to form a strong covalent linkage through deprotonation at the amino group. Even though the basic solution might possibly degrade the silica, the GPTS layer provides adequate protection.

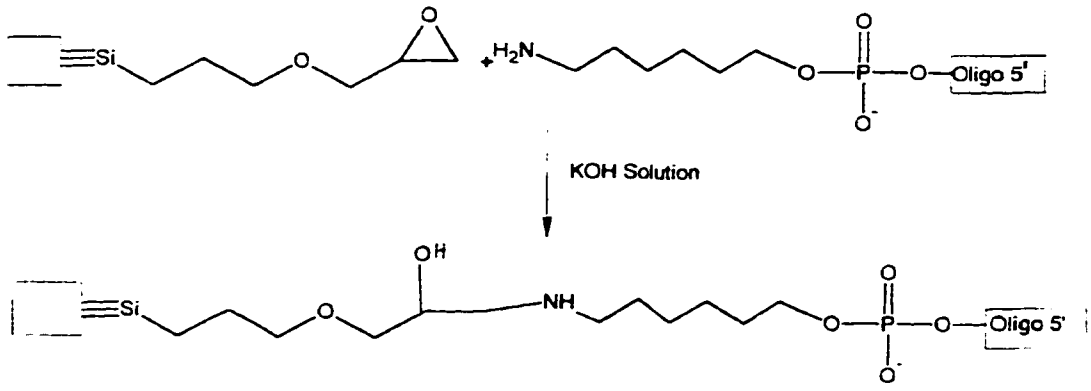


Figure 2.18: Binding of aminolinker-oligonucleotide to GPTS

(a) Diazonium salt/aminolinker method

A more robust modification of silicon surfaces by electrochemically reducing the hydrogen terminated silicon electrodes using 4-NO_2 and 4-bromobenzenediazonium salts has also been reported[41]. This modification requires no insulating (SiO_2) layer and it is also a quicker method compared to the previously described APTS and GPTS methods of chemically modifying silicon.

The silicon substrates (n-type) used in our study (without a thermally grown oxide layer) were also manufactured at Tronics Microsystems with chromium/gold ohmic back contact. These substrates were subjected to the same cleaning procedures; however, they are chemically treated with fluoride solutions[42] to reduce the surface.

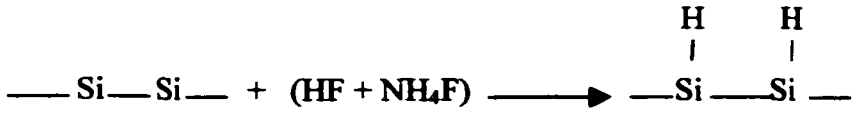


Figure 2.19: Reduction of silicon surfaces

Afterwards, direct molecular attachment of 4-bromobenzenediazonium to the H-terminated Si electrode can be achieved through electrochemical fixation and recorded and identified by cyclic voltammograms.

The summary of the Si surface reaction model is given in figure 2.20

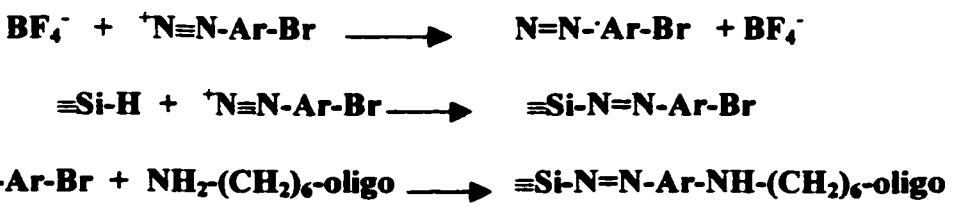


Figure 2.20: Fixation of diazonium moiety followed by immobilization of oligonucleotide Where Ar = aromatic 6C ring and ≡Si-H = reduced silicon triply bound to lattice of substrate.

The first two reactions of figure 2.20 shows the binding of the diazonium to the Si substrate. The last reaction shows the immobilization step of ss-oligonucleotides with a 5' aminolinker, where the primary amino group displaces the bromine atom.

3 Experimental Section

3.1 Materials and Chemicals

Table 1: Reagents used for APTS Silanization and ss oligonucleotides

Product	Company	Description/Model
3-aminopropyltriethoxysilane (APTS)	Sigma	98% Pure, stored at 4°C
Sodium Bicarbonate $NaHCO_3$	Sigma	High purity grade
Acetone	Sigma	HPLC grade
Methanol	Sigma	HPLC grade
Nitrogen gas	Prodair	High purity grade

Table 2: Reagents used for GPTS Silanization and ss oligonucleotides		
Product	**Company**	**Description/Model**
3-glycidoxytimethoxysilane (GPTS)	Sigma/Aldrich	98% Pure, stored under anhydrous conditions
N-N diisopropylethylamine (DPTA)	Sigma/Aldrich	99.5% pure, stored under anhydrous conditions
O-Xylene	SigmaAldrich	HPLC grade stored under anhydrous conditions
Potassium Hydroxide KOH	Fisher	Reagent grade
Acetone	Sigma	HPLC grade
Methanol	Sigma	HPLC grade
Ethanol	Sigma	HPLC grade
Ether	Sigma	HPLC grade
Nitrogen gas	Prodair	High purity grade

Table 3: ss oligonucleotides used		
Product	**Company**	**Description/Model**
TTT TTT TTT TTT TTT TTT TT Br 20 bases (oligodTBr)	Biocorp	Brominated at C6 of the Thymine 5' end
TTT TTT TTT TTT TTT TTT TT – $(CH_2)_6$-NH_2 ,20 bases (oligodTam)	Biocorp	Aminolinker synthesized at the 5' end
AAA AAA AAA AAA AAA AAA AA ,20 bases (oligodA)	Biocorp	
CCC CCC CCC CCC CCC CCC CC 20 bases (oligodC)	Biocorp	

Table 4: Other Miscallaneous reagents used		
Product	**Company**	**Description/Model**
Double distilled water	Millipore	Model: Milli-RX 20
Sulfuric acid	Baker	Reagent grade
Tris Buffer Saline Tablets	Sigma	Reagent grade
Hydrochloric acid	Baker	Reagent grade
Chromerge (chromic acid)	Fisher	Reagent grade

Table 5: Reagents used for Aryldiazonium salt modification		
Product	**Company**	**Description/Model**
Ammonium Fluoride	Baker	40% in solution
Trichloroethylene	Fisher	Reagent grade
Hydrofluoric acid	Fisher	Spectral grade
4-Bromobenzene Diazonium Tetrafluoroborate	Aldrich	Reagent grade
Acetone	Sigma	HPLC grade
Methanol	Sigma	HPLC grade

Table 6: Computer Hardware and Software used for radiolabelling		
Product	**Company**	**Description/Model**
Computer	MacIntosh	PowerMac 7500/100
Phosphoimager	Fuji Film	Fujix: Bio-imaging analyzer Bas 2000
Phosphoimaging Plate	Fuji Film	Fuji Imaging Plate for Bio Imaging Analyzer Type Bas-III
Analyzer (software)	Fuji Film	Bio-imaging Analyzer MacBas Ver. 2.2 & 2.5

Table 7: Reagents used for radiolabelling		
Product	**Company**	**Description/Model**
dATP - αP^{32}, dTTP - αP^{32}, dCTP - αP^{32}	ICN	Activity level 25Ci/mmol Half-life: 2 weeks
Terminal deoxynucleotidyl Transferase (TdT)	Life Technologies	
TdT Buffer 5X	Life Technologies	
Sodium Bicarbonate	Anachemia	
Potassium Hydroxide	Anachemia	

Table 8: Materials used for radiolabeling/immobilization/hybridization/cleaning

Product	Company	Description/Model
Separation columns	Pharmacia Biotech	ProbeQuant™ G-50 Micro-Columns
Centrifuge	Eppendorf	Model No. 5415C
DNA Thermal Cycler	Perkin Elmer	Model No. 480
Hybridization Oven	Tek-Star	
Gel Dryer	Biorad	Model No. 583
UV Spectrophotometer	Pharmacia Biotech	Gene Quant II RNA/DNA Calculaor
Shaker (petri-plates)	Thermodyne	Roto Mix Type 50800

Table 9: Materials and Reagents used for Electrophoresis Verification[43]		
Product	**Company**	**Description/Model**
TEMED (t-octylphenoxy polyethoxyethanol)	Pharmacia Biotech	
TBE buffer	Pharmacia Biotech	
Acrylamide	Pharmacia Biotech	
Bisacrylamide	Pharmacia Biotech	
APS (Ammonium Persulfate)	Pharmacia Biotech	
Gel loading buffer	Gibco BRL	
Power Supply	Biorad	Model 200/2.0
Supports for Electrophoresis	Biorad	Mini-protean II

The Si/SiO_2 substrates used are doped to a density of 10^{21} m^{-3} (P for n-type, Al for p-type).

Potentiostatic measurements were taken using the VoltaLab 40 and analysed on a pentium 166 PC computer using the Voltamaster 4 program analyzer.

The reactor used for the GPTS method was fabricated at Concordia University to our specifications.

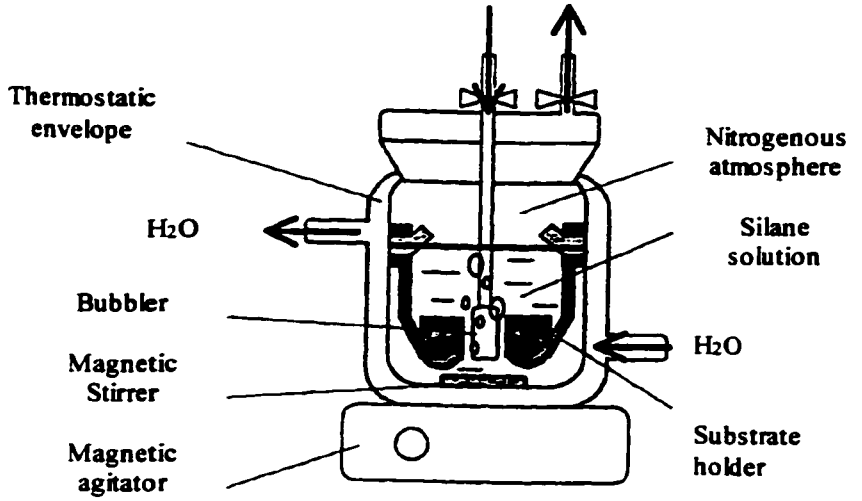


Figure 3.1: Reactor for GPTS silanization

3.2 Procedures

3.2.1 Cleaning of Glassware

All glassware is washed with a sulfochromic acid solution followed by thorough rinsing with double-distilled water.

Sulfochromic acid solutionwas prepared by gently adding a bottle of Chromerge to 2.5 L of sulfuric acid, and then mixing thoroughly.

3.2.2 Preparation of Tris buffer

Tris buffer is the electrolyte solution used for the electrical impedance measurements. It is prepared by adding two tablets of Tris buffer saline tablets to 90 ml of double distilled water. The Tris buffer pH is 7.1 with a final concentration of 10mM Tris-HCl and 50mM NaCl.

3.2.3 Silanization and Immobilization Method using APTS/oligodTBr Method

The substrates are first cleaned in boiling acetone for 5 minutes followed by boiling methanol for another 5 minutes. The substrates are allowed to air-dry (2 minutes) and are immediately placed in a sulfochromic acid solution for 4 minutes. This sulfochromic acid solution is used to hydrolyse the siloxane bonds on the surface of the silica. It is prepared by adding 5ml of a saturated aqueous solution of potassium dichromate, $K_2Cr_2O_7$, to 95 ml of sulfuric acid. After the acid treatment, the substrates are boiled for 10 minutes in distilled/deionized water and dried carefully with nitrogen gas. The substrates are then placed in the oven for 1 hour at 135°C[44,45].

The silanization of the substrates is accomplished by adding 40µl of a 20% (by volume in double distilled water) APTS solution for 45 minutes under humid

conditions to prevent the APTS solution from drying to rapidly. After 45 minutes, the substrates are thoroughly rinsed with distilled water and dried with nitrogen gas. At this point, an electrochemical measurement is taken (silanized substrate, before DNA immobilization).

Single-stranded DNA is then immobilized by adding 40µl of a 10^{-2} µg/µl in a 1M $NaHCO_3$ solution of oligodTBr. The substrates are left to react overnight under humid environment. The oligodTBr was purchased dry from Biocorp and the solution prepared by adding 600µl of a 1M $NaHCO_3$ solution giving a concentration of 2.0 µg/µl. At this concentration the solution can be stored at –4°C for later use or diluted to an appropriate concentration for immobilization.

The following day, the substrates are rinsed with double distilled water and are ready for electrochemical impedance measurements (parameters are in section 2.2 and 2.4) and/or hybridization with complementary strands.

3.2.4 Silanization and Immobilization method using GPTS/oligodTam

Procedures identical to those reported in the previous section are used to clean the glassware, including the reactor.[46,47,48] The substrates

are cleaned as described for the APTS method; cleaning in boiling acetone for 5 minutes followed by boiling methanol for another 5 minutes. The substrates are then allowed to air-dry (2 minutes) and are immediately placed in a sulfochromic acid solution for 4 minutes. After the acid treatment, the substrates are boiled for 10 minutes in double distilled water and dried carefully with nitrogen gas. The substrates are then placed in the oven for 1 hour at 135°C.

Immediately after the oven treatment, the substrates are placed in the reactor along with a magnetic stirrer and are transferred to a glove box for silanization. Before placing the apparatus directly in the glove box, it must be free from any form of humidity. The holding chamber of the glove box must be evacuated and then it is followed by a purge with nitrogen gas (high purity), this process is repeated once more. The reactor is ready to enter the glove box (pre-purged with nitrogen gas).

Inside the glove box, 111ml of O-xylene, 12.5 ml of GPTS (3-glycidoxypropyltrimethoxysilane), and 1.5 ml of DPTA (N-N diisopropylethylamine) are added to the reactor containing the substrates and stirrer. The cover of the reactor containing the gas bubbler is placed and sealed to ensure anhydrous conditions inside the reactor when it is removed from the glove box. The concentration of GPTS in the mixture is 10% by volume.

The reactor is heated at 70°C for 4 hours by circulating water in the outer jacket using a thermostated pump. The magnetic stirrer is placed to medium setting and the gas bubbler is attached to high purity grade nitrogen under mild circulation. After the reaction, the substrates are thoroughly rinsed with ethanol and then stored in ether for later use, or they can be used to immobilize the ss oligonucleotide. If the substrates were not stored after silanization they were used within 1 hour.

The ss oligonucleotides are immobilized by adding 40μl of a 10^{-2} μg/μl or 10^{-4} μg/μl aqueous solution of oligodTam in a 0.001M KOH. The substrates are left to react overnight under humid conditions. The oligodTam are purchased dry from Biocorp and solutions are prepared by adding 600μl of 1M $NaHCO_3$, resulting the oligodTam to have a concentration of 2.0 μg/μl. At this concentration they can be stored at –4°C for later use or diluted to an appropriate concentration for immobilization. The following day, substrates are boiled in double distilled water for 2 minutes and are ready for electrochemical impedance measurements and/or hybridization with complementary strands.

3.2.5 Bromo-Aryldiazonium method and immobilization with oligodTam

The bromoaryldiazonium salt[49] solution consists of 2mM 4-bromobenzene diazonium tetrafluoroborate in 0.1M H_2SO4 and 2% HF aqueous solution. The solution was de-aerated previous to use bubbling nitrogen gas for 20 minutes. This solution is used

for functionalizing the H-terminated silicon substrate with the bromo aryldiazonium moiety through reduction upon a cyclic voltametric cycle using the cell shown in figure 3.2.

Electrochemical impedance measurements were performed as previously described. The tris buffer electrolyte used for these measurements has also been previously described.

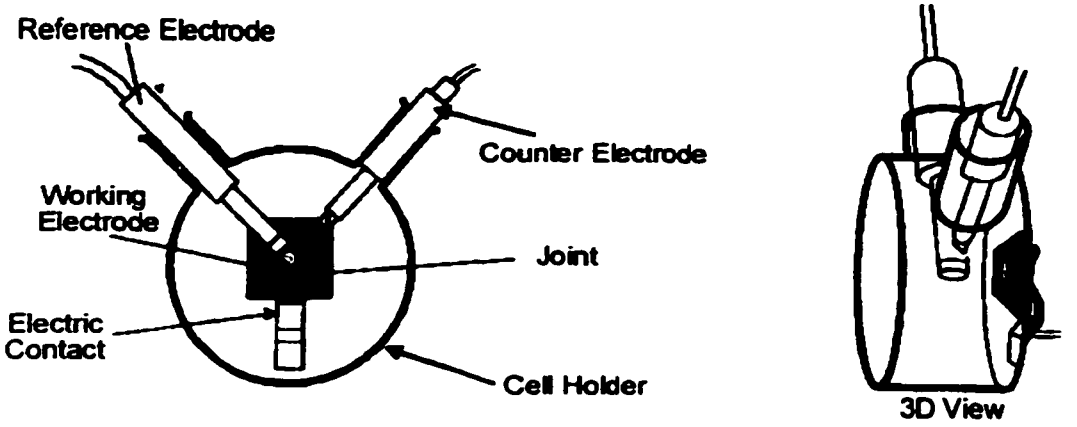


Figure 3.2: Electrochemical Cell for diazonium modification

3.3 Results and Discussion

The experimental results to be presented are as follows:

(a) Quantization of radiolabelled single-stranded oligonucleotides brominated at the 5' end deposited onto the surface of APTS modified substrates.

(b) Immobilization of oligonucleotides with an aminolinker onto the surface of the substrates silanated with GPTS. Quantification through radiolabelling and electrochemical impedence measurements. The effect of concentration on the amount of oligonucleotide immobilized is also reported. A study of the hybridization of the complementary strands and the removal of these strands through denaturation is also presented. Finally, a comparison was made between the electrochemical results and the amount of fixed ss-oligonucleotides immobilized onto the biosensor.

(c) A brief look at preliminary radiolabelling quantification results and electrochemical results using aryl diazonium functionalized silicon substrates. This method appears to be an efficient reproducible and rapid approach to DNA immobilization for sensor applications.

3.3.1 Radiolabeling, and preliminary results

The oligonucleotides used consist of 20 bases of single stranded dA, dC and uridine-brominated dT (dT-Br) or oligodTam DNA strands. The oligos were purchased dry from Biocorp Inc. and were dissolved in 600µl of a 1M $NaHCO_3$ solution. Each sample was then quantified by UV Spectrophotometer (Pharmacia Biotech) at 260nm. The following concentrations were measured.

Oligonucleotides (20 Bases)	Concentration (µg/µ l)
oligodTBr	2.03
oligodTam	2.03
oligodA	2.08
oligodC	1.93

The oligonucleotides were then ready to be labeled with the radioactive αP^{32} nucleotide or were stored in a freezer at –4°C for later use. 34µl of the 20 base oligonucleotide solution, 10µl of TdT buffer 5X (Life Tech.), 4µl of TdT (Life Tech.), and 2µl of αP^{32} – dNTP (ICN) were added to a 500µl microcentrifuge tube and left to react for 1 hour at 37°C, followed by 5 minutes at 70°C to deactivate the enzyme in a DNA Thermal Cycler (Perkin Elmer).[50]

The radiolabeled solution is then passed through a Probequant G-50 separation column (Pharmacia Biotech) in order to eliminate any free radioactive reagents. The labeled oligonucleotides were further diluted with 1M $NaHCO_3$ solution to a final

concentration of 0.1μg/μl (0.001M KOH solution for oligodTam). This concentration was subsequently diluted again with 1M $NaHCO_3$ solution to concentrations of 10^{-2} and 10^{-4} μg/μl, the concentrations with which the chips were either immobilized or hybridized.

3.3.2 Verification of Radio-Labeling

The labeling was verified by electrophoresis on a polyacrylamide minigel. The acrylamide solution was made by mixing 29g of acrylamide and 1g of bisacrylamide in 100ml distilled-deionized water (30% w/v). The acrylamide solution was then diluted to a final concentration of 8% (w/v).

The gel was prepared by mixing 10ml of 8% acrylamide solution, 50μl of 20% Ammonium persulfate (APS), and 5μl of TEMED. Each well receives 2μl of radiolabeled oligonucleotide solution to be analyzed along with 2μl of gel loading buffer. The gel runs in TBE buffer for 20 minutes, or until it runs 2/3 of the way through under a 180V tension.

In figure 3.3, radiolabeled DNA fragments from a phage Φ X-174 Hae III digest (right side) ranging from 1 kilobases to 72 bases was used as a standard. Using a logarithmic projection, it is noticeable that the radiolabeled αP^{32}-dTTP was successfully transferred to the 20 base oligonucleotides. The left hand run shows labeled oligodTBr after separation column cleaning, while the middle run shows the oligodTBr before the separation column cleaning.

Figure 3.3: Verification of labeled oligonucleotides (20 bases)

After verification is confirmed, a standard amount of 40μl of the radiolabeled oligonucleotides is added either to a pre-immobilized, or a clean surface substrate sample.

3.3.3 Immobilization of Radiolabeled Oligonucleotides

Immobilization was carried out by depositing 40µl of a 20 base single stranded oligodTBr (1M $NaHCO_3$) or oligodTam (0.001M KOH) onto the pre-treated substrates. 3 to 4 substrates were covered with a standard 100X20 mm tissue culture plates, along with a small water reservoir to keep samples under a humid conditions, and left overnight. The next day, substrates are measured (electrical impedance) and washed as later explained.

3.3.4 Hybridization of Radioactive Oligonucleotides

Hybridization was carried out by depositing 40µl of 20 bases single stranded oligodA (for complementary interaction with dT) or oligodC (for non-complementary interaction) on either pre-labeled dT, non-labeled dT, or with non-immobilized clean samples. 3 to 4 substrates were then covered with a standard 100X20 mm tissue culture plate, along with a small water tray to keep samples under humid atmosphere, and were left to hybridize overnight under a gentle agitation (Thermolyne) to allow maximum possible interaction with dT.

3.3.5 Cleaning Methods

Strong Wash: Two strong washing methods were used to test for covalent bonding with the surface. First wash is with a 0.05% Triton-X detergent

for 10 minutes where each sample is individually washed in separated scintillation vials followed by small rinse with distilled-deionized water and allowed to air-dry. A second wash includes a 95°C hot distilled-deionized water bath in a hybridization oven (Tek-Star) for 20 minutes followed by a small rinse, and allowed to air dry.

3.3.6 Measuring Radioactivity

After immobilizing or hybridizing samples and/or after a strong wash, each sample is doubly wrapped with Saran-Wrap, placed on a phosphoimaging plate (Fuji) and left to be exposed overnight. Reference standards were also concurrently used and prepared by adding 2.5µl of a 10^{-2} and 10^{-4} µg/µl of the radiolabeled oligonucleotides on a filter paper. The filter paper was allowed to air dry and was doubly wrapped with Saran-Wrap before being exposed on the phosphoimaging plate. The reference standards were used to determine the radioactivity of each sample. The quantification of the radioactivity then allows the determination of the amount of DNA in mass per unit area

After the overnight exposure, the phosphoimaging plate was read by the phosphoimager (Fujix, Bio-imaging analyzer) and analyzed (MacBas Ver2.2 and 2.5). This program allows calculations of the level of radioactivity of each sample and reference standards in terms of the area enclosed, and the radioactivity level per unit area. In addition, a background radioactivity count was also retrieved and subtracted from

each sample value offering an exact determination of radioactivity per unit area. Finally, the DNA surface density is given in terms of mass per unit area (ng/cm^2).

Each mass concentration is calculated with the following formula:

$$N = N_o (m/M)$$

- m is the radioactivity level per unit area minus background count in the central region of each sample.
- M is the radioactivity level per unit area minus background count for the reference standard. (same concentration as m)
- N_o is the amount of radioactive oligonucleotide originally added in the central region. (in terms of mass = 40µl added X concentration in µg/µl)

Notice that the unit of N is in terms of mass (converted to ng) found on the substrate. This value is then divided by the area enclosed by the radioactivity, giving a final mass surface density of ng/cm^2.

3.4 Quantization Results based on APTS/Immobilization/Hybridization

Previous work was conducted with oligodT and oligodTBr on substrates modified with 20% APTS showed no apparent flat band shifts between immobilized oligodTBr and hybridized oligodA. The concentration first worked with was 10^{-1} μg/μl oligodTBr and the same for oligodA. Both were in a 1M $NaHCO_3$ solution. It is important to note that hybridization of complementary DNA is dependent on the ionic strength of the sodium solution. In addition, the temperature also has an important effect on hybridization between complementary strands. At a certain temperature, the DNA can no longer rest in its duplex form and it will dissociate. This temperature is known as the melting temperature[51], T_m, and can be estimated with the following expression:

$$T_m = 81.5°C - 16.6(\log_{10}[Na^+]) + 0.41(\%(C+G)) - 0.63(\%F) - (600/n)$$

Where [Na+] is the molarity of sodium ions, %F is the percent of formamide and %(G+C) is the sequence composition with respect to G and C base pairing which forms three hydrogen bonds as compared to A and T which forms two. Therefore, the $NaHCO_3$ solution is necessary for efficient coupling of complementary sequences.

Cloarec[49] quantified the oligodTBr at 10^{-1} μg/μl; however, through electrical impedance measurements there was no apparent shift in the flat band potential. The immobilized oligonucleotide concentration was found to be 2,41 ng/cm^2. This represents approximately 2.5 X 10^{11} strands of DNA (1ng = 10^{11} strands) on the surface of the substrate. If we consider the 20 bases of oligodT to be a long cylinder, it has dimensions of 68Å in length and a diameter of 20Å.

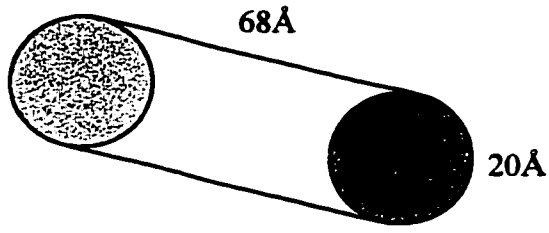
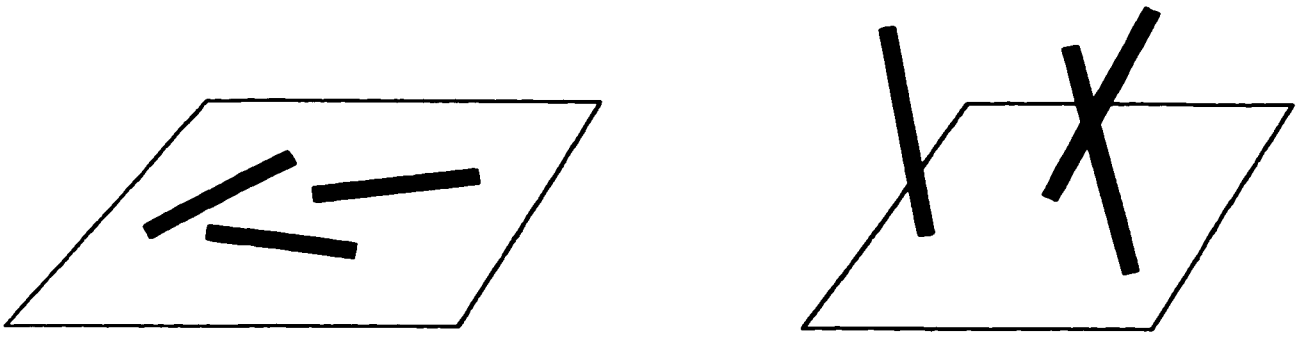


Figure 3.4: Schematic representation of a 20 base long single stranded oligonucleotide



(a) (b)

Figure 3.5: Some possible outcomes of immobilized oligodTBr, (a) strands are flat on surface, (b) ideal condition, strands are in an upright position.

Assuming that the strands do not overlap, as in case (a) of figure 3.5, multiplying the number of strands 2.5 X 10^{11} by the area of the rectangle taken from the cylinder shown in figure 3.4 (68Å X 20Å) a total of 1360Å or 0.034cm^2 is obtained which is 3.4% of the total area. (b) Assuming that the strands are upright (figure 3.5B), multiplying the number of strands (2.5 X 10^{11}) by the area of the base of the cylinder (figure 3.4) 314 $Å^2$ is obtained which is equal to 0.0079 cm^2 or 0.8 % of the total area. Since there is no apparent flat band potential shift under these conditions, decreasing the concentration of the oligonucleotide was proposed as a solution and this forms part of the basis for the work presented here.

The concentration of the oligodTBr was decreased to10^{-2}µg/µl. Figure 3.6 shows the variation in the amount of immobilized DNA before washing and after two strong washings.

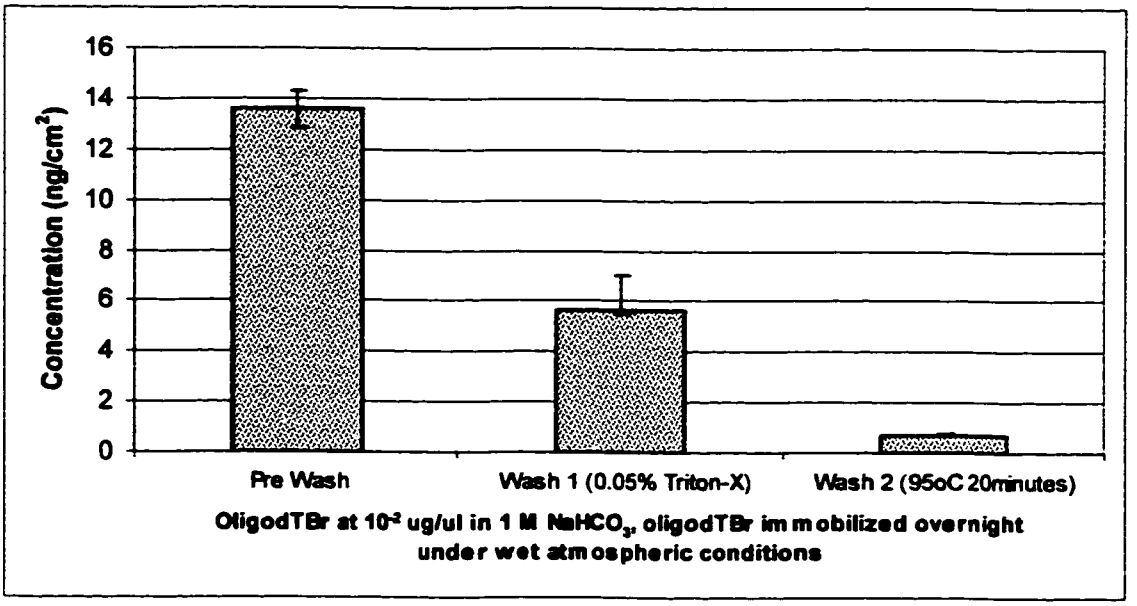


Figure 3.6: Surface Concentration of oligodTBr (4 substrates) immobilized on 20% APTS

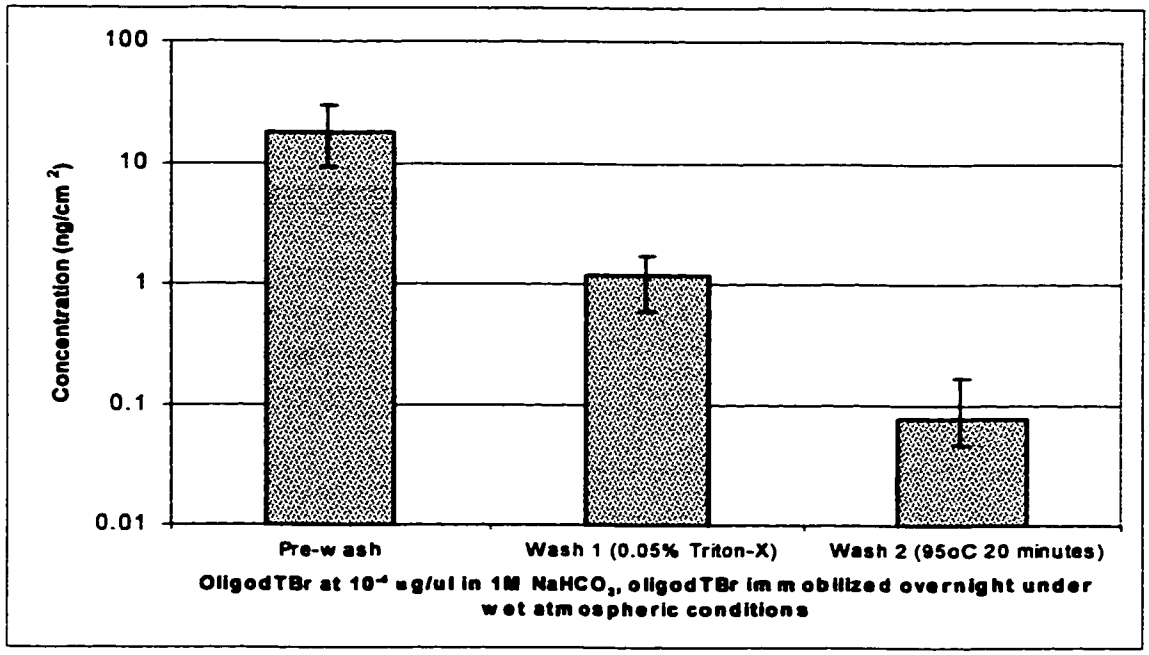


Figure 3.7: Surface Concentration of oligodTBr (8 substrates) immobilized on 20% APTS

Figures 3.6 and 3.7 show the concentration variations of oligodTBr immobilized on the substrates pre-treated with APTS. In both situations, the substrates were left to react overnight under wet atmospheric conditions. Quantization measurements were made prior to any cleaning of the substrates, followed by strong washings of Triton-X detergent agent[52] and 95°C hot water wash. In figure 3.7, the amount of oligodTBr after each washing substantially decreases, removing any unwanted oligonucleotide that may have been adsorbed onto the surface. This may indicate that most of the oligodTBr are physi- adsorbed onto the surface and not covalently bound.

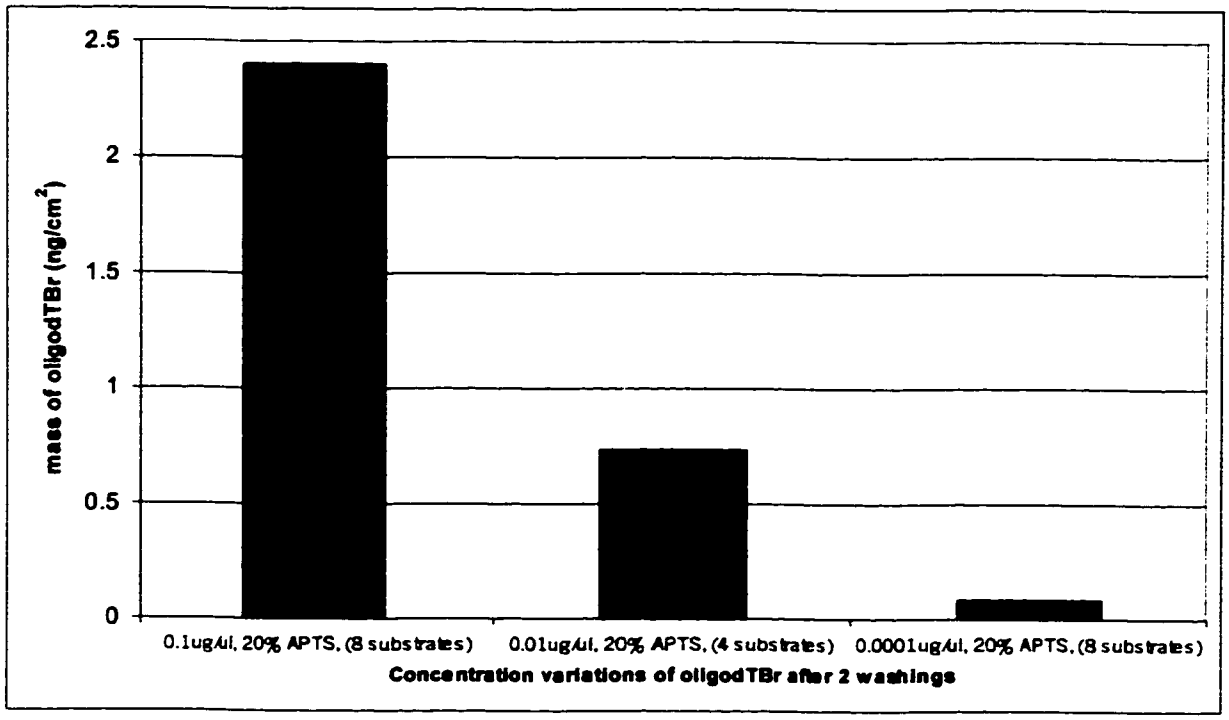


Figure 3.8: Concentration Variation

Figure 3.8 represents an overall view of the amounts of oligodTBr immobilized as a function of the initial solution concentration. With each ten-fold decrease in concentration there is a 3 to 4 fold decrease in the amount of ss-oligonucleotide fixed on the surface.

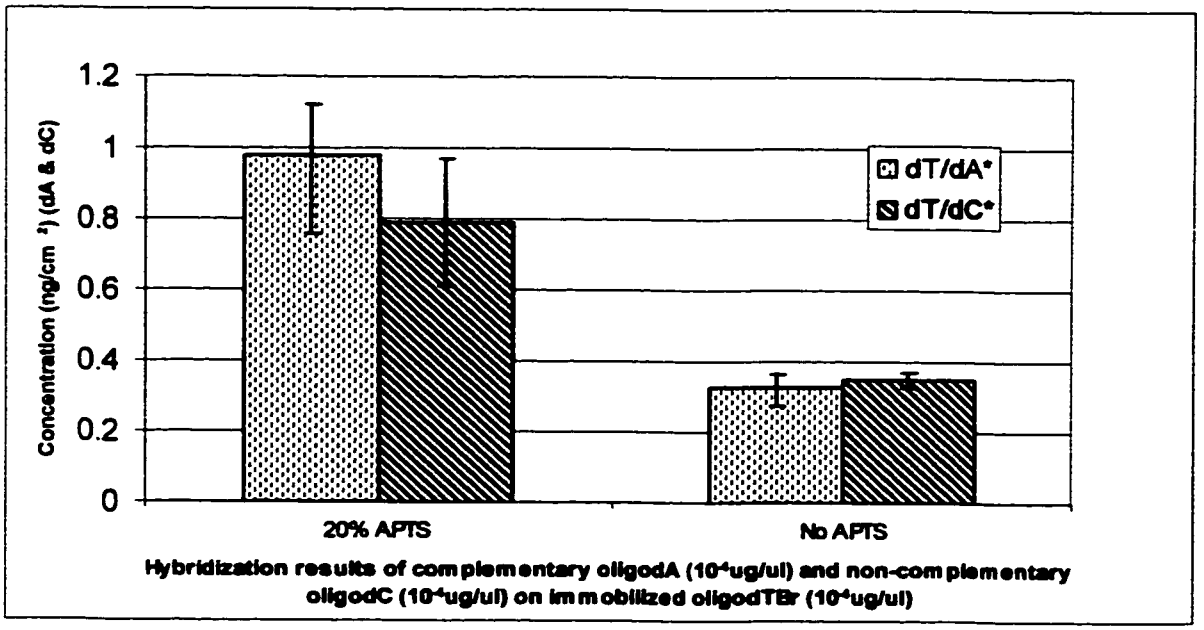


Figure 3.9: Hybridization results of complementary and non complementary strands

Hybridization was tested by adding 40 μl of oligodA (10^{-4} μg/μl in 1M $NaHCO_3$) to 4 substrates each treated with immobilized oligodT, with and without 20% APTS. Similarly, 40 μl of oligodC (10^{-4} μg/μl in 1M $NaHCO_3$) were added to 4 substrates each treated with immobilized oligodT with and without 20% APTS. The hybridization was accomplished at room temperature for 1 hour, followed by mild rinsing with double distilled water. Looking at the results shown in figure 3.9, several problems arise. First, there is no substantial difference between the complementary strand, oligodA, and non-complementary strand, oligodC. Second, the amounts of dA and dC are approximately 10 times greater than the immobilized oligodTBr. Finally, smaller but similar amounts

of dA and dC are found to remain on substrates that were not treated with APTS.

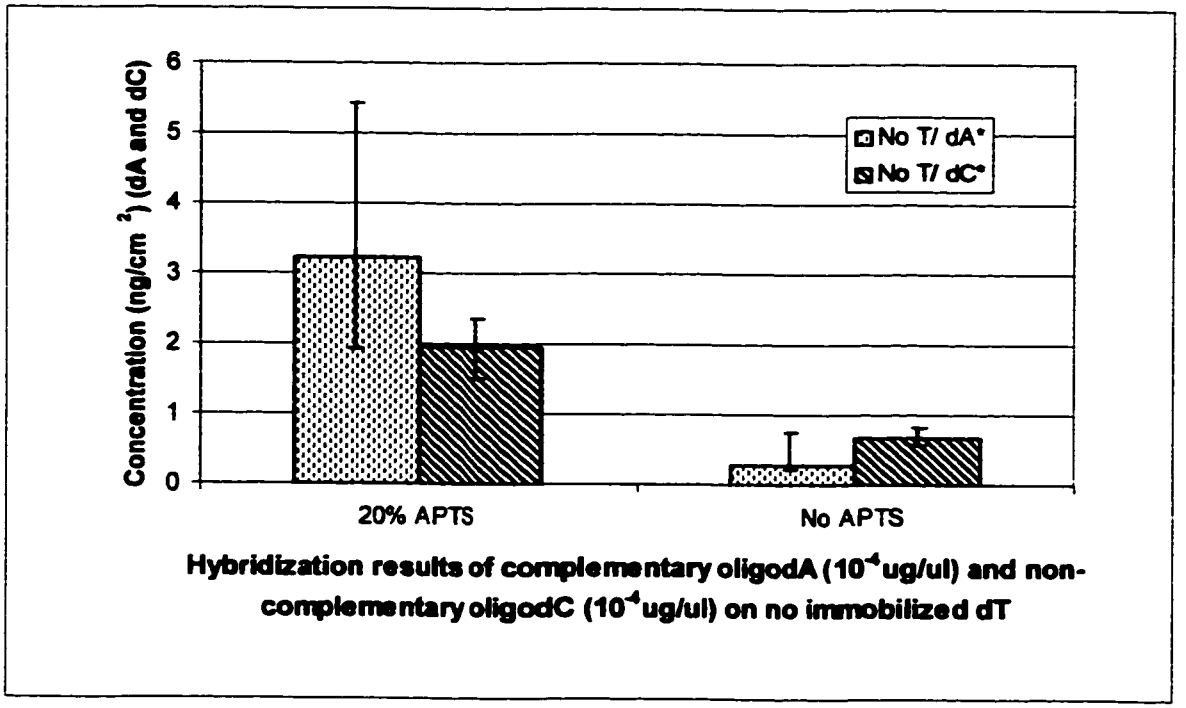


Figure 3.10:Hybridization of complementary and non-complementary strands on no dT

Figure 3.10 shows results similar to figure 3.4, but in this case, however, no immobilized oligodTBr was used. This gives very unusual results since we do not expect to find any hybridized DNA on the surface. This may suggest that the oligonucleotides are strongly adsorbed onto the surface (with or without immobilized oligodTBr and with or without APTS). These results provide an indication that the APTS added does not form a uniform monolayer and most of the oligonucleotides (oligodTBr, oligodA

and oligodC) are entrapped or embedded inside the APTS layer. This is also evident from the multiple washings performed at the immobilization level. Upon extended washings the oligodTBr will vanishes. Therefore, APTS does appear to be a good silanating agent and this approach was put to rest.

3.5 Immobilization Results based on GPTS and oligodTam

All immobilizations using oligodTam (40µl at the specific concentrations indicated) were left to react overnight under wet atmospheric conditions at an ambient temperature. The next day, they were placed in boiling distilled-deionized water for two minutes.

Impedance measurements were taken before and after the immobilization with oligodTam.

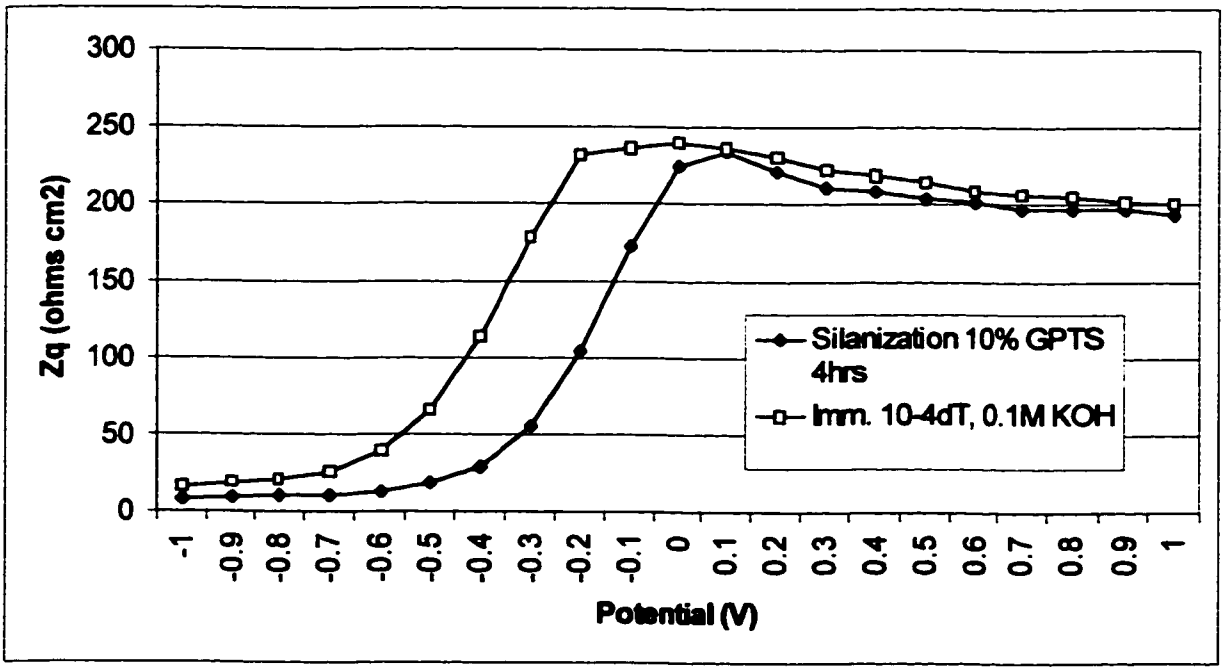


Figure 3.11: Impedance curve with 10% GPTS and 10^{-4} µg/µl oligodTam in 0.1M KOH

Figure 3.11 presents the impedance curves for these substrates in contact with the tris electrolyte. Measurements were repeated twice to insure good stability of the substrates and in each case they were identical. The immobilization of oligodTam results in a shift of approximately –200mV in flat band potential, V_{fb}, when compared the curves obtained after silanization. This corresponds to an increase of negative charges at the surface in contact with the electrolyte. This change may be due to either immobilization of oligodTam at the surface or due to the effect of the 0.1M KOH on the surface.

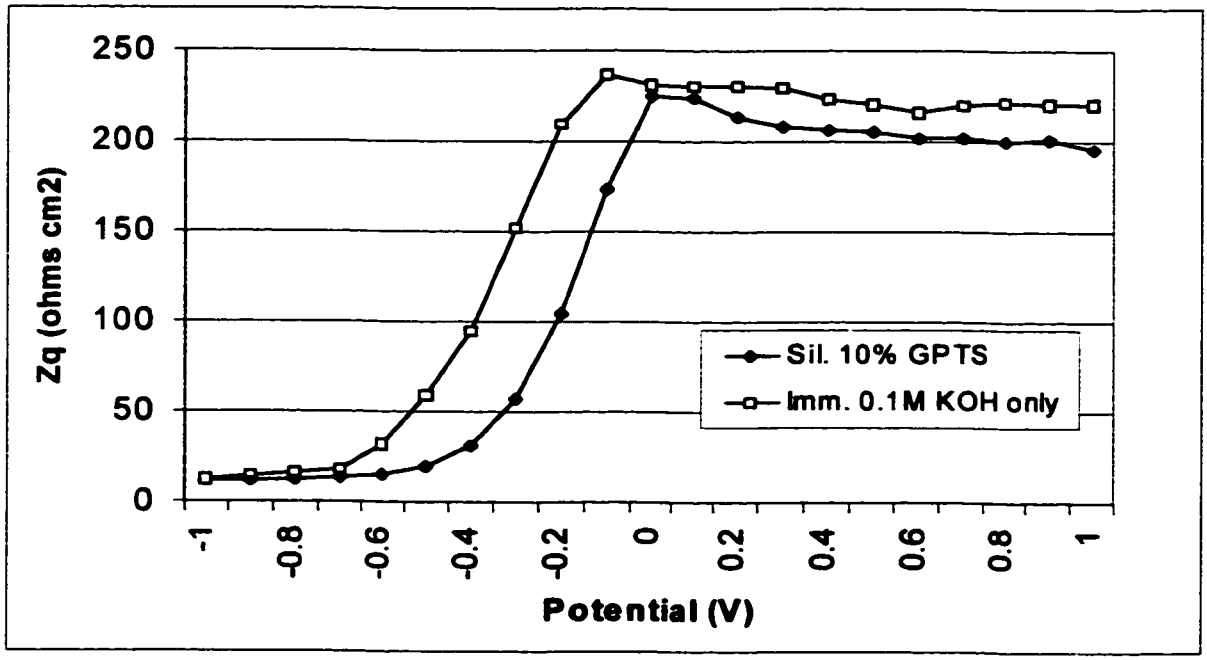


Figure 3.12:Impedence curve using 0.1M KOH as blank

40 µl of 0.1M KOH was added to GPTS modified substrate and left overnight under wet atmospheric conditions. Impedance measurements under these conditions (no oligodTam, KOH only), also show a shift in flat-band potential of approximately

-150mV. This variation may be explained by the diffusion and confinements of the ions (K^+ and OH^-) at the GPTS surface. It is also possible that the silane layer is altered by the action of KOH.

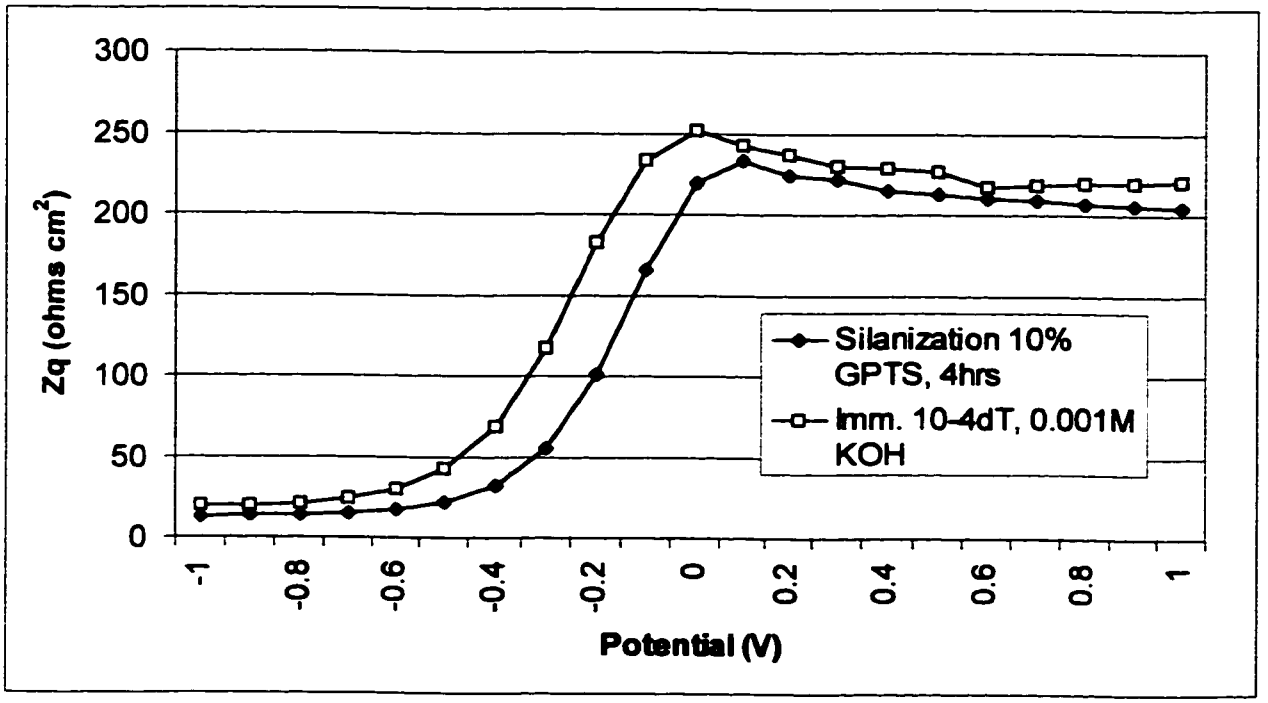


Figure 3.13: Impedance curve with 10% GPTS and 10^{-4} μg/μl oligodTam in 0.001M KOH

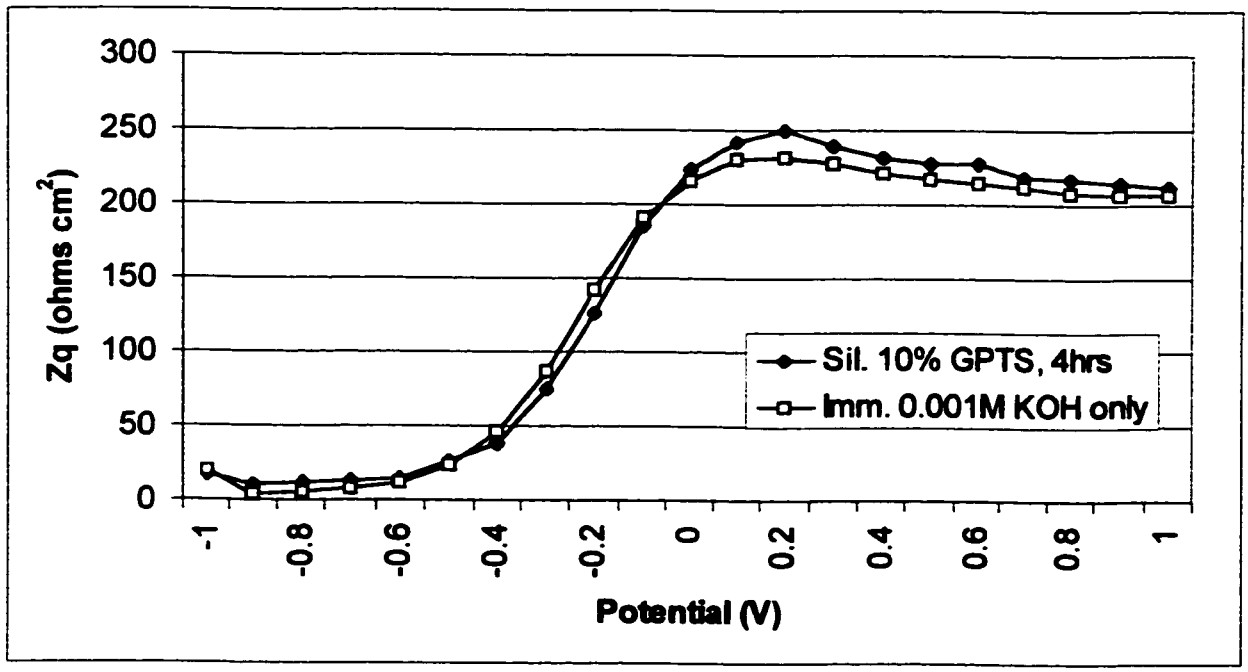


Figure 3.14:Impedance curve using 0.1M KOH as blank

A series of ten fold KOH dilutions were performed and an optimal concentration of 0.001M KOH was found to give a good result in terms of seeing V_{fb} shifts due solely to the immobilization of the oligodTam (figure 3.13 and 3.14). The difference in flat band potential with oligodTam is about –120mV as compared to –20mV found with 0.001M KOH solution alone. Therefore, a -100 mV shift can be attributed to oligodTam being directly immobilized onto the surface.

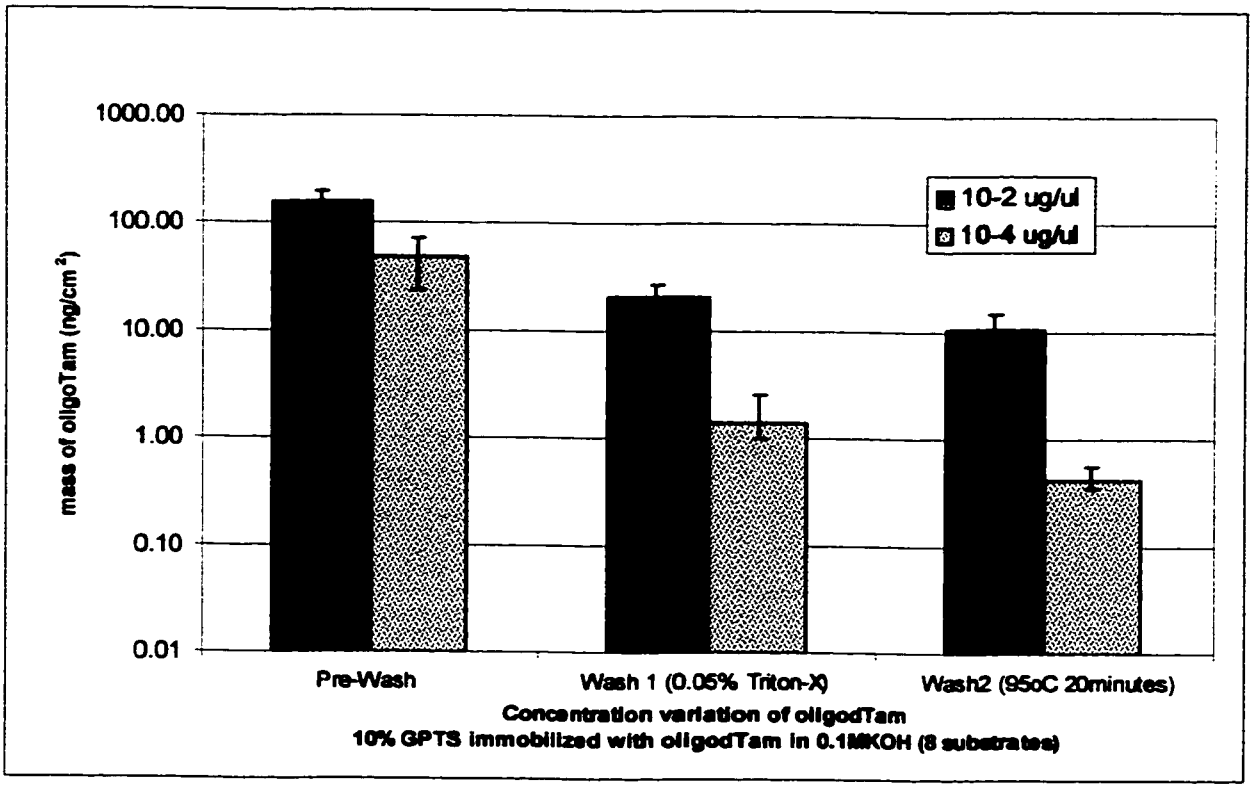


Figure 3.15: Immobilized OligodTam concentration variations (for 0.1M KOH Concentration)

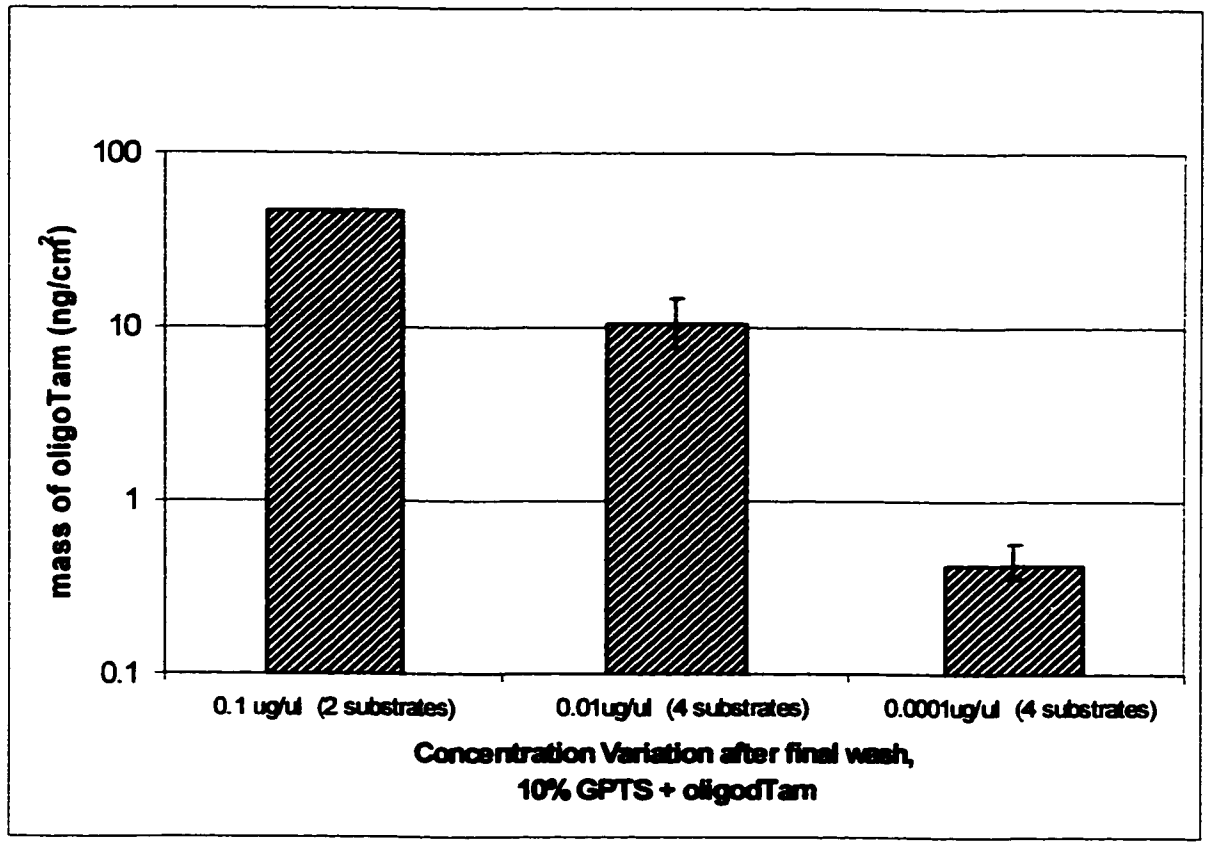


Figure 3.16: Immobilized OligodTam concentration variations as a function of initial solution concentration (for 0.1M KOH)

Figures 3.15 and 3.16 present the immobilized oligonucleotide concentration variation as a function of initial solution concentration, at 0.1M KOH, and 10% GPTS modified substrates. From figure 3.15, following the first and second washings, 60% of the initial amount of oligodTam (pre-wash) remains at the surface. This is a strong indication that perhaps the linkage between the GPTS monolayer surface and the aminolinker of the oligonucleotide is covalent. In figure 3.16, one sees that there is almost a ten fold increase in immobilized DNA for an initial concentration of 10^{-4} μg/μl

on substrates treated with GPTS as opposed to those treated with APTS (see figure 3.8). This suggests that a stronger interaction exists between the oligonucleotide and GPTS. Previous hybridization experiments by Cloarec[49], performed on GPTS/oligodTam modified substrates with 10^{-1} μg/μl oligodTam in 0.1M KOH were nonreproducible. Under these conditions, the amount of immobilized DNA was found to be 46.5 ng/cm^2 which is 20 times greater than that observed on substrates treated with 20% APTS. This also represents, assuming no overlapping of the strands, a coverage of 68% of the entire substrate. With this high surface concentration steric hinderance may well be preventing hybridization from occuring. It was therefore decided to reduce the oligodTam concentration to 10^{-4} μg/μl.

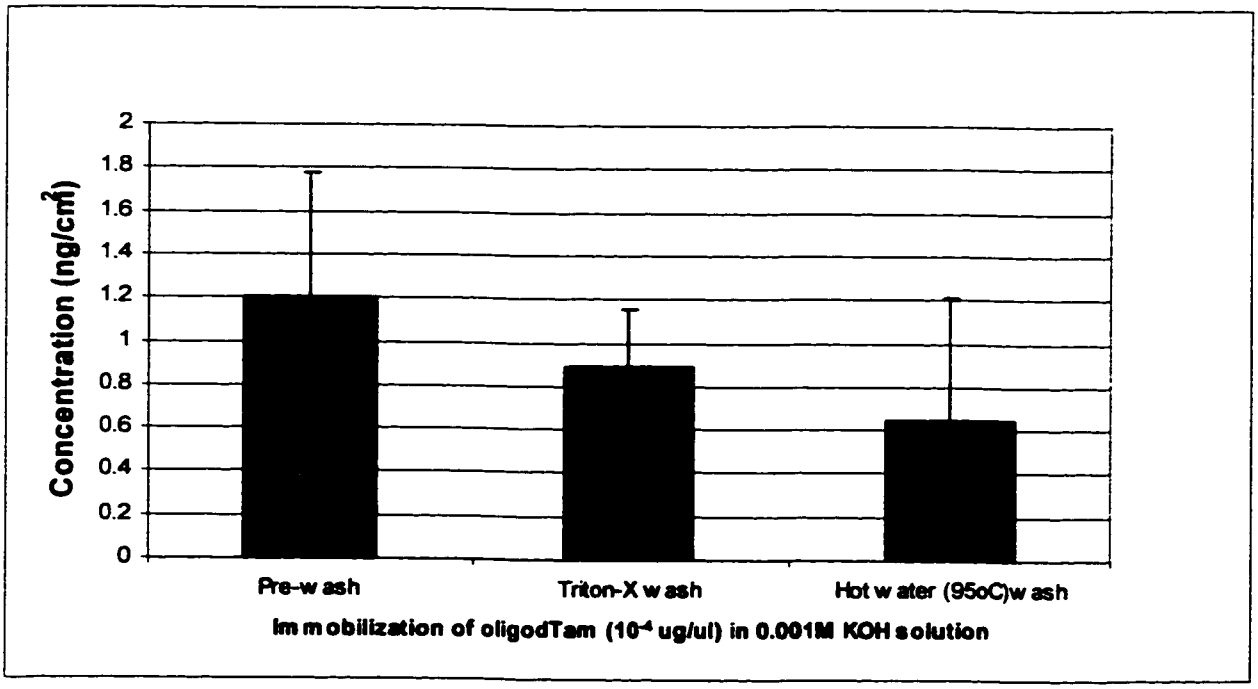


Figure 3.17: Immobilization of oligodTam in 0.001M KOH

Quantizations using 10^{-4} μg/μl oligodTam were carried out and after the second wash treatment a mass concentration of 0.64 ng/cm^2 was detected. The electrical measurements showed a V_{fb} shift of −100 mV.

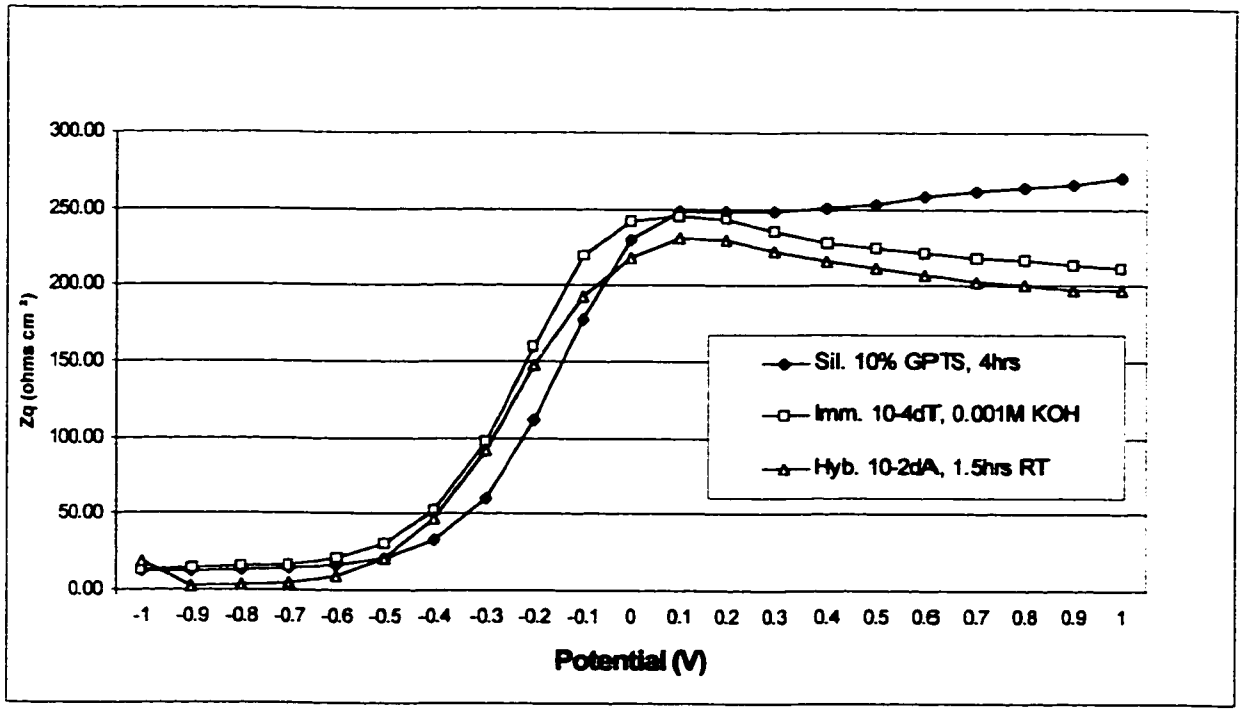


Figure 3.18: Immobilization and Hybridization with excess oligodA

The first impedance measurements for hybridization on 10 % GPTS modified substrates with immobilized oligodTam (10^{-4} μg/μl in 0.001M KOH) as shown in figure

3.18. 40 μl of an excess amount of oligodA (10^{-2} μg/μl in 1M $NaHCO_3$) were added to the substrates and left to hybridize for 1.5 hours, resulting in a weak flat band potential shift of –30 mV. This variation could be due to either the ionic affect such as the sodium ion (which does aid the hybridization process as explained earlier) or due to hybridization itself.

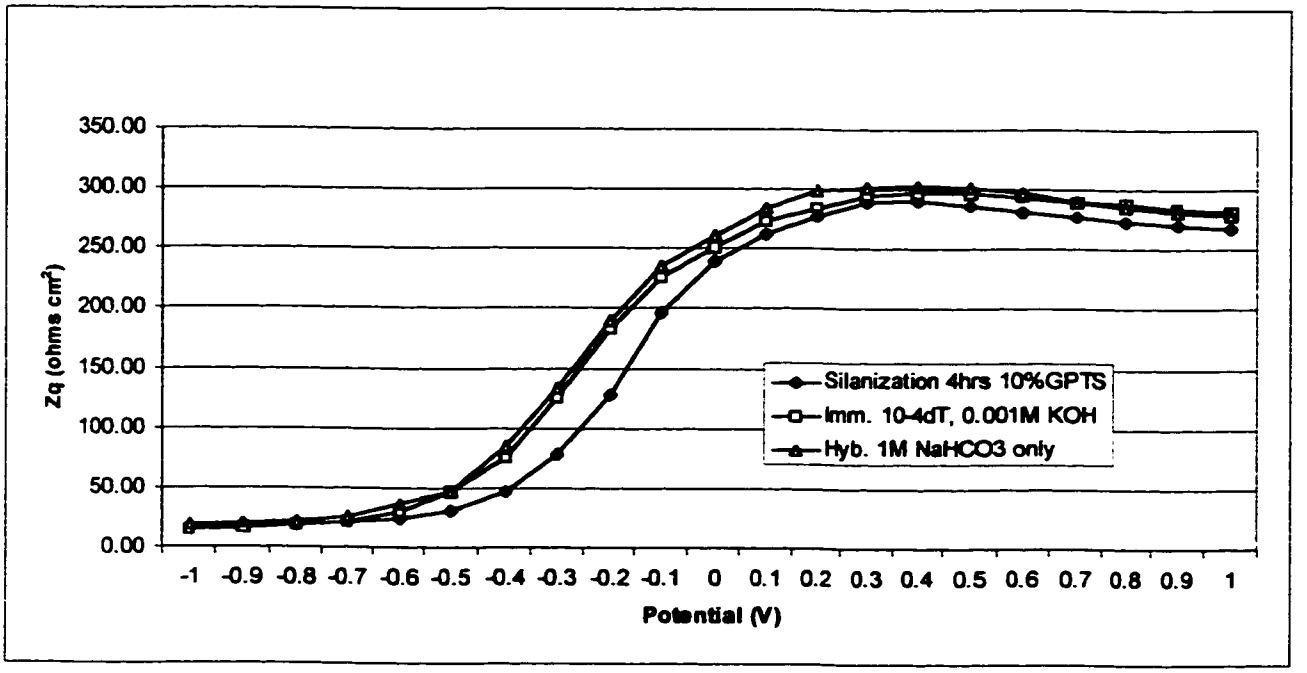


Figure 3.19: Hybridization with blank 1 M $NaHCO_3$ solution

This was tested and the results are shown in figure 3.19. 40 μl of 1 M $NaHCO_3$ blank solution (no dA) was deposited on an oligodT modified substrate and a small V_{fb} shift of –20 mV is observed between the hybridized curve and immobilized

oligonucleotide. This would indicate that the shift seen in figure 3.18 is mostly due to the ionic effect of the sodium bicarbonate solution. To increase the specificity of hybridization stringency, the temperature of the substrates undergoing hybridization was increased to temperatures above and just below the melting temperature (≈50°C) of the complementary strands.

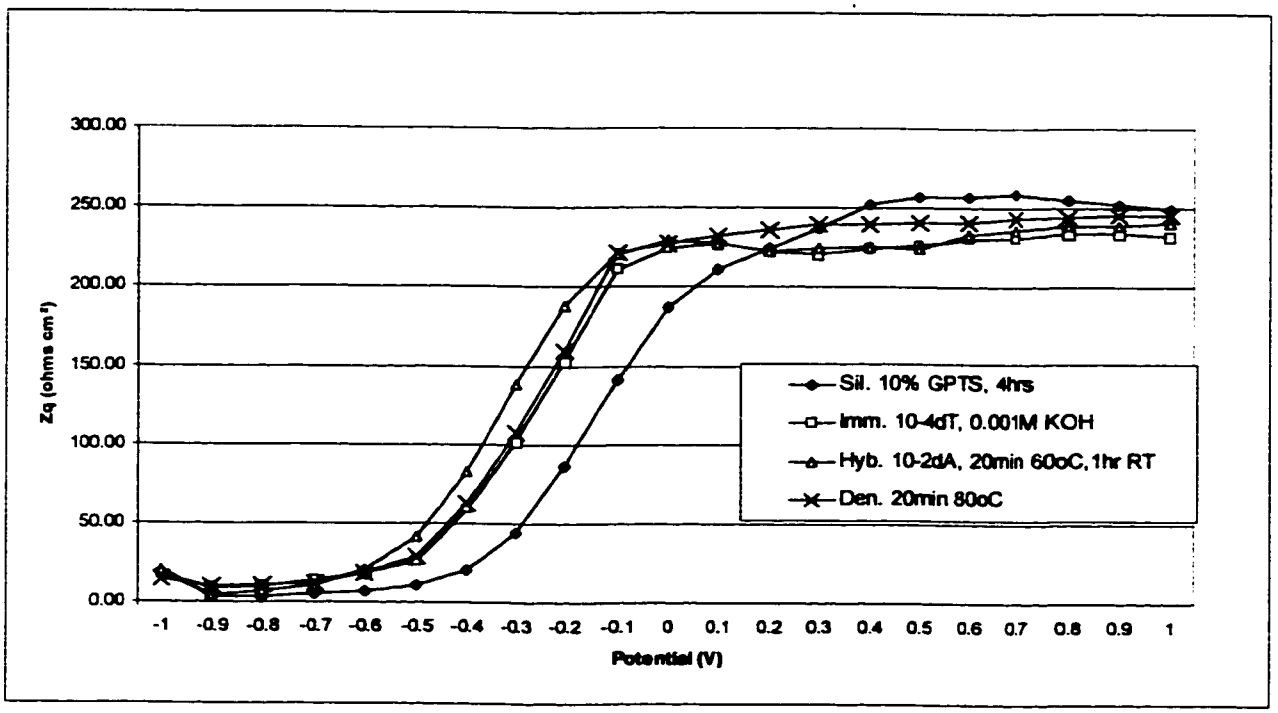


Figure 3.20 : Hybridization with oligodA (10^{-2} μg/μl in 1M $NaHCO_3$) and denaturation on pre-immobilized oligodTam (10^{-4} μg/μl in 0.001M KOH), 10% GPTS

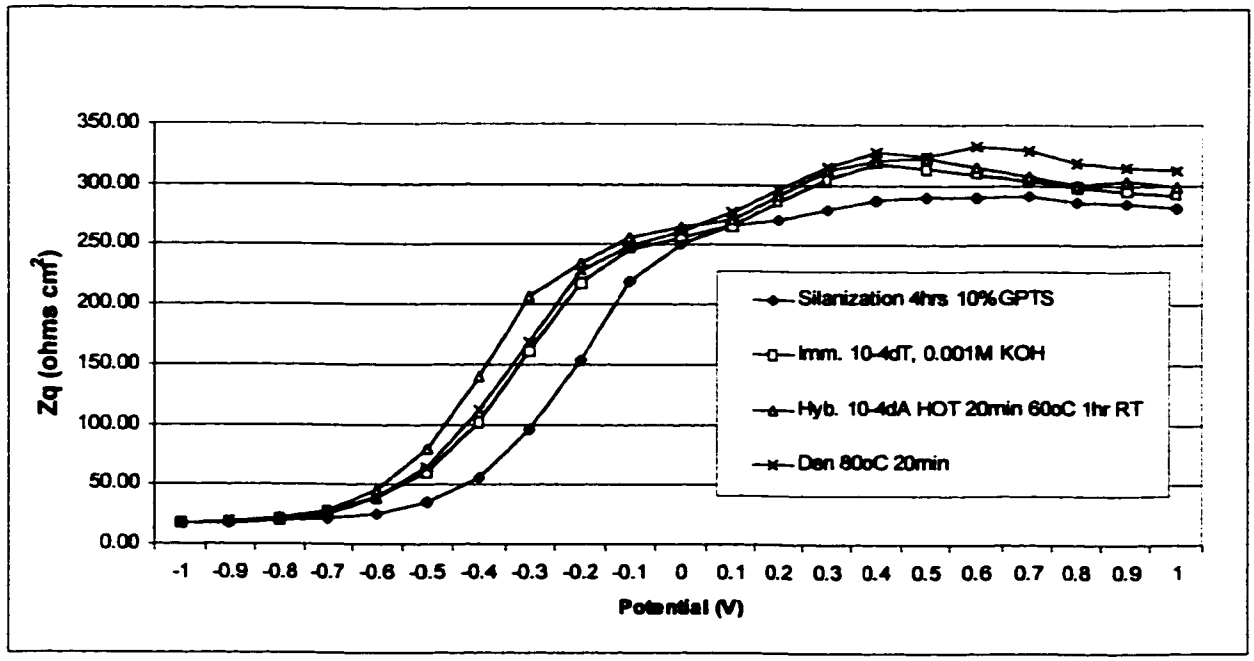


Figure 3.21 : Hybridization with oligodA (10^{-4} µg/µl in 1M $NaHCO_3$) and denaturation on pre-immobilized oligodTam (10^{-4} µg/µl in 0.001M KOH), 10% GPTS

Figures 3.20 and 3.21, present results obtained when hybridization is performed at a temperature of 60°C for 20 minutes. To do so the substrates were placed in an oven at 60°C for 20 minutes followed by cooling at room temperature for 1 hour. In both cases, where the concentrations of dA were 10^{-2} and 10^{-4} µg/µl (figure 3.21 and 3.22 respectively), a shift of about –80 mV was found with respect to the immobilization curve. These results show that hybridization is detected for a heat treatment at temperature above the T_m of the dA-dT duplex.

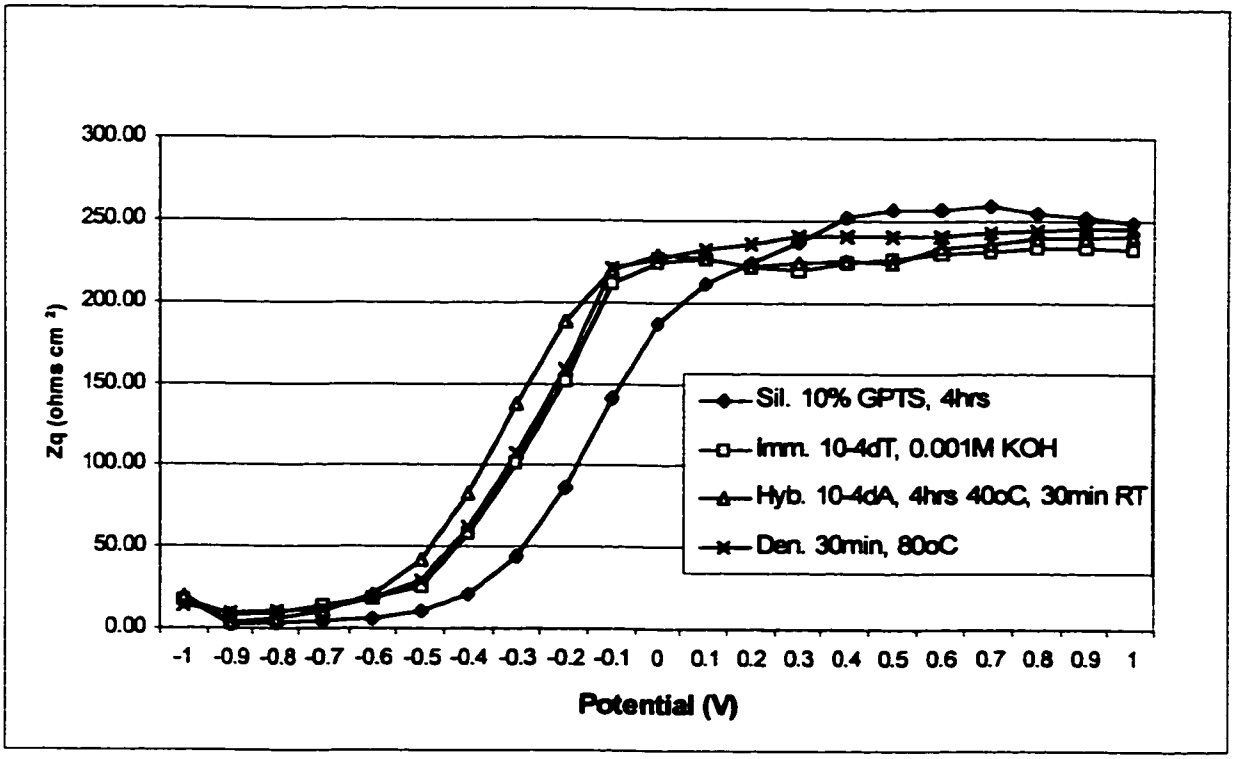


Figure 3.22: Hybridization treated at 40°C for 4 hours with oligodA (10^{-4} μg/μl in 1M $NaHCO_3$) and denaturation on pre-immobilized oligodTam (10^{-4} μg/μl in 0.001M KOH), 10% GPTS

Figure 3.22 shows the result obtained when hybridization is performed at a temperature lower than the melting temperature, and for a longer period of time. After 4 hours at 40°C, a flat-band potential shift of approximately –100 mV was obtained. These results are consistent with the direct detection of hybridization of the complementary and they are supported by the radiolabelling experiments performed to quantify the

hybridized dA sequences. Denaturation was performed for a longer period of time at 80°C. Even under these conditions, the double strands do not fully dissociate.

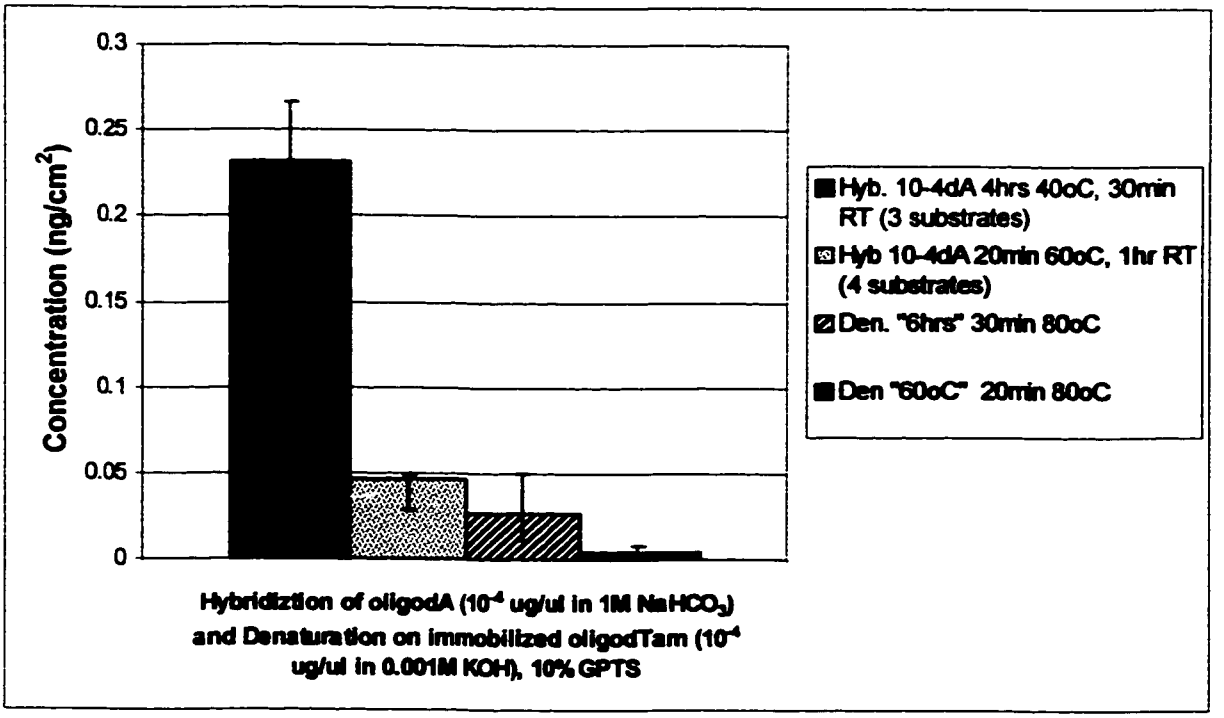


Figure 3.23: Hybridization (4 hours at 40oC and 20 min at 60oC) and denaturation of oligodA (10^{-4} μg/μl in 1M $NaHCO_3$) on immobilized oligodTam (10^{-4} μg/μl in 0.001M KOH), 10% GPTS

The quantization results match well with the electrical impedance measurements described above. Radiolabelling measurements were all at concentrations of 10^{-4} μg/μl oligodA in 1M $NaHCO_3$. The hybridized concentration was found to be 0.235 ng/cm^2

when hybridizing for 4 hours at 40°C and 0.048 ng/cm^2 when hybridizing for 20 minutes at 60°C. This represents a five fold increase and it is clearly reflected in the impedance measurements. In both cases, the hybridized oligodA is less than the 0.64 ng/cm^2 of immobilized oligodTam. The amount of hybridized DNA should not exceed the amount of immobilized DNA. In both cases, the denaturation process did not completely remove the oligodA. The 10% still remaining probably accounts for the small negative flat band shift that is still visible with respect to the immobilization curve.

3.6 Immobilization and Hybridization Results based on Aryl Diazonium fixation[54]

A scanning potentiostat (EG&G Princeton Applied Research Model 362) was used to apply d.c. potentials to the working electrode to perform the cyclic voltammograms. The current-voltage response was recorded on an XY recorder (Philips, Model PM 8143).

The silicon electrodes (without oxide) were chemically modified and reduced to obtain the desired H-terminated silicon electrode. The substrates were then placed in the 4-bromobenzenediazonium tetrafluoroborate solution and a reduction wave between -0.3V to −1.9V (current range set at 1mA) reduces $(N_2ArBr)^+$, binding it to the silicon surface (figure 3.24).

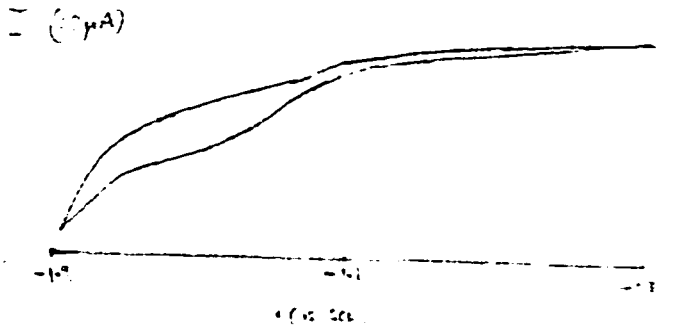


Figure 3.24: Cyclic Voltammogram of one substrate (100mV/s scan rate)

Proof of the surface modification was provided by X-ray photoelectron spectroscopy, XPS, performed on the modified silicon substrates (figures 3.25 and 3.26).

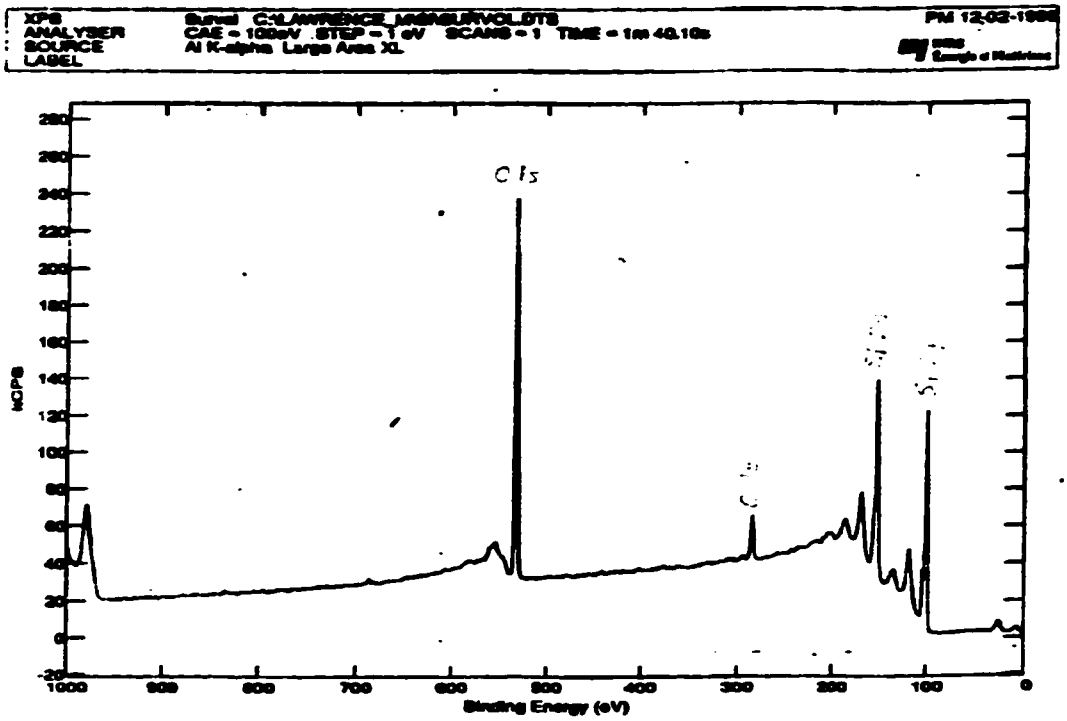


Figure 3.25: XPS of Si Substrate

Figure 3.25 shows results for an unmodified Si substrate and figure 3.26 shows the

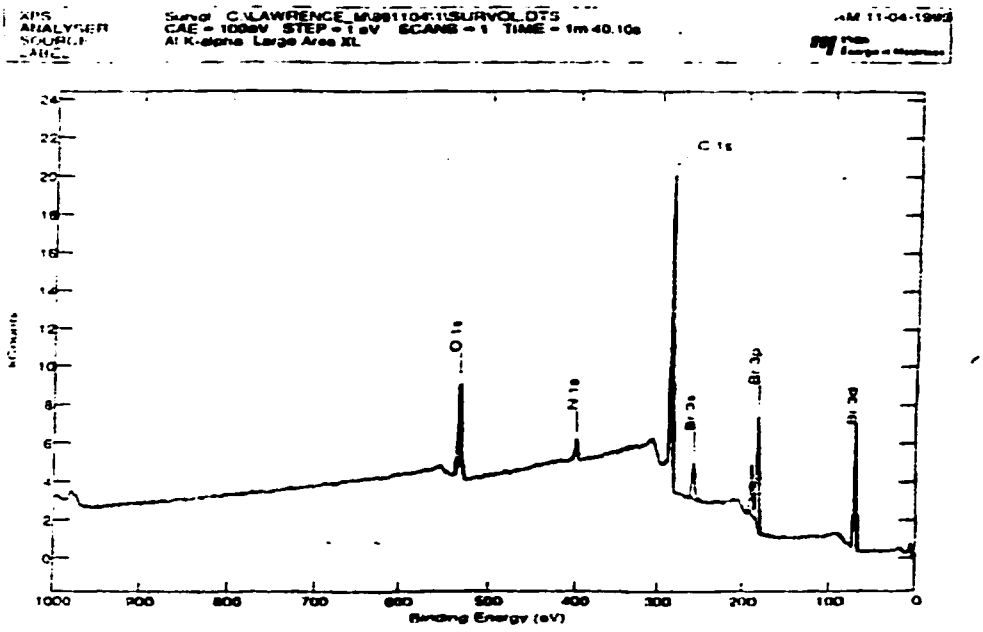


Figure 3.26: XPS after addition of bromo aryl diazonium

Effect of electrochemically modifying the silicon with the bromo aryldiazonium compound. The peaks due to Si are no longer visible, whereas the peaks due to the presence of bromine are clearly identified. Also noticable is the increase in the carbon peaks, the appearance of a peak due to nitrogen and the decrease of the oxygen peak when compared to the unmodified surface. The XPS measurements provide clear evidence for the presence of diazonium moiety at the surface of these substrate.

Electrical impedance measurements also confirms the evidence that the suface was modified and preliminary results show flat band potential shifts of about –80mV upon hybridization of dT with dA.

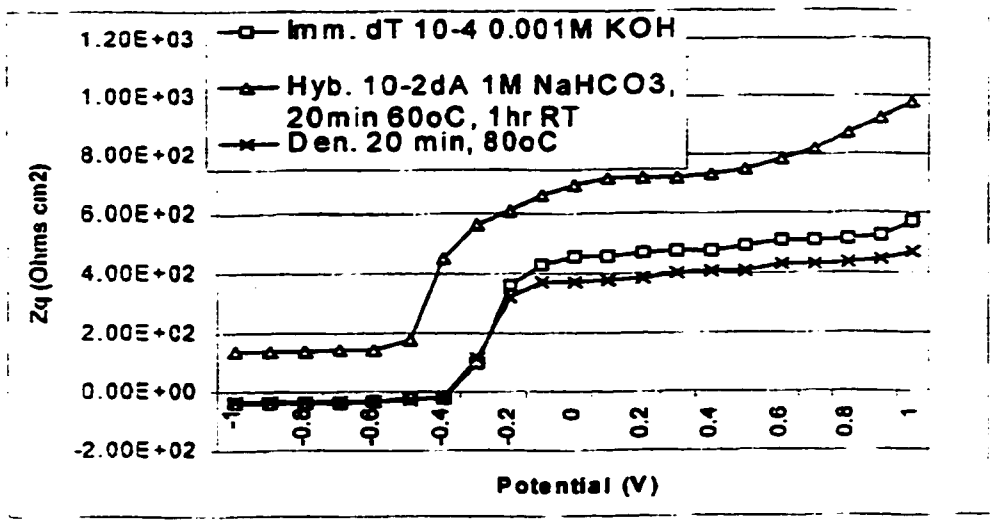


Figure 3.27: Impedance measurements of a substrate modified with diazonium salt. Immobilization, hybridization and denaturation curves.

In figure 3.27, 40µl of a 10^{-4} µg/µl oligodTam in 1M $NaHCO_3$ was immobilized overnight onto substrates electrochemically modified with the bromobenzene diazonium. The next day, substrates were placed in boiling distilled-deionized water bath to wash any unreacted excess and 40µl of a 10^{-2} µg/µl oligodA in 1M $NaHCO_3$ was left to hybridized at room temperature for 1.5 hours. This resulted in a –150mV flat band potential shift with respect to the oligodT immobilization curve (figure 3.27).

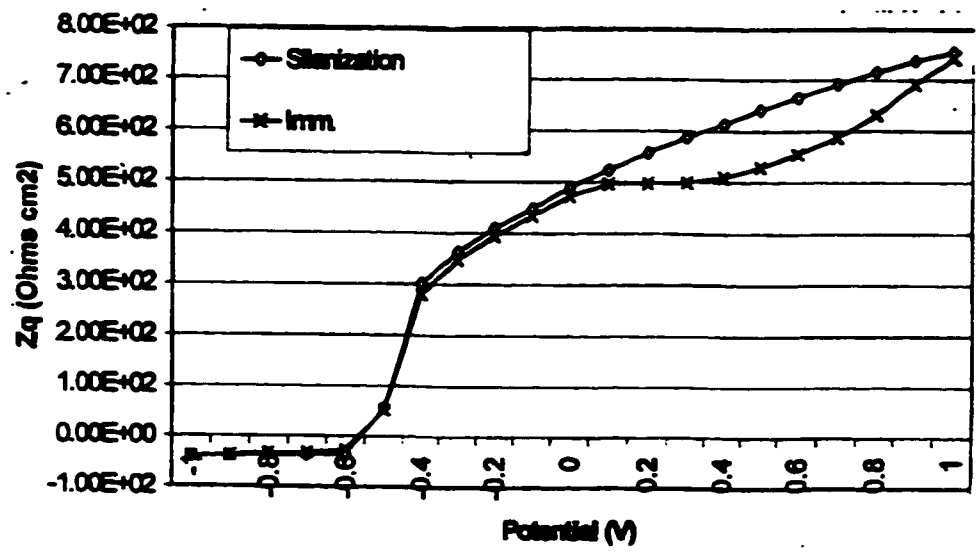


Figure 3.28: Impedence measurements using a 1M $NaHCO_3$ blank

Figure 3.28 shows the results obtained when 40µl of a 1M $NaHCO_3$ solution (no dT) was immobilized overnight. Without the oligodTam, no flat band shift is observed. This clearly indicates that immobilization is not due to any ionic effect of the solution. Quantization through radiolabeling shows a 50% increase in the amount of immobilized dT for the diazonium method compared to the GPTS method.

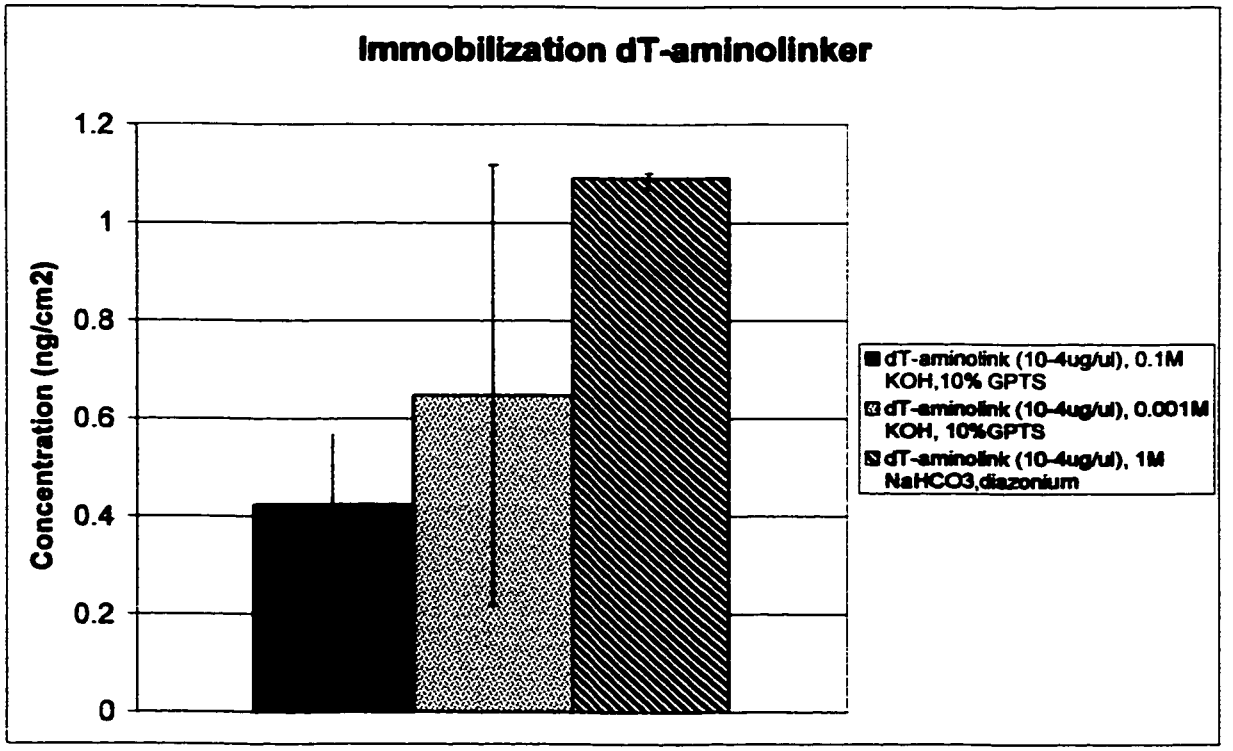


Figure 3.29: Immobilization comparisons between GPTS and diazonium salt

3.7 Conclusion

3.7.1 Silica Substrates Using APTS Silanization Agent.

From the quantization results obtained through immobilization and hybridization of oligonucleotides, it is seen that the fixation of APTS (20%) is of poor quality. The evidence lies in the immobilization using oligodTBr where after each washing the amount of oligonucleotides substantially decreases. This may indicate that the APTS layer acts to entrap the DNA rather than covalently binding to it. A stronger manifestation of the entrapment hypothesis lies in the quantization measurements found for the hybridization step. Following hybridization, minor washings were performed and a greater amount of the complementary oligonucleotide was found on the substrates compared to that forming the probe layer. Even with no immobilized DNA present, the complementary oligonucleotides were still in greater amount than the immobilized strands.

3.7.2 Silica Substrates Using GPTS Silanization Agent

GPTS works much better as a silanization agent. Electrochemical impedance measurements and quantization through radiolabeling show strong evidence for this. Consecutive washings at the immobilization level show quantitatively that the

oligonucleotides are fixed covalently.

With the addition of complementary strands (oligodA), hybridization produces a maximum flat band potential shift of -120 mV when treated at a temperature of 40°C for four hours. Applying these conditions, one-third of the immobilized oligonucleotides were hybridized.

Therefore, there is good indication that at this stage, detection of homo-oligomers (20 identical bases) was achieved using the GPTS method for the silanization. Subsequent works will involve hybridization with more complex oligonucleotides, using a variable oligonucleotide sequences for immobilization and hybridization and finally looking at one or more nucleotide mismatches and optimizing a method of detection at this level.

3.7.3 Diazonium salt method

The aryl diazonium method of functionalization appears to be the most efficient approach for chemically modifying Si substrate for DNA detection purposes. The diazonium method is quicker than the GPTS method to prepare the surfaces and it costs less since the diazonium method does not require that an insulating oxide layer be thermally grown onto the Si.

There is strong evidence (through XPS) that immobilization of oligonucleotides is strickly covalent and with rigorous washing methods, quantization results show 50% more immobilized oligonucleotides than the GPTS method (in 0.001M KOH solution). Preliminary results indicate that flat band potential shifts are obtained for hybridization.

Further research is required in both the GPTS and the diazonium method to eventually devise a biosensor that will be able to distinguish single-base pair mismatches. Secondly, impedance curves as a function of temperaturewill be used to ascertain nucleotide mis-matches through there melting temperature variations.

DNA biosensor based research faces endless opportunities and imposes an extraordinary need for maintaining contacts across a wide spectrum of developments in this field.

References

1. Cloarec. J.P., Martin, J.R., Lawrence, I., Polychronakos, C., Soutreyand, E., Lawrence, M.F., *Sens. and Actuat. A*, (Submitted Fall 1999)

2. Soutreyand, E., Cloarec. J.P., Martin, J.R., Wilson, C., Lawrence, I., Mikkelson, S., Lawrence, M.F., *J. Phys. Chem.*, **101**, (1997), 15, 2980-2985

3. www. ornl.gov/TechResources/Human_Genome/home.html

4. Murray, J.C., Buetow, K.H., Weber, J.L., Ludwigsen, S., Scherpbier-Heddema, T., et al., A Comprehensive human linkage map with centimorgan density., Cooperative Human Linkage Center (CHLC), *Science*, **265**, 2049-54, 1994.

5. Lander, E.S., Schork, N.J., Genetic dissection of complex traits, *Science*, **265**, 20237-48, 1994.

6. McLaren, N.K., Immunotherapy for IDDM. Endocrine Society meeting, Washington, D.C., 1995, S-25-2.

7. Hacia, J.G., Bfody, L.C., Chee, M.S., Fodor, S.P.A., and Collins, F.S., *Nature Genetics*, **14**, 441, 1996.

8. Chee, M.S., Yang, R., Hubbell, E., Berno, A., Huang, X.C., Stern, D., Winkler, J., Lockhart, D.J., Morris, M.S., and Fodor, S.P.A., *Science*, **274**, 610, 1996.

9. Sterngold, J., *The New York Times*, section 3 "Money and Business", August 18, 1996.

10. Bartlet, P.N., The Use of Electrochemical Methods in the Study of Molified Electrodes, *Biosensors, Fundamentals and Aplications*, New York, Oxford University Press, 1987, 211-246.

11. Killard, A.J., Deasy, B., O'Kennedy, R., and Shyth, M.R., Antibodies: Production, Function, and Applications in Biosensors, *Trends Anal. Chem.*, **14**, 257-266, 1995.

12. Yim, H.S., Kibbey, C.E., Ma, S.C., Kliza, D.M., Liu, D., Park, S.B., Torre, C.E., and Meyerhoff, M.E., Polymer Membrane-based ion, gas and bio-selective potentiostatic sensors, *Biosens. Bioelec.*, **8**, 1-38, 1993.

13. Lubbers, D.W., Fluorescence based chemical sensors, *Advances in Biosensors*, vol 2. JAI Press, Greenwhich, CT, 1992.

14. Grate, J.W., Martin, S.J., and White, R.M., Acoustic Wave microsensors, *Anal. Chem.*, **65**, 940-948 & 978-996, 1993.

15. Fodor, S.P.A., et al, Light-directed, spatially addressable parallel chemical synthesis, *Science*, **251**, 767-773, 1991.

16. Millan, K.M., and Mikkelsen, S.R., Sequence-selective biosensor for DNA based on electroactive hybridization indicators, *Anal. Chem.*, **65**, 2317-2323, 1993.

17. Zubay, G., Biochemistry 2nd Edition, MacMillan, 1988.

18. Sze, S.M., Physics of semiconductor devices, Wiley Inter Science, New York, 1981.

19. Bootsma, G.A., De Rooj, N.F., Van Silfhout, A., *Sensors and Actuators*, **1**, 111-136, 1981.

20. Bousse, L., De Rooj, N.F., Bergveld, P., *IEEE Transactions on Electron Devices*, vol ED-**30**, 10,1263-1270, 1983.

21. Kittel, C., Introduction to Solid State Physics, 7th, Wiley Inter Science, New York, 1996.

22. Bockris, J. O'M., Reddy, A.K.N., Modern Electrochemistry, voll-2, Plenum Press, 1970.

23. Sze, S.M., Physics of semiconductor devices, Wiley Inter Science, New York, 1981.

24. Sze, S.M., Physics of semiconductor devices, Wiley Inter Science, New York, 1981.

25. Nagasubramanian, G., Wheeler, B.L., Fan, F., and Bard, A.J., *J. Electrochem.*, **129**, 1742, 1982.

26. Weetall, H.H., *Appl. Biochem. Biotech.*, **41**, 157-188, 1993.

27. Iler, R.K., The Chemistry of Silica, *John Wiley & Sons*, New York, 1979.

28. Iler, R.K., The Chemistry of Silica, *John Wiley & Sons*, New York, 1979. pg 629.

29. Leyden, D.E., Chemically Modified Surfaces, volume 1 "Silanes, surfaces and interfaces", Gordon & Breach Science Publishers, 1990.

30. Caravajal, G.S., Leyden, D.E., Quinting, G.R., Marciel, G.E., *Anal. Chem.*, **60**, 1776-1786, 1988.

31. Keller, G.H., Cumming, C.U., Huang, D.P., Manak, M.M., Ting, R., *Ana/. Biochem.*, **170**, 441-450, 1988.

32. Kallury, K.M.R., MacDonald, P.M., Thompson, M., *Langmuir*, **10**, 492-499, 1994.

33. Pluedemann, E.P., Silane Coupling Agents, 2nd edition, Plenum Press, 1991.

34. Maskos, U., Southern, E.M., *Nucleic Acid Research*, **20**, 1679-1684, 1992.

35. Maskos, U., Southern, E.M., *Nucleic Acid Research*, **21**, 2269-2270, 1993.

36. Southern, E.M., Case-Green, S.C., Elder, J.K., et al, *Nucleic Acid Research*, **22**, 1368-1373, 1994.

37. Lamture, J.B., Beattie, K.L., Burke, B.E., Eggers, M.D., Erlich, D.J., Fowler, R., Hollis, M.A., et al, ., *Nucleic Acid Research*, **22**, 2121-2125, 1994.

38. Beattie, K.L., Eggers, M.D., Shumaker, J., Varma, R., Lamture, J.B., Hollis, M.A., Erlich, D.J., Rathman, D., *Clinical Chemistry*, **39**, 719-722, 1993.

39. Tripp, C.P., Hair, M.L., *J. Phys. Chem.*, **97**, 5693-5698, 1993.

40. Blitz, J.P., Shreedhara Murthy, R.S., Leyden, D.E., *Journal of Colloid and Interface Science*, **126**, 2, 387-392, 1988.

41. Henry de Villeneuve, C., Pinson, J., Bernard, M.C., Allongue, P., *J. Phys. Chem. B*, **101**, 14, 2415-2419, 1997.

42. Higashi, G.S., Chabal, Y.J., In Handbook of Semiconductor Cleaning Technology, Park Ridge, 1993.

43. Maniatis, Sambrook, Fritisch, Molecular Cloning, a laboratory manual, 1987.

44. Caravajal, G.S., Leyden, D.E., Quinting, G.R., Marciel, G.E., *Anal. Chem.*, **60**, 1776-1786, 1988.

45. Keller, G.H., Cumming, C.U., Huang, D.P., Manak, M.M., Ting, R., *Ana/. Biochem.*, **170**, 441-450, 1988.

46. Tripp, C.P., Hair, M.L., *J. Phys. Chem.*, **97**, 5693-5698, 1993.

47. Blitz, J.P., Shreedhara Murthy, R.S., Leyden, D.E., *Journal of Colloid and Interface Science*, **126**, 2, 387-392, 1988.

48. Southern, E.M., Case-Green, S.C., Elder, J.K., et al, *Nucleic Acid Research*, **22**, 1368-1373, 1994.

49. Cloarec J.P., Thesis, Concordia University, 1997

50. Maniatis, Sambrook, Fritisch, Molecular Cloning, a laboratory manual, 1987.

51. Meinkoth and Wahl, *Anal. Biochem.*, **138**, 267, 1984.

52. Williams, R.A., and Blanch, H.W., *Biosens. Bioelec.*, **9**, 159-167, 1994.

53. Meinkoth and Wahl, *Anal. Biochem.*, **138**, 267, 1984.

54. Henry de Villeneuve, C., Pinson, J., Bernard, M.C., Allongue, P., *J. Phys. Chem. B*, **101**, 14, 2415-2419, 1997.

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