Interfacial Oligonucleotide Chemistry Studied by 
an On-line Biosensor, Radiochemical Labelling 
and Nucleic Acid Microarrays 

by 

Linda Michelle Furtado 

A thesis submitted in conformity with the requirements 

for the degree of Doctor of Philosophy in the 

Department of Chemistry 

University of Toronto 

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Interfacial Oligonucleotide Chemistry Studied by an On-line Biosensor, Radiochemical Labelling and Nucleic Acid Microarrays


Linda Michelle Furtado

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ABSTRACT

This thesis presents the application of the thickness shear-mode (TSM) acoustic wave sensor to the study of interfacial oligonucleotide interactions. Radiochemical analysis of the TSM devices was performed and contrasted with confocal microscope analysis of the system immobilized onto glass slides.

Initial studies involved the investigation of HIV-1 TAR RNA and Tat peptide interaction. A 31-base sequence of the TAR RNA was immobilized to the TSM surface and challenged with a 12-amino acid peptide derived from the Tat protein such that the binding sites of both were incorporated. This was studied using both $^{32}\text{P}$-labelled TAR RNA and $^{125}\text{I}$-labelled peptide and compared with previously obtained data from the TSM sensor. The results showed that the TSM sensor is sensitive to actual TAR RNA/ Tat peptide binding interactions occurring at the interface; nonspecific binding of peptide was verified by radiochemical experiments, however, this was not observed with the TSM sensor.

The hybridization of a biotinylated 25-mer oligonucleotide probe with complementary, non-complementary and single-base mismatch 25-mer
oligonucleotide targets at the liquid-solid (neutravidin-modified) interface of a thickness-shear mode acoustic wave device was studied. Under ambient temperature conditions, different signals were obtained for the complementary and non-complementary cases. For non-complementary interactions, the system exhibits behaviour characteristics of the production of intermediate duplexes, which are decomposed by the re-introduction of buffer solution. The use of higher temperatures has the potential to permit the distinction of binding events involving a set of single-base mismatch 25-mers. The different responses observed were dependent on both the nature and the location of the instigated mismatch.

Regeneration of the probe-modified surface was achieved using λ-exonuclease, an enzyme that digests a single strand of double-stranded DNA starting from the 5'-end, cleaving single nucleotides as it progresses. Thus, with the 5'-end of the probe strand inaccessible through the biotin-neutravidin linkage, the target strand was effectively removed by λ-exonuclease digestion. This was confirmed by a series of radiochemical experiments involving oligonucleotides modified with 32P.

Investigation was conducted into applying this to high density oligonucleotide arrays. Fluorophore-labelled oligonucleotide probes were covalently attached to silanized glass slides via a disulfide linkage. The slides were treated to fluorophore-labelled targets of complementary and non-complementary sequences and λ-exonuclease digestion. The slides were scanned using a confocal microscope to verify these interactions. It was shown that this method of regeneration could be applied to microarray technology.
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I. INTRODUCTION

Recent years have seen enormous advances in our understanding of molecular genetics and genomics. Such comprehension is particularly important when it comes to the treatment of disease, whether of the genetic or infectious type. Analysis of gene sequences and an appraisal of gene regulation play crucial roles in both molecular diagnostics and in drug discovery. The detection of pathogens and genetic mutations at the molecular level creates the possibility of identification of a disease before any symptoms appear. Accordingly, the introduction of new pharmaceutical products, which are based on interaction with the gene expression system, have grown significantly especially with regard to antisense therapy and nucleic acid-protein complex - small molecule interactions.

Methods for the determination of gene sequences are based on conventional direct sequencing or on oligonucleotide-to-ssDNA hybridization. With regard to the latter, the target gene sequence is identified by an oligonucleotide or ssDNA probe that can form a duplex with its complementary nucleic acid. The formation of the latter is confirmed by radiochemical (e.g. $^{32}$P) or fluorescent labelling. This protocol has become widespread in the identification of genetic disease, for example, cystic fibrosis, and of viruses and bacteria. Despite these advances, there are a number of problems that are largely associated with the use of tagging agents. In this respect, even the advent of the so-called Gene Chip $^{TM}$, where the hybridization experiment is multiplexed, does not avoid the use of labels. Sequencing by hybridization in this case
employs fluorescent probes with detection by confocal microscopy. Moreover, in terms of drug discovery it is difficult to envisage incorporation of an analogous multiplexing strategy in the high-throughput screening of oligonucleotide-small molecule interactions. Accordingly, there is a genuine need for a continuous, label-free configuration for the detection of both nucleic acid hybridization and DNA-small molecule/protein interactions.

The architecture of a biosensor that is capable of signalling interfacial nucleic acid chemistry (the genosensor) consists of single or double strands of oligonucleotide, DNA or RNA attached to the surface of a transducer. Such structures have been the subject of a recent review by Yang et al.4 The research presented here involves transducers based on the thickness-shear mode (TSM) acoustic wave sensor. The TSM consists of a disc of piezoelectric material with metal electrodes on each face that can generate a standing transverse acoustic wave between opposite faces at ultrasonic frequencies. When exposed to a liquid these shear waves are coupled to the external medium through particular boundary conditions at the liquid-solid interface. Interestingly, in the present case, the oligonucleotide probe is situated in precisely the location where such coupling occurs.
1. NUCLEIC ACID CHEMISTRY

a. Structure of Nucleic Acids

Nucleic acids are comprised of pentose sugars, nitrogenous bases and phosphate. The bases for deoxyribonucleic acid (DNA) are the purines adenine (A) and guanine (G) and the pyrimidines cytosine (C) and thymine (T). Similarly, for ribonucleic acid (RNA), the bases are adenine, guanine, cytosine and uracil (U) (Figure 1.1.a). Another significant difference between DNA and RNA is that the pentose sugar for RNA is ribose and the sugar for DNA is 2'-deoxyribose (Figure 1.1.b). The bases are attached to the pentose sugars at the C1' position. Nucleic acid macromolecules are created by linking the sugar components via a phosphate group between the 3'-hydroxyl of one and the 5'-hydroxyl of the next (Figure 1.2).

b. The Double Helix

Watson and Crick rationalized the three-dimensional structure of B-DNA in 1953 by extracting information from X-ray diffraction photographs taken by Franklin and Wilkins. The double helix structural model they presented immediately implied the mechanism of replication of DNA. They deduced that DNA molecules are double-stranded moieties in which two opposing strands twist around each other and a common axis. The resulting helical structure has hydrogen bonding between adenine and thymine and between guanine and cytosine on the opposing strands (Figure 1.3). Thus, A and T and G and C are termed complementary base pairs. The specificity of
a) Bases

[Diagrams of Adenine (A), Guanine (G), Cytosine (C), Uracil (U), Thymine (T)]

Bases are attached to carbon 1 of the sugar moieties via nitrogen 9 of purines and nitrogen 1 of pyrimidines.

b) Sugars

[Diagrams of Ribose and Deoxyribose]

Figure 1.1. Base and sugar components of nucleic acids. Bases are attached to carbon 1 of the sugar moieties via nitrogen 9 of purines and nitrogen 1 of pyrimidines.
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base pairing in DNA results in the important consequence that the sequence of bases on one strand immediately infers the sequence of bases on the other. The paired bases are internal to the helix with the phosphate-deoxyribose chains creating the helical backbone. In contrast, most RNA molecules are single-stranded with double-stranded regions due to self-complementary segments of the single strand folding back on itself.

The primary structure is determined by the covalent bonds between adjacent nucleotides. The secondary structure of DNA - the double helix - is stabilized by a number of forces. Individually these forces are weak enough to allow conformational flexibility but are co-operatively strong enough to maintain a stable structure. Firstly, there are the hydrogen bonds between complementary bases. The bases are aromatic; resonance involves the π-orbitals of the C=O bonds and the p-orbital of the associated lone pair of cyclic nitrogen. These π- and p- orbitals become connected and all become involved in a single resonant π-orbital system. The resonance structure results in charge separation. Thus, in spite of being aromatic and uncharged, the bases are highly polar, allowing strong electrostatic, non-covalent interactions. This is very important when it comes to base-pairing. Aromatic systems are highly rigid and thus have reduced entropy costs of making a specific interaction with another molecule (relative to a non-rigid molecule), as there are no competing conformations. The lone-pair on the ring nitrogen is oriented away from the edge of the ring - an ideal arrangement for an H-bond acceptor, having no local interference. The lone pair on the exocyclic NH₂ is not available for H-bonding, yet, contributes to polarization of the ²N-H bonding. This, in turn, makes this a good H-bond donor. The H-bond donor on the exocyclic NH₂ is also parallel to the lone pair on the ring nitrogen. When this
interacts with another system in which the H-bond donor and acceptor are parallel, the entropy cost of making a second H-bond when a first one has already been made is enhanced. This co-operativity results from a reduction in entropy cost associated with the interacting pairs. For bases having exocyclic oxygen atoms involved in hydrogen bonding, the sp² orbitals are oriented in a plane 120° from the C=O bond making one parallel and in the same plane as the H donors and acceptors previously discussed, therefore, making the third H-bond of G:C pairs even lower in entropy cost. Although, the concentration of water in a living cell is ~40M, nucleotides will selectively bind to each other due to co-operativity. When nucleotides are hydrated by water, the enthalpy cost is proportional to the number of hydrogen bonds involved. Whereas, in double-stranded DNA, there is a high enthalpy cost due to the first hydrogen bond and less so for subsequent hydrogen bonds. The base pairs are similar in dimension and fit into the internal helical space in a like manner. There are also hydrophobic effects created by burying the planar, hydrophobic purine and pyrimidine bases within the helix. With the close packing of the base pairs, water is excluded from the interior of the helix resulting in a stable, hydrophobic environment. Precluding the bases from interaction with the external aqueous environment increases the stability of the helix. Weak van der Waals forces and dipole-dipole interactions add to the stability of the DNA structure through the stacking of neighbouring bases of the same strand on top of each other causing the planar faces of the bases to interact with each other in a complex way. These are referred to as stacking forces. The dipole moments of the bases also contribute to the anti-parallel interaction of opposing strands. The bases have large dipole moments and, where two bases are interacting,
the charge interactions are strongest when the dipole moments are aligned in the same plane. The self-pairing of bases results in unfavourable anti-parallel dipole interaction. Thus, DNA is rarely parallel stranded because the dipole-dipole-interactions would be unfavourable. The potential charge repulsion of the negatively charged sugar-phosphate backbone is compensated through interactions with metal cations and positively charged side chains of amino acids in proteins. This stabilizing effect is often referred to as the *charge effect*.

c. Stability of Nucleic Acids

A number of factors may disrupt the noncovalent forces holding the two strands together in the double helix conformation. When the two complementary strands of double-stranded nucleic acid are separated, the nucleic acid is considered *denatured*. Total denaturation of nucleic acids does not occur *in vivo* due to the carefully regulated environmental conditions in which the nucleic acids exist; however, it is common for local denaturations to occur during DNA replication, transcription and repair. Of course, *in vitro* it is possible to manipulate the environment of nucleic acids to such an extent that total separation of strands does occur.

For example, *pH* can be manipulated to denature the double strand by altering the ionization state of bases, sugars and phosphates. The charge repulsion between base pairs increases with increasing *pH* and, at values above ~11-12 becomes so great that it causes the two strands to separate. This is an effective means of both DNA and RNA denaturation, though, for RNA this must be carried out under very mild conditions in order to avoid hydrolysis of the RNA sugar-phosphate backbone.
the pH is lowered to below ~3, denaturation occurs as the result the purine-deoxyribose linkage being broken and the consequent release of bases. This is referred to as depurination. It is thus apparent that this method is not useful for experimental work requiring that the single strands remain intact.

Denaturation is commonly achieved by increasing the temperature to the level where all of the hydrogen bonds and stacking interactions are disrupted. The process of denaturation by this method is termed melting. This phenomenon can be observed by monitoring the absorption of UV radiation. There is a hyperchromic shift in the absorption of UV radiation as the double strand is melted due to the higher absorptivity of single-stranded nucleic acids relative to double-stranded nucleic acids. The observed increase in absorbance is directly related to the extent of denaturation.

d. Factors Affecting Nucleic Acid Hybridization

Hybridization experiments are designed with a specific target sequence in mind; in other words, this is the nucleic acid sequence that is being analyzed for its presence in a sample. With this in mind, probes are designed (oligonucleotides) or extracted (DNA of known sequence). The probes are often attached to a surface and exposed to a solution containing a sample of target oligonucleotides or mixture of DNA. For filter hybridizations, the target is attached to the filter and the probes are in solution but, for the purpose of this section, we will use the terms probes and targets as they apply to the first example.

It is important to consider the stability of the hybrids when designing nucleic acid hybridizations. Oligonucleotide hybrids are much less stable than hybrids of long
nucleic acids. This is reflected in lower melting temperatures. In addition, there are a
time number of factors which contribute to the stability of the chosen oligonucleotide
hybrid. These include the mismatches, rate of hybridization, and salt concentration.

The melting temperature, or \( T_m \), of oligonucleotide hybrids is defined as the
temperature at which 50% of the hybrids have dissociated. This is the point at which
equilibrium is achieved between dissociation and association of the hybrid. The
melting temperature is dependent on the length, base composition and base sequence
of the oligonucleotide duplex. The first two factors are more obvious in their effects -
the \( T_m \) is proportional to the length because \( T_m \) is a factor of the energy required to
break the interstrand interactions of the hybrid and \( T_m \) is a factor of the base
composition because G:C base pairs require more energy to dissociate than do A:T
base pairs. The relationship between base sequence and \( T_m \) is due to the stacking
forces between neighbouring bases. This factor is more significant in a very short
(11-23nt) molecule than in a long one where the stacking forces due to sequence is
more evenly distributed. For short oligonucleotides in 1M salt and for perfectly
matched hybrids, the \( T_m \) can be estimated using the relationship:

\[
T_m = 4 \left( G+C \right) + 2 \left( A+T \right)
\]

where \( T_m \) is the temperature (°C) at which 50% hybrids have dissociated and G, C, A,
and T are the number of guanine, cytosine, adenine and thymine bases, respectively, in
the oligonucleotide. For longer oligonucleotides, the relationship is replaced by:6
\[ T_m = 81.5^\circ C + 16.6 (\log_{10}[M]) + 0.41 (\%G+C) - 600/L \] (2)

where \( M \) is the molar concentration of the monovalent cation (e.g. NaCl), (\%G+C) is the content of guanine and cytosine residues and \( L \) is the length in nucleotides of the oligonucleotide. This relationship is applicable for oligonucleotides of 14 to at least 72nt. The above equations are useful for giving an estimate of melting temperatures, but the \( T_m \) found empirically is often higher by up to 10°C than that calculated. This variability has been suggested to arise through one of the strands of the hybrids being immobilized.\(^5\) For the best results, oligonucleotide hybridizations are generally carried out at 5-10°C below the \( T_m \).\(^6\) This permits the formation of perfectly matched hybrids while selecting against hybrids containing mismatches.

For systems involving oligonucleotide probes of up to about 200nt, the effect of base composition on \( T_m \) can be suppressed by incorporating quaternary ammonium salts into the hybridization mixture. Suppression is achieved through the selective binding of these salts to A:T base pairs and the subsequent prevention of melting at low temperature. Thus, the \( T_m \) of hybrids becomes proportional to length only and independent of base composition. For probes greater than 200nt, the \( T_m \) is already essentially independent of base composition. The type of salt added will also affect the magnitude of change to the \( T_m \). It is also important to note that there is no benefit to including quaternary ammonium salts for probes of up to about 16nt. The most significant use for this method of adjusting \( T_m \) is when several oligonucleotides probes are used at the same time.\(^6\) In this situation, there are several different \( T_m \)'s and it becomes difficult to decide at which temperature to carry out hybridization. By
removing the effect of base composition, the T_m for each probe becomes dependent only on the length and can be found from published sources\(^7\&\) of T_m values. The starting point for the optimization of the incubation temperature, T_i, is about 10°C lower than the calculated T_m.

The thermal stability of short oligonucleotide hybrids are particularly affected by mismatches.\(^9\) It can be estimated that for every 1.7% mismatching in the hybrid, the T_m is lowered by 1°C.\(^10\) Even so, it is apparent that the position, type and number of mismatches factor into the magnitude of the effect of mismatches on the T_m. In bulk solution, the T_m is reduced to a greater extent if the mismatch is at the center of a hybrid than if it is at the end. The type of mismatch affects the stability and thus the T_m because some mismatch pairs are more stable than others. The order of stability of mismatch pairs, from most to least stable and in relation to T:A or A:T base pairs, is T:A, A:T > G:T, G:A > A:A, T:T, C:T, C:A.\(^11\) For hybrids with more than a single mismatch, the hybrid is more stable if the mismatches are clustered than if they are dispersed over the length of the hybrid.

For short oligonucleotide hybrids (less than ~20nt in length), the T_m can be lowered by as much as 10°C by a single mismatch.\(^9\) The difference in thermal stability between the fully complementary hybrid and one with a mismatch can be utilized to detect mutations.\(^12\)

The rate of hybridization should be considered when performing hybridization experiments. For short oligonucleotide probes and targets and a single type of probe, only short periods are necessary. This is possible when the targets are in high concentration when applied to the probes attached to the surface. As well, diffusion of
these relatively small targets to the probe is rapid and the targets should not reassociate in solution, thus, there is no competing reaction in solution to reduce the concentration of the probe. Lastly, being short lengths of oligonucleotides and a single sequence of probe, the complexity is low. For situations incorporating mixtures of oligonucleotides, the complexity and, thus, the time required for hybridization both increase according to the number of different probes.

The hybridization of oligonucleotides follows pseudo-first order kinetics so the time required for half completion of the reaction can be calculated from the following equation:  

$$t_{\frac{1}{2}} = \frac{N \ln 2}{3.5 \times 10^5 \times L^{0.5} \times C_o}$$  

(3)

where \(t_{\frac{1}{2}}\) is the time(s) required for the reaction to be 50% complete, \(N\) is the complexity of the probe, \(L\) is the length of the probe in nucleotides and \(C_o\) is the concentration of the probe (mol. nucleotide L\(^{-1}\)), note concentration expressed in terms of the number of strands, not nucleotides. This relationship is applicable for oligonucleotides as short as 11nt. It should be noted that the actual rate of hybridization on surfaces is slower than the rate calculated from the above equation.

It is also apparent that the thermal stability of oligonucleotide hybrids are dependent on the salt concentration in the hybridization solution (Equation 2). However, this factor is not generally utilized to manipulate the \(T_m\) of hybrids.
e. DNA-Protein Interactions

There has been considerable study of DNA-protein interactions. Both structure and sequence are factors in DNA-protein interactions. Proteins which bind to DNA can be classified according to function:

1) regulatory proteins - these proteins participate in the general initiation of transcription through binding at particular signal sequences, but, for the most part, they are involved in controlling the processing of a particular gene through interaction with dsDNA at highly specific sequences;

2) DNA cleavage proteins or nucleases - this group of proteins include examples which exhibit high sequence specificity (restriction enzymes) and examples which exhibit low sequence specificity (DNase I);

3) repair proteins - these proteins are involved in the recognition and excision of DNA lesions resulting from various types of damage and may also incorporate proteins involved in joining together the breaks in damaged DNA strands;

4) proteins which unwind or unravel DNA prior to replication in order to rectify topological problems;

5) structural proteins - these provide scaffolding for folded or packaged DNA in order to maintain their integrity, an example of this type of protein would be histones in chromatin;

6) processing proteins - this group uses DNA as a template for nucleic acid synthesis, as exemplified by the polymerases; these proteins do not utilize sequence-specific recognition but instead require a specific structural form of dsDNA.
For proteins and nucleic acids to interact, there must be a loss of Gibbs free energy on the formation of a complex. Gibbs free energy ($G$) is dependent on both entropy ($S$) and enthalpy ($H$) according to the relationship:

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

The enthalpy is related to the many short-range, non-covalent interactions involved in the complex formation. The entropy, however, is dependent on solvent interactions with the protein and nucleic acid before complexation and the complex after its formation, particularly at the interacting surfaces of the two molecules. For example, the interaction of protein and nucleic acid may result in a significant number of ordered water molecules being displaced on complexation, contributing to a positive change in the entropy term (which decreases the Gibbs free energy). The recognition process determines whether or not the interaction is favourable and can be divided into two components. The first concerns the global architecture of the protein in relation to the target nucleic acid and is referred to as shape recognition. The arrangement of secondary structure elements determines the shape of the interacting surfaces and, thus, shape recognition. Helices and helix-turn-helix elements have been shown to be particularly important for protein docking due to the numerous contacts to sugar phosphate which are possible from amino acids. The $\alpha$-helix is also especially prevalent as a major groove interaction element. Since DNA structures are considered to be essentially uniform, diverse DNA-binding proteins employ similar architectural strategies to achieve interfaces which are complementary in shape. This
is not necessarily the case with RNA due to the various shapes - loops and bulges - which it can incorporate. The second component is chemical recognition at an atomic level and is determined by the stereochemical arrangement of amino acid side chains on the interacting surface of the protein. Hydrogen bonds, van der Waals forces, hydrophobic interactions, global and local electrostatic interactions contribute to the aspect of chemical recognition.\textsuperscript{13,15} It appears that the orientation of the protein for specific contacts in the major groove of DNA are facilitated by contacts with the phosphate backbone. There are also common features among DNA-binding proteins in which certain amino acids are known to make contacts with individual bases (i.e. both arginine and glutamine are able to make bidentate contacts to individual bases).

A single binding site does not generally have an adequate number of contacts with the nucleic acid to have sufficient specificity and affinity for a unique target site. This problem is addressed by incorporating more recognition elements by adding arms or tails which recognize other features of the nucleic acid (particularly in the minor groove of DNA), doubling up on recognition elements through hetero- and homo-dimerization and increasing the number of nucleic-acid binding domains through tandem repeats of a single binding motif or coupling different types of motif.\textsuperscript{13,15}

Protein and nucleic acid binding interactions have largely been considered from the perspective of the protein recognizing the nucleic acid. This perspective neglects the possible significance of the structural diversity of the target sites. Depending on the local base stacking (determined by base sequence), the degree of hydration can induce the double helix to adopt discrete conformations. These conformations are characterized by the dimensions of both the minor and major grooves, the
displacement and orientation of base pairs in relation to the helix axis, the helical periodicity and the global bend of the double helix. Since these characteristics are determined by the base sequence, the structure and flexibility of the double helix is continuously variable and must, therefore, be significant in the recognition of proteins. The two most commonly encountered conformations are the A-form (RNA) and B-form (DNA). The A-form is characterized by a very deep, narrow major groove and a very shallow, wide minor groove. This allows easy access for protein interaction with the minor groove. However, the B-form is characterized by a quite deep, wide major groove and a deep, narrow minor groove. The major groove is much wider than the minor groove and more easily accommodates protein secondary structure. The pattern of hydrogen bonds in the major groove is unique for each base pair, whereas, the hydrogen bonds in the minor groove is not. Therefore, the major groove of the B-form is best tailored for sequence-specific interactions. Proteins interact with both the base pairs and the phosphate backbone permitting both direct and indirect recognition of the base sequence. The importance of nucleic acid conformations in the recognition process is evident in that sometimes bases are highly conserved between different targets of the same protein. However, in other cases they are not. For some proteins the ability to distort the nucleic acid is essential to the binding event; for these cases, the flexibility of the nucleic acid is important.

The role of solvent is not yet well-defined, however, it is known that there are ordered water molecules present at the protein-nucleic acid interface. The number and role of these molecules also differ from complex to complex - water molecules may contribute to the structure of the nucleic acid, reducing the polarity of the target site,
and/or participate in fast exchange with the bulk solvent at the protein-nucleic acid interface. It has been suggested that the protein specifically recognizes the associated water network in addition to the structure of the nucleic acid. As mentioned previously, water molecules may also have an important role in the thermodynamics of protein-nucleic acid binding event. Displacement of a number of water molecules at the binding sites of the protein and nucleic acid occurs upon complexation. Complex formation may be favoured by the resulting positive change in the entropy term (i.e. $\Delta G$ becomes more negative). In addition, the water molecules which remain at the interface engage in a network of hydrogen bonds which may also contribute favourably to the enthalpy term (i.e. negative $\Delta H$ contributes favourably to $\Delta G$).

In spite of all that is known about protein-nucleic acid interactions, there is no defined recognition code. It has been proposed that certain amino acids are employed to contact certain base pairs. However, with the great variety of uses for each amino acid, the possibility of predicting the contacts involved becomes exceedingly complicated. For members of the same family of proteins, patterns of contacts emerge and it is, thus, possible to make predictions based on family characteristics. Another limitation to the derivation of a definitive recognition code is that the roles of nucleic acid structure and hydration are not well-understood and, hence, are difficult to predict.
As previously mentioned, proteins participate in a number of functions with respect to nucleic acids. DNA exonucleases are involved in replicating, repairing and recombination of DNA.\textsuperscript{16} Lambda ($\lambda$) exonuclease is a 24kDa protein encoded by bacteriophage $\lambda$.\textsuperscript{17} This enzyme is essential for phage DNA recombination through the double-stranded break repair and single-stranded annealing pathways.\textsuperscript{18-23} $\lambda$-Exonuclease binds a free end of double-stranded DNA and digests one of those strands in the 5' to 3' direction. This enzyme is highly processive (>3000bp) and releases 5' mononucleotides and the non-hydrolyzed complementary single-stranded DNA (ssDNA),\textsuperscript{16,24} digesting the one strand at a rate of ~12nt per second.\textsuperscript{27} The ssDNA that is released has a free 3'-hydroxy end which is thought to interact with the lambda $\beta$ protein to initiate recombination.\textsuperscript{28}

Recent investigation resulted in the elucidation of the crystal structure of $\lambda$-exonuclease.\textsuperscript{29,30} It was found that this enzyme consists of three subunits that form a toroid, with a tapered channel passing through the middle. From the elucidated structure, it is apparent that the wide end of the channel (diameter of ~30 Å) is large enough to accommodate dsDNA but the narrow end of the channel (diameter of ~15 Å) can accommodate only ssDNA. This coincidence of dimensions implies that the enzyme encloses its substrate, threading the dsDNA into the center of the toroid, with the ssDNA product exiting out the back of the tapered enzyme channel (Figure 1.4.). The enzyme remains strung on the non-digested strand of DNA, once cleavage has begun, translocating along the dsDNA until the protein reaches the terminus of the DNA or the protein dissociates into monomers.\textsuperscript{31}
The confined digestion of one of the two DNA strands is explained by this proposed structure of the λ-exonuclease-DNA complex. The three subunits of the trimer are aligned parallel to the long axis of the dsDNA. This results in the correct alignment of the active sites with respect to only one strand of the dsDNA because the active sites face the other strand in the opposite orientation.\(^9\)

It has been well-established that this enzyme has a preference for a phosphorylated 5' end over a hydroxylated 5' end. Further research of the interaction of this enzyme with the ends of DNA has been carried out by Mitsis and Kwagh.\(^{12}\) They found that the enzyme actually protects ~13-14 bp of the double-stranded DNA but little, if any, of the nucleotides of the single-stranded DNA product. They were also able to show that the \(k_{\text{cat}}\) varies with different end structures, increasing in the order 5' overhang \(\ll\) blunt \(\ll\) 5' recessed. However, by finding that \(k_{\text{cat}}/K_m\) remains relatively constant, they suggested that these end structures may be equally efficient as substrates for λ-exonuclease. Even so, when the 5'-end is recessed by more than 100 nt, the activity of the enzyme is inhibited.
Figure 1.4. The structure and interaction of λ-exonuclease with DNA, as proposed by Kováč and Matthews. The threefold axis of the protein and the long axis of the DNA coincide. The toroidal structure accommodates dsDNA at one end of the tapered channel but only ssDNA at the other.
The human immunodeficiency virus type 1 (HIV-1) is strongly regulated at the transcriptional level by the interaction of the transcription activation (Tat) protein with the trans activation-responsive element at the 5'-end of the viral messenger RNA transcript (TAR).\textsuperscript{33-36} The highly conserved TAR element is composed of a 69-nucleotide RNA which incorporates a trimucleotide bulge region and a six-base loop.\textsuperscript{37} The Tat protein is an essential protein involved in the propagation of HIV-1. It is an 86 amino acid protein which is encoded by the HIV-1 gene. The primary structure of Tat can be divided into an acidic region, a cysteine-rich region and a basic region.\textsuperscript{38,39} The cysteine-rich domain contains a binding site for two metal ions\textsuperscript{40} and, along with the acidic domain, is essential for the transcription-activating function of Tat. The basic motif, situated toward the C-terminus mediates specific binding to TAR RNA.\textsuperscript{41} When Tat binds to TAR, it activates transcription of RNA. Transcription of the gene results in the production of Tat which serves to activate further transcription. Thus, the TAR-Tat binding interaction establishes a strong feed-forward loop resulting in both massive induction and further propagation of the HIV-1 gene.\textsuperscript{42} Much has been learned about the structural aspects from basic biochemical studies and from NMR spectroscopy with respect to this specific interaction,\textsuperscript{43-45} which is crucial to the activation process.\textsuperscript{37,46-53}

The UCU bulge of TAR has been found to be an important molecular recognizable site for the Tat protein.\textsuperscript{54} Deletion of this bulge results in the loss of the binding affinity of TAR for the protein and, consequently, transcription activation.
These three unpaired pyrimidines are responsible for widening the major groove - access to the major groove being a significant element of nucleic acid-protein interaction, in a general sense.\textsuperscript{37,50,53} This makes the major groove more accessible to other molecules and allows for specific interaction between the trinucleotide bulge bases and the base pairs on either side of the bulge with the Tat basic domain.

Electrostatic forces derived from phosphate residues and positively charged sites on the protein further stabilize the TAR-Tat combination.\textsuperscript{37,47-50}

The TAR RNA-Tat system has attracted much attention in drug discovery research.\textsuperscript{45} In fact, a number of small molecules which block the binding of Tat to the TAR RNA have been developed.\textsuperscript{56-62} A key issue with regard to the characterization of binding events between the RNA and Tat, peptides, and small molecules is the choice of a technique capable of signaling the appropriate chemistry. Accordingly, this research presents the use of an on-line thickness shear mode (TSM) transverse acoustic wave device for the detection of nucleic acid-ligand interactions that avoids a number of these problems. This method, described in sections 4 and 5, is based on transverse acoustic waves which are launched into a substrate at ultrasonic frequencies. When exposed to liquid, coupling at the liquid-solid interface results in the propagation of acoustic energy into the external medium. A particularly interesting feature of the physics of this process is that receptors, such as oligonucleotides, are situated in precisely the location where acoustic coupling occurs.
Figure 1.5. (a) Secondary structure of HIV-1 TAR RNA for positions +1 through 57. The RNA moiety is divided into sections as follows: four stem regions, I (bases 5-9 and 50-54), II (bases 10-15 and 44-49), III (bases 17-21 and 29-43), IV (bases 25-28 and 35-38); a three-base pyrimidine bulge; and six-member loop; and unpaired nucleotides. (b) Chemically synthesized biotinylated RNA with the three-base pyrimidine bulge is comprised of 31 nucleotides.
Figure 1.6. Schematic of HIV Tat-1 protein (a) and primary sequence of Tat (b). Arrows indicate sequences of chemically synthesized Tat₁₂. In this peptide, position 58 (proline) is replaced by glycine.
2. DNA BIOSENSORS

The definition of a biosensor is "an analytical device that combines the specificity of a biological sensing element with a transducer to produce a signal proportional to target analyte concentration". This signal may be produced in response to changes in a variety of parameters, such as proton concentration, absorption, heat/light emission, reflectance, etc. The transducer then converts this signal into a response which can be measured as, for example, current, potential, light absorption, etc.

Of all biomolecules, it may be contended that deoxyribonucleic acids are the most significant. As described in section 1.a., DNA has a unique complementary structure, with the bases adenine and guanine pairing with thymine and cytosine, respectively. This characteristic of DNA has been the basis for genetic analysis. DNA-based detection methods are based on the fact that single strands of DNA, in effect, seek out their complementary sequence and hybridize with it.

Recent advances in nucleic acid research and work on the Human Genome Project have instigated interest in the development of real-time detection of hybridization in a biosensor format. This has been explored by attaching DNA to the surface of a transducer and allowing complexes to form with target strands, drugs or proteins in solution phase resulting in the generation of a device response. Nucleic acid hybridization sensors have been investigated using optical, electrochemical, and acoustic wave technologies.

It should be noted that, though these devices may be operated in solution, the
Hybridization on a sensory surface is a solid-phase reaction. Hybridization in solution occurs at a much greater rate than hybridization on solid supports. However, the kinetics and mechanism of hybridization at the solid-liquid interface are not yet well-understood. This is partially due to the wide variety of methods used for measurement and the difficulty in predicting the process from theory because the exact amount of immobilized probe is not known. In addition, the issue of strand association between strands immobilized at a solid support and strands free in solution is not fully understood, particularly when, by some methods, it is not known how many attachments exist between the immobilized DNA and the solid support. If there are more than one points of attachment, the strand may be inaccessible for hybridization.

a. Optical Devices

Optical techniques involve the use of fiber optics, surface plasmon resonance, biomolecular interaction analysis and Raman spectroscopy.

Piumno et al. were the first to develop the use of an optical fiber for direct detection of DNA hybridization. Single-stranded thymidyl acid icosanucleotides (dT$_{20}$) were grown onto the surface of optical fibers through a covalent 1,10 decanediol bissuccinate spacer arm terminated with 5'-O-dimethoxytrityl-2'-deoxothymidine. The optical fiber surfaces were first derivatized using γ-aminopropyltriethoxy-silane (APTES). The immobilized dT$_{20}$ oligomers were able to hybridize with complementary dA$_{20}$ oligomers. This interaction was detected by the use of a fluorescent molecule, ethidium bromide, which intercalates between bases of
dsDNA but does not associate with ssDNA. The authors reported a detection limit of 86 ng·mL\(^{-1}\) and linearity to 800 ng/mL, with an analysis time of 46 min by the non-optimized procedure. The sensor was shown to be reproducible, renewable and to have a long lifetime. The sensitivity of ethidium bromide may reach 5 to 50 ng DNA under ideal conditions. The major disadvantages of this method are that ethidium bromide is extremely harmful and must be used with considerable care and that byproducts of nucleic acid synthesis remain on the fiber surface along with the desired complete nucleic acid sequence.

Surface plasmon resonance (SPR) devices employ an evanescent wave sensor or resonant mirror. Detection of DNA hybridization has been reported by Watts et al.\(^1\) The sensor surface was modified with streptavidin and biotinylated oligonucleotide probes were immobilized to the sensor surface. Hybridization of the immobilized probes to 40-mer oligonucleotide targets were followed in real-time. This method displayed sequence specific interaction, as well as the possibility for regeneration. The sensor response was found to be dependent on both the concentrations of the probe and target oligonucleotides. Quantitative endpoint analysis provided a limit of detection of 9.2 nM of 40-mer oligonucleotide target.

Biomolecular interaction analysis (BIA), in particular the BIAcore (Pharmacia Biosensor) protocol, is also based on surface plasmon resonance. The refractive index and/or optical mass at the sensor’s surface, and changes therein, are monitored over a period of time; the results are plotted as resonance units (RU) plotted against time (called a ‘sensorgram’). Changes are proportional to the mass of molecules bound to the surface, and thus, for hybridization interactions, the refractive index should change
in proportion to the number and size (mass) of target strands. This method may provide both stoichiometric and kinetic information for interactions occurring at the surface. Both Wood\textsuperscript{29} and Nilsson \textit{et al.}\textsuperscript{28} have applied this technology to the study of DNA. The first published work described the covalent coupling ligand to the sensor chip surface, which had been derivatized with dextran and also had one face bound to a 50 nm gold surface. Four separate reaction chambers were created. Reactants were delivered to individual chambers through fluid channels by operator-programmed software. The reaction surfaces were brought into contact with the glass interface of the optical system and monochromatic light was shone on the surface. The light was reflected from the reaction surface in relation to the difference in refractive indices of the liquid layer and the glass. The resulting internal reflectance was monitored during the reaction, in addition to electronically monitoring the change(s) in evanescent wave energy generated at the gold surface. The results showed that nonspecific adsorption of DNA to the sensor surface was not observed.

Nilsson and co-workers used this technology for the detection of DNA hybridization with a slightly different approach. The first difference was that they did not covalently attach the DNA probes as Wood did. They immobilized DNA fragments to the sensor surface using the streptavidin-biotin interaction. They also focused on events other than the "hybridization/no hybridization" binary results; these include DNA strand separation, DNA hybridization kinetics, multi-step solid-phase gene assembly and the performance of different enzymes used for the synthesis and manipulation of DNA.

Surface-enhanced Raman scattering (SERS) spectroscopy was employed by
Vo-Dinh et al. for DNA hybridization detection. The authors reported that single strands of DNA can be labelled with SERS probes and be used in hybridization analyses. This method has the advantage that it does not require the use of radioactive labels and it has the potential for high sensitivity and selectivity.

b. Electrochemical Devices

Millan et al. proposed a DNA biosensor which employs a glassy carbon working electrode as a transducer. Single-stranded probes dT₃₀TdG₉₀ were immobilized onto the electrodes subsequent to activation with 1-[3-(dimethylamino)-propyl]-3-ethylcarbodiimide hydrochloride via the guanine residues. Detection of hybridization was achieved through the use of tris(1,10-phenanthroline)cobalt(III) perchlorate and tris(2,2'-bipyridyl)cobalt(III) perchlorate, which reduce, reversibly, to their cobalt(II) forms. These complexes act as indicators of hybridization because they interact differently with ssDNA and dsDNA. A carbon paste electrode has been similarly used to look at selective detection of the cystic fibrosis αF508 sequence. Hashimoto and co-workers have also described electrochemical applications for the detection of DNA hybridization using DNA-probe-modified gold or graphite electrodes. DNA probes are immobilized onto these electrodes via a 5' mercaptohexyl group. These devices have the advantage of simple construction, requiring only two electrodes and a voltmeter. However, there are significant associated disadvantages: poor sensitivity and interference by nonspecific interactions with proteins and non-complementary DNA.

Early investigation into the electronic structure of DNA was carried out by
Ladik and co-workers.$^{173}$ Many others have recognized the potential to use this property in the analysis of DNA-DNA interactions.$^{174-178}$ It has since been discovered that dsDNA conducts electricity more efficiently than ssDNA. This property has been utilized by Clinical Micro Sensors (CMS), headed by T. J. Meade and J. F. Kayyem, in the development of a handheld DNA sensor based on the inherent conductive properties of DNA.$^{179,180}$ The CMS method involves the immobilization of labelled probe DNA onto an electrode. Samples containing whole cells and viruses can be added directly to the device because the underlying gold electrode is protected from interacting with other redox species floating in solution. This is an obvious advantage of this technology over methods requiring stringent sample preparations.

c. Acoustic Wave Devices

Acoustic wave devices involve the use of piezoelectric transducers, which offer the advantages of a “solid-state construction, chemical inertness, durability, and ultimately the possibility of low cost mass production.”$^{94}$ These devices have been used as mass sensors and hybridization detectors.$^{74-76}$ The kinetics of hybridization at a solid-liquid interface have also been measured using an acoustic wave device.$^{79,81}$ Okahata et al. have also employed this technique for quantitative analysis of sequence-specific binding of peptides to dsDNA.$^{95}$ Recently, Niikura et al. have demonstrated the usefulness of this technique for directly monitoring polymerase chain reactions.$^{96}$ In addition, genetic afflictions such as Tay-Sachs$^{83}$ and damage by UV radiation$^{87}$ have been studied by acoustic wave sensors.

Most acoustic wave devices employ AT-cut quartz wafers as transducers.
Measurement is achieved by applying an external voltage across the thickness of the wafer by electrodes placed on opposing crystal faces. This voltage, in turn, causes deformation of the crystal wafer, resulting in relative motion between the two faces of the crystal, termed “thickness shear” motion. Crystal relaxation and oscillation at the resonance frequency is maintained by an external circuit. Changes in the resonance frequency (Δf) are commonly monitored as this is directly related to added mass for gas-phase systems, as described by Sauerbrey (see section 4).

Fawcett et al. first reported the detection of nucleic acid hybridization. Single-stranded polyadenylic acid (polyA) was covalently attached to quartz crystals and the added mass, from exposure to polyuridyl acid (polyU), was calculated from the measured change in frequency. These frequency changes were compared with values from control crystals immobilized with non-complementary strands. Though the crystals were exposed to liquid solutions for the various protocols, the measurements were performed in air after the crystals were dried. The results were shown to be both quantitative and qualitative, as well as a useful non-radioactive means of hybridization detection. Since this initial work, a number of similar studies have been reported.

Other electrode configurations result in different crystal motions. An interdigitated array of electrodes on the crystal surface results in the generation of mechanical waves which are transmitted to a receiver electrode array. Mass deposition can be measured through changes in surface acoustic wave (SAW) speed and amplitude as the generated waves are affected by interactions at the surface.

Andle et al. were the first to report the use of an acoustic wave device in
liquid-phase. The authors described the covalent immobilization of ssDNA and the
detection of the hybridization of poly(dA) with bound poly(dT). Su et al.\textsuperscript{80,85,102} have
presented the use of thickness shear mode acoustic wave devices in liquid-phase.
Some of this work involved the adsorption of \textsuperscript{32}P-labelled ssDNA to palladium oxide
electrodes as well as nylon membranes under various solution conditions.
Quantification of the attached nucleic acid was carried out using both phosphor
imaging and scintillation counting. It was noted that there was a discrepancy between
the values found from radiochemical labelling and acoustic wave analysis. This was
attributed to changes in interfacial viscosity resulting from interactions with the
adsorbed probe. Further work showed that this device could be applied to the
detection and quantification of anticancer drug binding to DNA.\textsuperscript{80} This work also
indicated that this device could be used to study the kinetics of nucleic acid-small
molecule interactions. The authors also introduced the real-time measurement of
parameters other than resonance frequency, such as the motional resistance, during
interfacial hybridization. Overall, operation of these devices in liquid phase involves
some uncertainty\textsuperscript{104}; the sensitivity is reduced by the damping of the measured wave
component normal to the surface. This has led to the study of the interfacial
parameters of surface viscosity, free energy, and surface morphology and how they
relate to the measured response.\textsuperscript{105}
3. OLIGONUCLEOTIDE AND DNA PROBE ARRAYS

A critical component in obtaining genetic information is the ability to screen a DNA sequence. The basic experimental protocols employed in the past have been the electrophoresis gel assays of Sangar et al.\textsuperscript{106} and Maxam and Gilbert.\textsuperscript{107} Although there have been advances with respect to throughput in these strategies, such as the use of capillary zone electrophoresis, the separation-based protocol is still relatively time-consuming and, therefore, rate-limiting with regard to the overall sequencing effort.

An elegant alternative to the above is the use of so-called gene microchips or, more accurately, oligonucleotide and DNA probe arrays. This approach represents an exquisite aggregation of sequencing by hybridization (SBH), light-directed spatially addressable interrogation, combinatorial chemical synthesis, confocal fluorescence microscopy, robotics and the polymerase chain reaction (PCR). The technology offers significant advantages in terms of avoidance of time-consuming protocols through multiplexing, the ability to produce probe sequences of any type, the future possibility to generate inexpensive re-usable chips and capability for incorporation into micro-fabrication and micro-device technology.

a. Sequencing by Hybridization

The principle of this approach is based upon the fact that any linear DNA sequence incorporating the four bases is composed of overlapping, shorter sequences. Using this concept as a basis, SBH employs hybridization of a set of oligonucleotide
probes (e.g. 8- to 20-mers) with sub-sequences in a particular target DNA fragment or sample. In practical terms, the DNA target bound on a surface or present in solution is allowed to interact with a set of conveniently labelled probes, which may also be surface-attached or reacting from solution. In order to achieve analytical specificity, complete discrimination in hybridization between fully complementary target-probe sequences compared to combination involving mismatches must be affected. The number of possible probes is large for probe sequences longer than heptamers (the possible number of probe sequences is $4^n$ where $n$ is the base length of the probe). As an example, if a 12-mer target DNA sequence AGCCTAGCTGAA, is allowed to perfectly hybridize with all 65,536 possible octamer probes only five will combine with the target - TTCAGCTA, TCAGCTAG, CAGCTAGG, AGCTAGGC and GCTAGGCT will combine with the target. Consideration of overlapping sequences reconstructs the assembly of bases complementary to the above-specified 12-mer (TTCAGCTAGGCT). Obviously, increasing probe length could lead to enhanced specificity on hybridization, but there is a limit in producing a domain size for the rapidly increasing number of probes required. Finally, it should be mentioned that it is not usually the case that all possible probes are employed in this type of study. The nature of the set of probes used, or their length, depends very much on the types of mapping or sequencing information that is required at the outset.

b. Fabrication of ssDNA and oligonucleotide arrays

The use of directed light to produce oligonucleotide arrays combines photolithographical and combinatorial chemical synthetic technologies. Although
the early emphasis appeared to center on the surface immobilization of peptides, recent times have seen greater concentration on oligonucleotide chemistry.\textsuperscript{112} The production of oligonucleotide arrays by the imposition of directed light beams involves, initially, the derivatization of a glass substrate with linking molecules, such as those based on multi-layer forming aminopropyltriethoxysilane, which are in turn capped with a photolabile protecting functionality. Removal of the latter by light directed through a mask generates an area of reactive hydroxyl groups. These are then exposed to solutions of, for example, 5'-photolabile N-acyl-deoxynucleoside-3'-O-phosphoramidite.\textsuperscript{113} Next, light is directed to a different or adjacent area of the substrate, again through a photolithographic mask prior to analogous immobilization of a different nucleoside. This whole process can be repeated to produce any number of areas containing one of the four bases. These steps are shown in Figure 1.7.

Subsequent steps in producing the oligonucleotide array involve turning of the substrate in a perpendicular fashion followed by appropriate photo-deprotection to effect round 2 of the process. Figure 1.8. illustrates the whole procedure for the immobilization of the 256-possible tetramers. Clearly, although the number of probes associated with particular base sequences increases in a polynomial fashion in terms of oligo base length, the number of chemical cycles required is $4 \times n$, i.e., for tetramers, 16 steps. Accordingly, even the generation of the $4^{20}$ 20-mers requires only 80 cycles and can be conducted in a matter of hours. The main points of this technology are that the precise location of a particular oligonucleotide, and of its sequence, is known accurately and, secondly, because of the use of the photolithographic process, high-density arrays can be achieved. However, because sequences are being
synthesized directly onto the surface and the yield of each chemical reaction is less than 100%, incomplete sequences may interfere with results. The limit of resolution is obviously limited by the wavelength of the light, and its diffraction, resulting in approximate micrometer areas. If domains of 50 μm are envisaged, 40 000 oligonucleotide sites can be imposed per square centimeter. In reality, to avoid 'cross-talk' between domains and to produce distinct areas for confocal microscope detection, spaces are often placed between oligo domains. This photolithography-combinatorial chemistry strategy has been commercialized by the Affymetrix Corporation.114

An alternative approach to the fabrication of ssDNA arrays is that based on physisorption of nucleic acid to the substrate surface.115 This strategy possesses the obvious advantage that significantly longer, chemically pure probes (i.e. incomplete probe sequences removed) can be employed, resulting in a higher level of probe specificity. The protocol varies but, essentially, solutions of dsDNA can be applied in relatively small domains to poly-L-lysine-coated glass slides. Presumably, the forces binding the nucleic acid to the substrate are largely electrostatic in nature. In order to achieve application of DNA to a small area, sophisticated robotic ‘spotter’ instruments are employed that can supply extremely small volumes in an automated manner. The final step is to denature the DNA by increased temperature exposure of the wafer, resulting in a population of single-stranded nucleic acid. This approach has been pioneered and commercialized by Synteni company.116
Figure 1.7. Steps involved in photolithographic addition of oligonucleotide bases to a substrate surface. (P represents a photolabile protecting moiety).
Figure 1.8. Combinatorial chemical synthesis of all possible 256 oligonucleotide tetramers.
c. Hybridization of Target DNA to Probe Arrays

Whether the probe array has been manufactured by light-directed chemical synthesis or robotic printing, hybridization methodologies and subsequent detection are somewhat analogous. For example, Affymetrix has developed a fluidics station that allows the reproducible hybridization of target DNA to oligonucleotides on the chip surface. With respect to this instrument, it is crucial to control temperature in a uniform mixing protocol. Many of the appropriate steps involved in the processing of each microarray are fully automated and involve very low volumes of solution for application and washing. Nearly all studies of completed hybridization of probe arrays have involved the use of confocal fluorescence microscopy to detect the level of duplex formation in a particular array cell. This protocol mandated the fluorescence-tagging of the target DNA. There are obvious assumptions here, namely that the species used for fluorescence-labelling does not compromise the hybridization reaction and that the detected level of duplex formation accurately reflects the nature of complementarity in each cell. In order to detect the fluorescence radiation emanating from each cell, a confocal microscope capable of sensing multiple-emission wavelengths is generally employed. This instrument must be capable of the discrimination of fluorescence associated with labelled hybridization from other sources and pixel resolution at the several micrometer scale. Incident light from an argon ion laser excites the appropriate tag through a confocal optical system in a configuration whereby the chip is moved relative to the scanner. Emitted light is utilized for image processing.
d. Some Applications of Nucleic Acid Microarrays

This section outlines a small selection of examples where oligonucleotide microarrays have been employed in bio-analytical chemistry. Not surprisingly, there has been considerable interest in the use of microarrays for the diagnosis of genetic diseases. For example, blood obtained from β-thalassemia patients can be used to develop diagnostic tests for mutations within the first exon and first intron of the β-globulin gene. DNA samples were hybridized with 10-mers immobilized on a microchip having cell dimensions of 40x40x20 μm and 100x100x20 μm. The results indicated a significant difference in hybridization levels for matched and mismatched sequences. The authors of the work concluded that identification of gene mutations in patients was unequivocal in yielding yes/no diagnostic answers. As an aside here, it is worth noting that various researchers have concluded that single-base mismatches at the center of the duplex can be detected with facility, whereas flanking bases often cause difficulty in terms of their mismatch distinction. Another example is that concerning the now well-known, early-onset breast cancer gene BRCA1. The protein coding region of this gene contains 5 592 base-pairs in 22 coding exons spread over 100 kb of genomic DNA. Because many mutations have been associated with the malfunctioning gene, it has become necessary to screen the relatively large gene for all possible heterozygous mutations in patients. In elegant studies, it has been shown that high-density arrays consisting of over 96,600 oligonucleotides, 20 bases in length and in tandem with 2-colour fluorescence analysis (use of two tagging agents) can successfully detect a number of mutations in a particular fragment of the BRCA1 gene. In the real screening of patients known to harbour genes with mutations, the
success rate for microarray detection was over 90%.

Other studies have been concerned with efforts to obtain basic sequence information. For example, the simultaneous analysis of the entire human mitochondrial genome using DNA arrays of about 135,000 probes has been achieved. To give the reader a feel for the accuracy and time involved in this type of analysis, the authors pointed out that the throughput of conventional gel-based sequencing could result in reading two mitochondrial genomes a day, at best. In contrast, using 5 microarrays per hour yields the possibility to examine 50 genomes in one day. There are also significant reductions in the sample preparation time in concert with a highly reliable analysis. As for the study mentioned above, use of the 2-fluorophore protocol enhanced the detection of sequence polymorphisms significantly, and provided single base resolution. Finally, DNA microarrays have been employed successfully to monitor metabolic and genetic control of gene expression on a genomic scale. Brown et al. used a probe array to identify genes where expression was affected by deletion of a transcriptional co-repressor or overexpression of a transcriptional activator. This highly detailed work was conducted with respect to the organism, Saccharomyces cerevisiae, in terms of the metabolic shift associated with gene expression from fermentation to respiration. As a result of their work, the authors concluded, prophetically, that mutations in specific genes encoding candidate drug targets can serve as surrogates for an ideal chemical inhibitor or modulator of their activity. DNA microarrays can be employed to screen for patterns in the alteration of gene expression prior to assaying for chemicals that produce particular patterns.
e. Summary

There is no doubt that photolithography-combinatorial synthesis of oligonucleotide arrays is appealing in terms of its elegant simplicity, however, the configuration, by definition, is restricted to the use of relatively short oligonucleotides. Despite the possibility of fabricating huge arrays, this factor can lead to a lack of specificity in the characterization of relatively large genomes. Other aspects of this technique are the lack of ability to produce arrays with variable base-length DNA, at least in a straightforward manner, and the fact that increased chain length of oligos suffers from lack of probe purity due to incomplete strands.

Although some of these issues are resolved in the case of robotic printing, sensitivity may be an issue for this technique because of potential difficulties connected with the availability for hybridization of multi-point attached probes on the poly-L-lysine surface. In somewhat analogous work with the more traditional membrane-oligonucleotide probe arrays, such a lack of probe availability for reaction is known to occur. The effect does not constitute a real practical hindrance here, because of the extremely high sensitivity of $^{32}$P radiochemical labelling.

Both strategies described above generally involve the mandatory use of fluorescent tagging agents. The future might see attempts at label-free detection of hybridization events. A somewhat earlier attempt at this sort of approach was the atomic force microscopy study of protein-based microarrays.\(^2\)

Surface chemistry, in particular, the immobilization of nucleic acids, is an area that clearly requires development. Better control of surface chemical aspects could lead to increased oligonucleotide coverage, enhanced orientation with respect to
hybridization and optimization of packing density. Furthermore, at the present time microarrays are quite expensive to produce which is reflected in the purchase price, and to a large degree are considered to be expendable devices. The future may see new approaches for the ‘regeneration’ of microarrays using chemical or other protocols.

Finally, in the future, we are undoubtedly going to witness a scaling down in size and, of course, cost of the whole DNA microchip configuration. We are some time away from having Gene Chip™ systems at the end of every molecular biologist’s bench top, however, the excitement generated by this technology will clearly spawn efforts to increase general availability and ease of use.
4. THE TSM SENSOR

The sensor utilized for this work incorporates transducers based on the thickness-shear mode (TSM) acoustic wave sensor. The TSM consists of an AT-cut piezoelectric quartz crystal with metal electrodes on each face that launch transverse acoustic waves into the substrate at ultrasonic frequencies. In 1880, Jacques and Pierre Curie discovered that applying mechanical stress to the surfaces of certain crystalline materials (of which quartz is one) produced a proportional electric potential across the crystal. The converse effect, that application of a voltage across the crystal would result in mechanical strain, was predicted by Hankel and demonstrated by the Curies to be true.

Thus, a quartz crystal behaves as an acoustic wave transducer, transforming electrical energy to mechanical energy and vice versa. The crystal symmetry, the angle of cut relative to the crystallographic axes and the configuration of the excitation electrodes used to apply the electric field across the crystal influence the electromechanical coupling and stresses resulting from the applied electric field. The various modes of electromechanical coupling result in diverse modes of propagation, particle displacements and resultant acoustic waves. A TSM device employs AT-cut quartz, obtained by cutting wafers of quartz at an angle of 35°15′ with respect to the z-axis, with metal electrodes deposited on opposite sides of the wafer (Figure 1.9.a.). Displacement of the crystal lattice is induced when an alternating potential is applied to the electrodes producing a shear-mode bulk acoustic wave propagating in the thickness direction, parallel to the electric field and perpendicular to the atomic
Figure 1.9. (a) Thickness shear mode (TSM) device and (b) acoustic wave propagation.
displacements (Figure 1.9.b). At resonance, the resultant standing wave has displacement maxima occurring at the crystal faces. The resonant frequency of the acoustic wave depends on the physical characteristics of the quartz and the thickness of the crystal wafer as these properties directly affect the velocity of the wave through the crystal as well as the wavelength for the standing wave. It has been shown that a rigidly adhered, thin coating on quartz can be treated as an extension of the quartz material and, therefore, as an increase in the thickness of the quartz wafer. The change in mass due to this added layer can be quantitatively calculated from the corresponding shift in resonant frequency produced. Sauerbrey originally described this mass-frequency phenomenon in 1959.7 The Sauerbrey equation was derived for an AT-cut crystal to describe the relationship between the resonant frequency and the deposited mass:

$$\Delta f = \frac{2f_0^2 \Delta m}{A \sqrt{\rho_p \mu_p}}$$

(5)

where $\Delta f$ is the measured or expected frequency shift, $f_0$ is the fundamental resonant frequency of the crystal prior to mass loading, $\Delta m$ is the total change in mass due to loading, $A$ is the surface area of the piezoelectrically active area in cm$^2$, and $\rho_p$ and $\mu_p$ are the density and shear modulus of quartz, respectively. The density and shear modulus values for AT-cut quartz are $\rho_p = 2.648$ g cm$^{-3}$ and $\mu_p = 2.947 \times 10^{11}$ g cm$^{-1}$. This gives a predicted detection limit on the order of $10^{-12}$ g. Sauerbrey modelled the resonance frequency shift caused by a mass loading as an increase in the effective
thickness of the piezoelectric quartz resonator oscillating in the gas phase with the added mass being assumed to have the same density and acoustic properties as quartz. This assumption is invalid resulting in significant differences with respect to predicted frequencies when added films are relatively thick and/or are of different acoustic impedance. For example, it has been shown repeatedly that device frequencies respond to changes in film viscoelasticity even when the sensor is operating in the gas phase.\textsuperscript{129} Even so, it is possible to use the Sauerbrey equation for \textit{rigid} mass loading to a limit of $\Delta m/m_o = 2\%$ for most piezoelectric materials, where $m_o$ is the mass per unit area of the unloaded crystal and $\Delta m$ is the change in mass.\textsuperscript{126}

\textbf{a. Operation of the TSM Sensor in the Liquid Phase}

Although it was thought that damping viscous forces would prohibit the use of TSM devices in liquids, in 1980 it was shown that the system could be operated in contact with solution if one side of the crystal was maintained under gas.\textsuperscript{131} When operated in liquids, however, the behaviour of the TSM device becomes much more complicated; it does not adhere strictly to the Sauerbrey equation. In addition to mass gain or loss on the device surface, viscous forces couple the liquid medium to the oscillating surface. Accordingly, such a coupling process results in the transfer of acoustic energy into the liquid in terms of a damped transverse wave. Coupling between the crystal motion and the adjacent liquid generates acoustic waves with decay lengths on the order of 1 $\mu$m. A schematic representation of this mechanism together with some of the factors that govern the behaviour of the system is depicted in Figure 1.10. In modelling the TSM sensor, bulk and interfacial properties have been
Figure 1.10. Factors affecting the responses of the TSM device.
included to account for the change in the resonant frequency upon liquid loading. These include viscosity, density, conductivity, viscoelasticity, surface free energy, surface roughness, surface stress, molecular slippage, viscous energy losses and dielectric effects. Various theories to date are briefly reviewed below and summarized in Table 1.

There have been a number of attempts to provide theoretical treatments of fluid coupling to a TSM structure, including the case where a molecularly recognizable film is imposed on the device surface. Nomura and Minemura were the first to present a theory based upon empirical formulation which related the change in resonant frequency, \( \Delta f \), to the density and specific conductivity of the adjacent liquid.\(^{131}\) Nomura and Okuhara furthered the theory by demonstrating that, in an organic liquid medium containing no electrolytes, the change in resonant frequency is directly influenced by the viscosity and density of that medium.\(^{132}\) Both of these observations have since been attributed to field fringing at the electrode edges.

In 1985 both Bruckenstein and Shay\(^{133}\) and Kanazawa and Gordon\(^{124}\) published descriptions which predicted the change in resonant frequency of a piezoelectric crystal immersed in solution. Bruckenstein and Shay used a diffusion-momentum analogy, modelling the effects of viscosity and density on \( \Delta f \), to calculate the effective thickness of the coupled fluid layer. In the treatment of Kanazawa and Gordon the device was assumed to be an elastic solid oscillating in a surface shear mode and the liquid was treated as a purely viscous fluid. Through a no-slip boundary condition, a resonance configuration was obtained by matching the shear forces at the quartz-fluid interface. Although the Kanazawa and Gordon and the Bruckenstein and Shay models
<table>
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<th>Authors</th>
<th>Equations</th>
<th>Considerations</th>
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<tbody>
<tr>
<td>Nomura and Minemura$^{131}$</td>
<td>$\Delta f = A_1 k_L^{0.511} - B_1 (\rho_L - 1)^{1.02}$</td>
<td>Empirical formulation. Conductivity and specific gravity.</td>
</tr>
<tr>
<td>Nomura and Okuhara$^{132}$</td>
<td>$\Delta f = A_2 \eta_L^{2.5} + B_1 \rho_L^{0.5} - C$</td>
<td>Empirical formulation. Viscosity and density.</td>
</tr>
<tr>
<td>Bruckenstein and Shay$^{133}$</td>
<td>$\Delta f = -2.26 \times 10^{-6} f_0^{3/2} (\eta_L \rho_L)^{1/2}$</td>
<td>Viscosity and density of bulk.</td>
</tr>
<tr>
<td>Kanazawa and Gordon$^{134}$</td>
<td>$\Delta f = f_0^{\Delta n} \left( \frac{\eta_L \rho_L}{\eta_Q \rho_Q} \right)^{1/2}$</td>
<td>Viscosity and density of bulk.</td>
</tr>
<tr>
<td>Schumacher et al.$^{143}$</td>
<td>$\Delta f = A_3 \Delta m_L = A_3 \frac{\rho_L \varepsilon^2}{2}$</td>
<td>Surface roughness.</td>
</tr>
<tr>
<td>Heusler et al.$^{138}$</td>
<td>$f_0 - f_m = A_4 (P - P_m)^2$</td>
<td>Surface stress.</td>
</tr>
<tr>
<td>Hager$^{139}$</td>
<td>$\Delta f = -k_1 \Delta (\eta_L \rho_L)^{1/2} + k_2 (\Delta \varepsilon_L)$</td>
<td>Hydrodynamic coupling analysis and liquid dielectric constant.</td>
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Table 1.1. Models of liquid phase TSM acoustic wave sensors.

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<th>Authors</th>
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<th>Considerations</th>
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<tr>
<td>Yao and Zhou(^{142})</td>
<td>(\Delta f = A_5 + A_6 \eta_L^{0.5} + A_7 \eta_L^{0.5} - A_8 \varepsilon_L)</td>
<td>Hydrodynamic coupling analysis and liquid dielectric constant.</td>
</tr>
<tr>
<td>Shana and Josse(^{140})</td>
<td>(\frac{\Delta f}{f_0} = \left(\frac{f_0 \eta_L \rho_L \pi \eta Q \rho Q}{\pi} \right)^{1/2}</td>
<td>\text{Piezoelectric effects.}</td>
</tr>
<tr>
<td>Martin et al.(^{136})</td>
<td>(Z_f = \frac{N \pi}{4 \kappa^2 \omega_s C_o \left( \frac{G \rho_f}{C_{66} \rho_q} \right)^{1/2} \tanh\left( \frac{\mu \theta T_f}{G} \right)^{1/2} \right)}</td>
<td>\text{Rheological properties.}</td>
</tr>
<tr>
<td>Shana and Josse(^{140})</td>
<td>(\frac{\Delta f_s}{f_0} = \frac{\kappa^2}{\pi^2} \frac{\varepsilon_{22}^2 \kappa^2}{\pi^2} \frac{\sigma_2}{\varepsilon_{22}^2 + \varepsilon_L^2 + \sigma^2 (\varepsilon_{22} + \varepsilon_L)^2} )</td>
<td>\text{Acoustoelectric coupling.}</td>
</tr>
<tr>
<td>Muramatsu et al.(^{141})</td>
<td>(R_m = \frac{A}{K^2} (2 \pi f_0 \eta_L \rho_L)^{1/2} )</td>
<td>\text{Equivalent circuit.}</td>
</tr>
<tr>
<td>Martin et al.(^{136})</td>
<td>(R_m = \frac{\eta Q}{\mu Q C_1} \left( \frac{\sigma}{\sigma_s} \right)^2 + \frac{\sigma_s L_1}{N \pi} \left( \frac{2 \mu \rho \eta L_1}{\mu \rho Q} \right)^{1/2} )</td>
<td>\text{Equivalent circuit.}</td>
</tr>
</tbody>
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are based on different properties, the relationships between $\Delta f$ and the characteristics of the quartz and bulk solution are the same. Kanazawa and Gordon proposed that the change in resonant frequency results from coupling between the oscillating crystal and the liquid. A damped standing shear wave, which propagates into the liquid in the direction perpendicular to the quartz crystal surface, is produced (Figure 1.10.). The decay length of the standing wave is proportional to the square root of the viscosity of the bulk solution and also dictates the thickness of the "rigid" liquid layer adjacent to the crystal surface. Both models impose a no-slip boundary condition. This implies that the transverse displacement of the surface of the crystal is in tandem with that of the contacting liquid layer. This boundary condition is strictly valid for solutions which are perfectly insulating and the electrode surface is perfectly smooth. This leads to the prediction that the resonant frequency changes in proportion to the liquid's acoustic impedance. It was ultimately suggested that only a thin layer of liquid adjacent to the crystal surface is displaced, hence, the device response is a function of the properties of this adjacent layer. The combination of both models serves to connect Sauerbrey's original gas-phase mass response and the properties of a thin-film liquid boundary. Nevertheless, these models only incorporate the mechanical properties related to the resonant frequency and overlook the impact of the electrical properties of the TSM device and, hence, cannot fully predict the behaviour of the TSM sensor in liquid. It is also important to consider the effects of chemical reactions at the surface and processes which change the properties (such as mass, structure, surface free energy, interfacial viscosity, and surface morphology) of the surface and/or adjacent liquid layer.
The first comprehensive two-layer model by Reed et al. involved the equations for the shear stress and the electrical displacement being solved for the imposition of a film on one face of the device. Again, the no-slip boundary condition was invoked. Importantly, both the quartz of the sensor and the added film were regarded to be viscoelastic. The real part of the complex viscosity represents an energy dissipation; whereas the imaginary part is connected to an energy storing process. This model was extrapolated from the detailed matrix equations for quartz and incorporates the piezoelectric effect of the quartz transducer as well as the viscosity and shear modulus of the adjacent liquid medium. The conditions of no-slip and no stress at the free surfaces as well as the application of a driving voltage to both sides of the quartz crystal were imposed boundary conditions. The energy dissipated in the crystal is compensated for by incorporating the viscous component of the Hooke’s Law equation. This model closely resembles Kanazawa and Gordon’s for the conditions where the adjacent layer is treated as infinitely thick and purely viscous.

Martin et al. replaced the adjacent viscous layer of the above model with an equation for liquid loading derived from the Navier-Stokes equation. The no-slip boundary condition was again invoked and the deposited film was considered to be an infinitesimally thin film. Mass loading and viscous loading could be measured simultaneously using this model. The results were converted to a device impedance form and parameters presented as an extension to the well-known Butterworth-van Dyke model. Heusler et al. suggested that parabolic dependence of the resonant frequency on the hydrostatic pressure at the surface of the crystal could be related to the energy stored in the quartz, when taking into consideration the effect of surface
stress on the resonant frequency. Hager's model is based on the dielectric effect and velocity of the adjacent layer at the surface of the crystal in addition to the energy losses to the viscous medium. Shana and Josse imposed electrical and mechanical boundary conditions for the interface of the crystal and adjacent liquid layer. Solving the characteristic equations for the layers led to the derivation of an equation for the observed shift in resonance frequency. Muramatsu et al. contemplated the resistance of an equivalent circuit in the derivation of a relationship between the frequency shift and the density and velocity of the adjacent liquid medium. This proposed relationship is based on a study involving alcohol:water solutions and remains valid when only one side of the crystal is exposed to liquid of similar viscosity but becomes nonlinear when both sides are in contact with solution or when exposed to highly viscous solutions. Yao and Zhou put forth a model that is dependant on the effects of the liquid in terms of its dielectric constant and conductance.

Properties of the crystal/liquid interface are overlooked in these early models; surface roughness, interfacial viscosity, surface free energy, and interfacial slippage are boundaries that require consideration for developing a comprehensive model. For example, the complexity of motion of the adjacent liquid medium generated by the vibration of the surface increases with surface roughness. The ideal shear standing wave shown in Figure 1.10. may be perturbed by the production of turbulent flow, eddies, or compressional waves caused by the motion of protruding features of the surface and from the trapping of liquid in the topography of the surface. Schumacher and co-workers recognized this and developed a model that attributed anomalously large shifts in resonance frequency to surface roughness. The model involved
treating the surface as if it were comprised of hemi-cylinders of diameter \( e \) and the trapped liquid as a rigidly attached layer of thickness \( e/2 \). This trapped liquid was directly correlated to the additional change in resonant frequency. Further investigation of the effect of surface roughness by Martin and co-workers led to the development of a theory that this layer of trapped liquid could account for any anomalous shifts in resonance in frequency. The authors suggested that it was, thus, not necessary to involve the possibility of liquid ordering or slippage occurring at the quartz-liquid interface. It was also put forth that, for surfaces with topographical features considerably less than the decay length of the standing wave, the surfaces may be considered hydrodynamically smooth. Hence, any shifts in resonance frequency must be the result of changes in the density:viscosity product. The coupling of a smooth surface to the liquid is significantly more important than its wettability. With the increase of the depth of topographical features on the surface, factors such as the generation of compressional waves and trapping of liquid become more significant in interpreting the response of the TSM device. The authors also stated that liquid trapped in the surface features becomes less significant as the hydrophobicity of the surface is increased.

Yang and Thompson conducted a study comparing rough and smooth surfaces.\(^{14}\) This rendered evidence that surface stress, in addition to shear and non-shear coupling phenomena, contributes considerably to the response of the TSM device. In a number of subsequent investigations by Thompson and co-workers, it has been shown that the molecular boundary conditions existing at the solid-liquid interface of the TSM device determine its response.\(^{145-148}\) These studies produced the
central doctrine that propagation of acoustic energy could be perturbed by a partial slip boundary condition at the solid-liquid interface, supporting the concept of interfacial viscosity at a solid-liquid junction, with one component in motion.

Duncan-Hewitt and Thompson\textsuperscript{149}, solving a similar set of equations, proposed a four-layer model which included the crystal and three fluid layers (Figure 1.11.). The three fluid layers were associated with interfacial liquid ordering caused by the solid surface. The first layer is the quartz crystal. For the purposes of this model, the electrodes deposited on the crystal surface are assumed to be thin and elastic and to behave as layers of quartz. The layer adjacent to the crystal surface is an ordered layer of liquid. The depth of this region is determined by interaction at the solid-liquid interface and can be predicted using contact angle measurement data. It has been shown that the apparent viscosity of water increases to one-and-a-half times that of bulk water in quartz capillaries\textsuperscript{150} and, for the purpose of this model, both the viscosity and density of this ordered layer of liquid are considered to be five times that of the bulk liquid. The third layer is a thin transitional layer that has an estimated thickness of one to five molecular diameters. The contact angle of the surface and, thus, the hydrophobicity of the system can be utilized to predict the composition and behaviour of this layer. If the surface is highly hydrophilic, the density of this layer approaches that of the bulk solution, whereas, if the surface is hydrophobic, the density approaches that of the vapour phase. The fourth, and final, layer in this model is the bulk solution. This layer has an approximate thickness of 3 \( \mu \text{m} \). This four-layer model incorporated the assumptions that each layer was purely viscous and no slippage exists at the liquid-liquid interfaces. For the solutions of the differential equations to be
Figure 1.11. Four-layer model
valid, each layer was considered homogeneous with defined boundaries, however, in truth, transitions across and between layers are gradual. Using contact angle measurements, Thompson and Duncan-Hewitt were able to associate the viscosity and density of these layers with interfacial energetics. The authors demonstrated that predicted outcomes using their model were comparable to actual experimental results. Thompson and Duncan-Hewitt derived this model using strict linear descriptors for piezoelectricity to solve for the interfacial viscosity in terms of an activation barrier to interfacial flow. This model was used in conjunction with the interfacial viscosity molecular theory proposed by Krausz and Eyring and contact angle measurements to demonstrate that the TSM response could be related experimentally to the surface free energy of the device. The surface free energy can also be coupled to interfacial slip properties of the device. Thus, Duncan-Hewitt and Thompson were able to show that interfacial parameters do have a role in the determination of interfacial viscosity. The characteristics of the liquid medium immediately adjacent to the solid surface are not the same as those of the bulk liquid. It has been shown that these characteristics may influence the performance of the entire system.

Thompson and Duncan-Hewitt weighed the legitimacy of imposing the no-slip boundary conditions, as generally encountered in previous models. Tolstoi and Blake initially suggested the possible existence of slippage at a solid-liquid interface. Israelachvili gave evidence for this phenomenon through surface microscopy of thin films. Krim et al. were able to apply this phenomenon in their representation of the TSM device behaviour. Hayward similarly concluded the presence of interfacial slippage in a recent derivation of a TSM model in which the components of the
equivalent circuit are linked to the dissipation of acoustic energy.\textsuperscript{159} In 1994, Ferrante et al.\textsuperscript{160} modelled the TSM oscillating in an infinite fluid. For the first time, the no-slip boundary condition was not employed; in its place a complex slip parameter was introduced. The authors used impedance analyses of both hydrophilic and hydrophobic surfaces, exposed to a series of glycerol solutions to derive a model. A new boundary condition, the interfacial slip parameter, \( a \), was introduced for a two-layer model of the TSM device in liquid. This model requires that the sensor surface does not perturb the structure of the contacting liquid. The interfacial slip parameter is the ratio of the particle displacement of the contacting liquid to the particle displacement of the sensor surface. The interfacial slip parameter was determined by theoretical treatment of real impedance values of the TSM device obtained from experiments. The authors modelled the molecular slippage to explain the effect of viscosity on interfacial slip. It was found that the displacements of the liquid and solid particles coincide if the inter-particle forces are very strong. When this is the case, the value of \( a \) would be 1 and represents the absence of interfacial slippage. However, when the inter-particle forces are zero, there would be no coincidence in displacements of the two phases. When this is the state, the displacement of solid particles does not induce the displacement of adjacent liquid particles. The value of \( a \) generally lies between the extremes of 0 and 1. As the viscosity of the water:glycerol approached that of water, some slip was observed, however, as the viscosity was increased and approached that of glycerol, very little slip was observed.

Hayward and Thompson\textsuperscript{61} have recently introduced a universal model for the TSM operating in both gas and fluid phases. A four-layer model was developed to
represent a real coated sensor. This is based on the fundamental equations of motion and piezoelectricity. The model involves the combination of sets of equations dealing with the three "solid" layers (quartz, electrode, attached film) and those for the liquid phase. All of the various layers are considered to be viscoelastic and slip at three interfaces is included. The underside of the crystal is regarded as a free surface with an ideal electrode of no mass or thickness. The various differential equations required are given in descriptional terms in Table 2. Notably, the viscous element in Hooke's equation allows energy dissipation in the crystal in agreement with the introduction of a series resistance in the Butterworth-van Dyke equivalent circuit model of the resonator. The model is developed with equations involving both particle displacement in all the solid phases and the liquid phase. Solutions to these expressions requires a set of assumptions and equations for nine boundary conditions. With respect to the former the following are employed:

1. There are no electric fields in the layers attached to the quartz crystal.
2. Similarly, these layers are not piezoelectric.
3. The bottom of the device is a free surface with an ideal electrode, that is one with neither mass nor thickness.
4. All layers are considered to have elastic and viscous properties.
5. Flow is incompressible.
6. There are no external forces.
Table 1.2. Basic equations for the development of a theory for the propagation of acoustic energy into liquid.

**Solid phase**

<table>
<thead>
<tr>
<th>Equation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\sigma_{ij} = \rho \ddot{u}_j$</td>
<td>Newton's second law</td>
</tr>
<tr>
<td>$D_{li} = 0$</td>
<td>Gauss' law</td>
</tr>
<tr>
<td>$\sigma_y = c_{ijkl}S_{kl} - e_{ik}E_k + \eta_{ijkl}S_{ij}$</td>
<td>Hooke's law</td>
</tr>
<tr>
<td>$D_i = e_{ik}S_{ik} + e_{ik}E_k$</td>
<td>Dielectric equation</td>
</tr>
<tr>
<td>$S_{ii} = 0.5(u_{i,\ell} + u_{\ell,i})$</td>
<td>Strain and deformation</td>
</tr>
<tr>
<td>$E_k = -\phi_k$</td>
<td>Field equation</td>
</tr>
</tbody>
</table>

**Liquid phase**

<table>
<thead>
<tr>
<th>Equation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\rho \dot{u}_{ij} + (\rho u_i u_j) = 0$</td>
<td>Continuity equation</td>
</tr>
<tr>
<td>$\sigma_{ij} = -p\delta_{ij} + n_{ij}(u_{ij} + u'<em>{ij}) + (\zeta - 0.67\eta</em>{ij})\delta_{ij}\dot{u}_{ij}$</td>
<td>Hooke’s law</td>
</tr>
<tr>
<td>$\sigma_{ij} + F_i = \rho_{liq}(u_i + u'_{ij})$</td>
<td>Newton's second law</td>
</tr>
</tbody>
</table>

* $\sigma$, stress; $u$, displacement; $S$, strain; $e$, piezoelectric coefficient; $D$, electric displacement; $E$, electric field; $e$, dielectric coefficient; $\phi$, potential across device; $p$, pressure; $\rho_{liq}$, density of liquid; $F$, external force; $\zeta$, second viscosity term; $\eta_{liq}$, liquid viscosity; $\delta_{ij}$, Kronecker delta.
Nine boundary conditions are invoked to produce the required nine equations. A set of three equations is derived from the interfacial slip parameters between successive layers which allow for phase shifts between the displacements at each interface. A second set of three equations arises from the necessity for the continuity of shear stress throughout the system. A seventh equation stems from the no-stress condition at the underside of the crystal. The remaining two equations are the result of the introduction of electrical forcing functions which cause the crystal to oscillate. This model was found to be in agreement with data acquired from the study of water:glycerol solutions. In addition, correlations were found with the models of Sauerbrey\textsuperscript{127}, Lu \textit{et al.}\textsuperscript{130}, Kanazawa and Gordon\textsuperscript{134} and Ferrante \textit{et al.}\textsuperscript{160}. Hayward and Thompson also implemented concepts from the models of Reed \textit{et al.}\textsuperscript{135}, Duncan-Hewitt \textit{et al.}\textsuperscript{149} and Ferrante \textit{et al.}\textsuperscript{160}. The liquid at the interface of the crystal and the liquid was considered to be viscoelastic, ordered and to have slip, respectively. The stiffness of the fluid medium was shown to have a proportional relationship, whereas, its viscosity was shown to have an inverse relationship with the series resonant frequency. Combining these two parameters yielded a complex shear viscosity parameter. This parameter proved to be interchangeable, though not equivalent, to the previously introduced interfacial slip parameter. It was concluded that both viscoelasticity and interfacial slippage are essential, the former parameter being especially significant for more viscous liquids and the latter for less viscous fluids.

All the equations described above (in qualitative terms) are solved with the electric field solution being transferred into the conventional crystal impedance. The
theoretical treatment outlined above is important in terms of the understanding of the
responses of a molecularly recognizable TSM placed in water or other liquids. Addition
of material to the surface of the device in liquid is clearly expected to perturb a number
of physicochemical factors, all of which influence the series resonance frequency and
equivalent circuit parameters. This will include slippage, interfacial viscosity, the
viscoelastic properties of the attached layers and acousto-electrical properties.
Accordingly, the notion that the TSM can be employed universally to “calibrate”
added mass during electrochemical deposition experiments is invalid, if the imposed
material possesses acoustical properties vastly different from quartz or metal. This
result clearly implies that a re-evaluation of the “Measuring the Masses” concept
espoused in recent times is required.162

When the TSM device is used in a conducting medium, the generated acoustic
wave interacts with the ions and dipoles of the liquid through viscous and
acoustoelectric coupling. In particular, the acoustoelectric coupling results from the
interaction of the generated evanescent wave with the charged and dipolar species in
the adjacent solution. The TSM response is affected by the energy expended through
this interaction and is, thus, influenced by the specific conductivity and the dielectric
constant of the surrounding fluid. The relationship between the series resonant
frequency and these electrical properties have been addressed by Nomura et al.131 and
Yao et al.142, respectively (Table 1). The equation developed by Josse and
coworkers140 described the change in $f_s$ of a TSM device with one side immersed in a
dilute conductive solution (Table 1). This was achieved by applying appropriate
electrical and mechanical boundary conditions at the interface and by solving the
resulting equations. The conductivity of the liquid medium and the dielectric constants for both quartz and liquid were incorporated.

In the development of the TSM sensor, other factors have been suggested to affect its responses. It has been proposed that an electrical double layer forms at the solid-liquid interface. In addition, it has been suggested that there is a blurring of the electrical field into areas not bounded by the electrodes, creating a fringe effect of the electrical field. Adamson\textsuperscript{163} suggested that the thickness of the electrical double layer is much less than the decay length of the electric field (0.01 \(\mu\text{m}\) vs. 0.2 \(\mu\text{m}\)). This can be considered as an additional capacitance to the TSM sensor. Rodahl \textit{et al.}\textsuperscript{164} proposed that coupling is possible between surface charges which have been acoustically induced and charged species in the contacting solution.\textsuperscript{164} However, they also suggested that interferences from surface charges or other microscopic phenomena, such as an electrical double layer, were not significant factors in determining the influence of electrical properties on the resonant frequency. Changes in the parallel resonant frequency come about because the area of the electrode is effectively enlarged by contact with the conducting liquid medium. Hence, the contacts to the electrodes and the fringing of the electrical fields affect the dissipation of energy at the sensor surface and, thus, the resonant frequency response of the TSM sensor.

The stray capacitance and the resistive pathways caused by the solution via its dielectric and conducting properties were additional considerations in the authors' research. A capacitor is formed by the two electrodes sandwiching the quartz crystal, resulting in a shunt capacitance parameter, \(C_s\). This parameter is influenced by a
stray capacitance caused by the fringing field which is, in turn, influenced by the geometry and the dielectric constant of the media that the field lines pass through. The capacitance is directly proportional to the magnitude of the dielectric constant. The TSM sensor response would be affected by a significant increase in this capacitance.
5. Network Analysis of TSM Sensors

a. Oscillator Circuit Method

The oscillator, or active, method had been used exclusively to monitor TSM response until 1990.\textsuperscript{43-45,165} This method incorporates the device as the frequency-determining component in an oscillator circuit, in the feedback loop of an amplifier. Early examples include the simplified single-transistor oscillators utilized by Nomura\textsuperscript{12} and Yao\textsuperscript{12}. Recent models include a series of two Transistor-Transistor Logic (TTL) inverters that provide a zero phase shift between the input and output voltage and make up the oscillator architecture. Oscillation occurs when the following two conditions are met: (1) the feedback voltage is equivalent to the input voltage and, thus, the loop gain of the circuit is equal to one and (2) there is a zero phase shift around the complete circuit. The electrical capacitance and, thus, dual resonance are dependent on the configuration of the electrodes on the crystal. The phase shift is zero for the series resonant frequency, $f_s$, and the parallel resonant frequency, $f_p$, however, in the oscillator setup, the first condition can only be satisfied at one of these resonant frequencies. The circuit is generally configured to oscillate at the series resonant frequency, $f_s$. Various oscillator circuits have been designed to meet different requirements such as sensitivity to temperature or control of the actual voltage between the electrodes for electrochemical purposes.

The oscillator method has several inherent limitations. Particularly apparent is the fact that, by configuring the circuit to only one resonant frequency, the crystal is only partially characterized. In addition, this resonant frequency is not fully
representative of the TSM device operation, being sensitive to manipulations of the properties of the capacitor in series with the quartz crystal. This method of analysis also breaks down when the TSM device is immersed in solution or exposed to a highly viscous liquid medium due to the cessation of the oscillation of the circuit. This occurs because the phase shift of the crystal decreases to a negative value at high viscosity, thus, the second condition of operation is no longer met. Measurement of the output voltage\textsuperscript{47-50} and the feedback voltage of the amplifier\textsuperscript{84} and the use of an impedance analyzer to measure the motional resistance (\(R_m\))\textsuperscript{167} have been implemented in the attempt to compensate for the primary limitation. Even so, with only two measured parameters, the response of the TSM device cannot be fully characterized.

Thompson and co-workers improved on these concepts by incorporating a network analyzer\textsuperscript{144,145,148,149}. Network analysis is the determination of the complex electrical impedance of a device or electrical circuit as a function. It involves fitting of different theoretical models to that device/circuit to characterize its behaviour in terms of discrete electrical components. The analyzer applies ac voltage signals at a range of frequencies to the device under test (DUT) at a constant power. This is compared to the reflected power. The difference between the two (and any shifts in the phase of the reflected vs. transmitted signals) give the complex impedance \(Z\) of the DUT. The instrument then displays \(|Z|\) and \(\arg Z\) vs. \(f\). Figure 1.12. depicts the values of the magnitude and the phase of impedance, calculated from the voltage and current at each frequency point. Eight parameters can be directly extrapolated from these plots. Additional parameters can be determined using the impedance plots in equivalent circuit analysis. The network analysis method provides a more complete
Figure 1.12. Magnitude and phase of impedance.
characterization of the TSM device.

b. BVD Equivalent Circuit

The TSM sensor can be replaced as an element of an oscillator circuit by a theoretical combination of simple circuit elements so that the overall circuit exhibits the same impedance behaviour as the sensor. The electrical behaviour of a piezoelectric crystal was described by Cady\textsuperscript{167} and Bottom\textsuperscript{168}. A single equation, in terms of the voltage across the crystal, was derived to describe the density of the current conducted by the crystal. This was achieved by considering the motion of each point of quartz plate. The resultant parameter, the admittance, is a function of both the frequency and properties of the crystal. The impedance is the reciprocal of the admittance. The TSM device response corresponds to the derived Butterworth-van Dyke (BVD) equivalent circuit (Figure 1.13.); each element of the circuit is related to certain physical characteristics of the operating sensor. It consists of a series branch containing a resistor ($R_m$), inductor ($L_m$) and capacitor ($C_m$), all in parallel with a second branch containing only a capacitor ($C_n$). The motional elements of the circuit, $R_m$, $L_m$ and $C_m$, account for the electro-mechanical coupling and vibrational motion. The two arms work in tandem in that, at resonance, the motional arm dominates the impedance, whereas, off resonance, the electrostatic element prevails in the impedance. The internal and external dissipation of electrical energy in the TSM is described by the motional resistance, $R_m$. Internal friction, producing thermal energy, contributes to internal energy losses, whereas, the device mounting and the contacting liquid medium contribute to external energy losses via acoustic wave decay. The
Figure 1.13. Butterworth-van Dyke equivalent circuit parameters.
motional inductance, $L_m$, pertains to the inertia related to mass displacement during oscillation and involves the mass of the quartz wafer, its coating and the effect of the contacting liquid. The resilience or elasticity of the quartz crystal in motion is represented by the motional capacitance, $C_m$. The electric capacitance, $C_e$, is the capacitance of the two parallel electrodes of opposing faces of the quartz wafer. The circuit elements can be stated in terms of the physical characteristics of the quartz crystal and each element has its own impedance, as follows:

$$R_m = \frac{e^2 \sigma_d}{8 A e^2} \quad Z = R_m$$  \hspace{1cm} (6)

$$L_m = \frac{e^2 \rho}{8 e^2} \quad Z = j \omega L_m$$ \hspace{1cm} (7)

$$C_m = \frac{8 A e^2}{\pi^2 e \mu Q} \quad Z = \frac{1}{j \omega C_m}$$ \hspace{1cm} (8)

$$C_e = \frac{k e_0 A}{e} \quad Z = \frac{1}{j \omega C_e}$$ \hspace{1cm} (9)

where $k$, $\sigma_d$, $\rho$, $e$, and $\mu_Q$, represent the dielectric constant, the damping coefficient, the density, the piezoelectric stress constant, and, the elastic constant of the quartz crystal, respectively; and $e$ and $A$ represent the thickness and area of the crystal; the permittivity of free space is represented by $e_0$.

Electrical elements of measured impedance and phase curves of the TSM
device are fitted to the equivalent circuit model which is then used to extract information about the interfacial interactions of the solid and liquid phases. For example, Figure 1.14 depicts a number of characteristic frequencies which can be derived theoretically in terms of the circuit elements from the impedance and phase curves. Impedance is a characteristic of an ac circuit that consists of capacitance or inductance and is analogous to the resistance in dc circuits. The impedance of each of the circuit elements can be stated in terms of angular frequency, $\omega$, where $\omega=2\pi f$ and $f$ represents the frequency of the voltage source (Figure 1.15.). The impedance of the entire BVD circuit, $Z$, is complex-valued and can be described as the impedance of its four individual components, as follows:

$$Z = R + jX$$

where,

$$X = \frac{\omega L}{\omega C}$$

The impedance, $Z$, can also be defined as the ratio of the voltage across the crystal to the current flowing through the crystal. The resistance, $R$, is the real component of the impedance and the reactance, $X$, makes up the imaginary component, as $j$ is the complex unit $\sqrt{-1}$. The reactance, $X$, is a function of the angular frequency of oscillation, $\omega$, and the magnitudes of the circuit elements $L_m$, $C_m$ and $C_o$. When $X$ is positive, the inductance dominates and, thus, the circuit is termed to have inductive reactance; when $X$ is negative, the capacitance is dominant and the circuit is termed to have capacitive reactance. When the voltage and the current are in phase (i.e. the two
The admittance diagram of the TSM sensor which depicts the relative positions of the characteristic frequencies. The admittance ($Y$) is made up of two components, susceptance ($B$) and conductance ($G$) such that $Y = G + jB$.
Figure 1.15. The impedance-frequency profile of the TSM sensor operating in liquid. Important impedance points ($Z_{\text{min}}$ and $Z_{\text{max}}$) and characteristic frequencies ($f_n$, $f_s$, $f_{\theta x}$, $f_p$, and $f_x$) are indicated. Note that $\theta = \tan^{-1}\left(\frac{X}{R}\right)$. 
components of $X$ are counterbalanced), meaning $X=0$ and $Z=R$, the circuit is behaving as a pure resistor. When this occurs, the circuit is considered to be in resonance; the corresponding frequency, $f_r$, is the resonance frequency of the circuit. Using equation (11) and the relationship $\omega = 2\pi f$, resonance frequency for the circuit involving the elements $R$, $L$ and $C$ connected in series is:

$$f_r = \frac{1}{2\pi} \frac{1}{\sqrt{LC}}$$  \hspace{1cm} (12)

For the BVD equivalent circuit of the TSM device, the total impedance of the device, $Z_{AB}$, is derived from the individual impedances of the circuit elements:

$$Z_{AB} = \frac{R_m + j(\omega L_m - \frac{1}{\omega C_m})}{1 - \omega^2 L_mC_o + \frac{C_o}{C_m} + j\omega R_mC_o}$$  \hspace{1cm} (13)

When the oscillator is at resonance (i.e. the voltage and current are in phase), setting the imaginary component of equation (13) to zero gives an equation which reduces to a quadratic equation by assuming the device is operated in air. When the TSM is operated in the gas phase, the motional resistance, $R_m$, is approaching zero. Thus, assuming $R_m=0$, the resultant quadratic equation gives two roots, $f_R$ and $f_A$, where $f_R$ represents the resonant frequency and $f_A$, the anti-resonant frequency.

$$f_R = \frac{1}{2\pi} \sqrt{\frac{1}{L_mC_m}}$$  \hspace{1cm} (14)
\[ f_\Lambda = \frac{1}{2\pi} \sqrt{\frac{1}{L_mC_m} + \frac{1}{L_mC_o}} \]  

(15)

\[ f_\Lambda = f_R \left(1 + \frac{1}{2r}\right) \]  

(16)

where \( r = C_o/C_m \). The above equations indicate that \( f_R \) is independent of \( C_o \), whereas \( f_\Lambda \) is dependent on \( C_o \). As well, equation (16) indicates that \( f_\Lambda \) is always greater than \( f_R \).

When the device is not operated in the gas phase, the motional resistance is no longer negligible because of viscous damping and large energy loss, and thus, \( R_m \neq 0 \). The corrected resonance and antiresonance frequencies are called the series resonance frequency, \( f_s \), and the parallel resonance frequency, \( f_p \), respectively.

\[ f_s = f_R \left(1 + \frac{r}{2Q^2}\right) \]  

(17)

\[ f_p = f_R \left(1 + \frac{1}{2r} - \frac{r}{2Q^2}\right) \]  

(18)

where,  

\[ Q = \frac{\omega L_m}{R_m} = \frac{1}{\omega R_m C_m} \]  

(19)

The quality factor of the oscillating device, \( Q \), gives an indication of the device’s ability to sustain its oscillation and each crystal has a unique value. It is physically defined as:
The frequencies $f_0$ and $f_c$ are determined at zero-phase and $Z$ at pure resistance. Although they can be measured separately using the oscillator method, the network analysis method permits simultaneous determination. The characteristic frequencies at maximum and minimum impedances, termed $f_{Z_{\text{MAX}}}$ and $f_{Z_{\text{MIN}}}$, respectively, can also be derived via circuit analysis.

\[
Q = 2\pi \frac{E_{\text{Stored}}}{E_{\text{Dissipated}}}
\]  

\[
f_{Z_{\text{MAX}}} = f_R \left(1 + \frac{1}{2r} - \frac{r}{2Q^2}\right)
\]

\[
f_{Z_{\text{MIN}}} = f_R \left(1 - \frac{r}{2Q^2}\right)
\]
II. RESEARCH OBJECTIVES

Recent research has focussed on the use of DNA sequences as disease markers, pharmaceutical targets for drugs and antisense therapy. In addition, the Human Genome Project aims to sequence the complete human genome and, eventually, to relate segments to morphology and function. As such, there is a great desire to develop a means of detecting nucleic acid or DNA interactions with other molecules, such as other nucleic acids, DNA, RNA, proteins, and small molecules, with specificity. An important aspect of this sensor would be the ability to reuse it. This is particularly important for improving reproducibility as most available biosensors which incorporate disposable surface-modified components cannot be guaranteed to be exactly the same between experiments. Another essential factor in the development of this sensor is the ability to monitor interactions in real-time.

For the use of the TSM sensor as a nucleic acid sensor, the probe must be attached to the sensor surface. This has been accomplished with some success using an avidin-biotin protein-substrate binding event, as the nucleic acid probe can be easily modified to have a terminal biotin. However, this was found to exhibit poor reproducibility. Thus, research was undertaken to study parameters which may affect the adsorption of the protein - electrolyte concentrations (NaCl at 0, 70 and 650 mM), addition of a detergent, surface morphology (rough or polished), electrode metal (gold or silver) and cleaning procedure (plasma and non-plasma) were studied. The plasma-cleaned and non-plasma-cleaned surfaces were characterized by stationary
contact angle measurements. Following this study, the adsorption of avidin was compared with that of neutravidin, a non-glycosylated derivative of avidin. X-ray photoelectron spectroscopy was performed on both hydrophobic and hydrophilic surfaces with either no protein, avidin or neutravidin adsorbed onto the surface. Once the optimal experimental conditions were established, work continued using the thickness shear mode (TSM) acoustic wave sensor to study nucleic acid interactions with small molecules.

Work previously carried out using the TSM acoustic wave sensor showed that oligonucleotide-small molecule interactions, specifically, the TAR RNA/Tat peptide, could be detected. However, some unexpected trends in the TSM response instigated further investigation. The first part of this work involved a comparison of radiochemical experiments with results from the thickness shear mode (TSM) acoustic wave sensor. The TSM response to non-specific versus specific interactions was of particular interest. In order to accomplish this, the biotinylated TAR RNA (B-TAR) and Tat₁₂ peptide were radiochemically labelled with ³²P and ¹²⁵I, respectively. The B-TAR was immobilized to the TSM device via neutravidin adsorbed to the surface and quantified. This surface was then exposed to solutions of Tat₁₂ peptide and then the amount of peptide was quantified. In order to determine the amount of non-specific adsorption possible, the neutravidin-modified surface was exposed to Tat₁₂ and the peptide on the surface was quantified. These results were then compared with corresponding TSM response values.

The desired nucleic acid sensor requires the ability to differentiate specific interactions. Hence, a study ensued involving comparisons of the TSM sensor
response for complementary, non-complementary and single-base mutated oligonucleotide interactions with an immobilized oligonucleotide probe. All of the complementary, single-base mutated and probe oligonucleotides were 25-mers; one of the non-complementary oligonucleotides was also a 25-mer, the other was an 11-mer. The temperature conditions were manipulated to induce greater differentiation between TSM sensor responses for complementary versus single-base mutated interactions with the probe.

It is possible to denature dsDNA by altering a number of experimental conditions. These include temperature, ionic strength, and solvent composition (i.e. incorporate urea). However, the TSM sensor also responds to changes in these parameters and the TSM device cell is corroded by non-aqueous solvents. In addition, high temperatures may also affect the stringency of the neutravidin-biotin binding by denaturing the protein. Thus, it is not practical to consider manipulating these factors to melt the oligonucleotide hybrids. In living organisms, enzymes are used to melt dsDNA for replication and repair. Upon careful inspection of our probe-target system and commercially available DNA digestion enzymes, λ-exonuclease was chosen for its ability to digest a single strand of dsDNA in the 5' to 3' direction, leaving the opposing strand intact. This enzyme would, in theory, digest the target strand and leave the probe strand intact on the surface. The TSM response was studied and compared to radiochemical data obtained by performing similar experiments with 32P-labelled target strands.

Recent research in genetics and genomics have led to the development of DNA and nucleic acid microarrays. Regeneration of the microarray probe surface has
become an issue of concern and quite desirable, particularly for reasons of reproducibility. Research parallel to the set of experiments set up on the TSM acoustic wave device and using radiochemistry, was carried out using glass slides, similar to those used in DNA/nucleic acid microarray research. These slides were derivatized with a thiol-functionalized silane in order to covalently attach thiol-modified oligonucleotide probes. The probes were also functionalized with a fluorophore and, thus, could be analyzed with a confocal microscope, as in microarray technology. Hybridization was initiated using targets modified with a different fluorophore and was measured using a confocal microscope. Digestion by λ-exonuclease was also characterized by confocal microscopy.
III. EXPERIMENTAL

1. REAGENTS

All reagents mentioned below were freshly prepared from the specified commercial product without further treatment. ImmunoPure neutravidin was purchased from Chromatographic Specialties (Brockville, ON, Canada). The A, G, C and T CPG columns and phosphoramidites and biotin phosphoramidite were purchased from Applied Biosystems and BioCan Scientific. The reagents for the DNA/RNA synthesizer: tetrazole/acetonitrile; 1-methylimidazole/THF; acetic anhydride/pyridine/THF; and iodine/water/pyridine/THF, and anhydrous acetonitrile were purchased from Applied Biosystems and aqueous ammonia was purchased from Sigma-Aldrich Canada. The solvents aqueous ammonia, trifluoroacetic acid, acetonitrile used for purification were obtained from Sigma-Aldrich and prepared in the appropriate concentrations by dilution with deionized water. The triethylammonium acetate (TEAA) for purification was made from glacial acetic acid and triethylamine (Sigma-Aldrich) and deionized water. A buffer solution of 10 mM Tris-HCl (Canadian Life Technologies), 70 mM NaCl and 0.2 mM EDTA (Sigma-Aldrich) was used to prepare neutravidin and oligonucleotide solutions for experiments. A buffer solution of 67 mM Glycine-KOH (Sigma-Aldrich), and 2.5 mM MgCl₂ (Sigma-Aldrich) and pH 9.4 was used to prepare oligonucleotide and λ-exonuclease for experiments. Lambda exonuclease was purchased from Canadian
Life Technologies (product #28023-018). [γ-32P] ATP was purchased from Mandel Scientific Company Ltd. (product NEG 502Z). 10X T4 PNK reaction buffer (contains Tris-HCl, MgCl2, dithiothreitol) and T4 Polynucleotide Kinase were purchased from New England Biolabs. Solutions of 3N NaOAc and phenol/chloroform/isoamyl alcohol were prepared from reagents purchased from Sigma-Aldrich. CHROMATIDE Alexa Fluor 546-14 dUTP (product #C-11401) and CHROMATIDE BODIPY 630/650 dUTP (product #C-11395) were purchased from Molecular Probes Inc. The labelling kits (including 10X NE Reaction Buffer, CoCl2, and deoxynucleotidyl transferase) were purchased from New England Biolabs Ltd. (3-mercaptopyrrol)-trimethoxysilane, aqueous ammonia, anhydrous ethanol, and acetic acid were purchased from Sigma-Aldrich Canada and used as received. Sodium bicarbonate/sodium carbonate (500mM, pH 9.0) was prepared from chemicals purchased from Sigma-Aldrich. Tween 20 was purchased from Sigma-Aldrich.

Non-biotinylated, 5'-thiol-modified-F1, 3'-biotin-F1, F2 and MN-F2 used for 32P labelling and/or fluorophore labelling for radiochemical and fluorescent analysis, as well as F3 and F4, were synthesized by the Centre for Applied Genomics, The Hospital for Sick Children, Toronto, ON, Canada. The purified sequences were freeze-dried and stored at 4 °C until required.

Biotin-TAR RNA and Tat12 peptide were provided by Parke-Davis pharmaceutical research, Ann Arbor, MI, USA.
2. OLIGONUCLEOTIDE SYNTHESIS

Oligonucleotides synthesized for this research were:

F1:  \[5'\text{-biotin-TATAAAAAGAGAGAGGATGAGTC}\ - 3' \]
F2:  \[5'\ - \text{GACTCGATCTCTCTCTCTTTTATA} - 3' \]
F2-T4C:  \[5'\ - \text{GACTCGATCTCTCTCTCTTTTATA} - 3' \]
F2-T4G:  \[5'\ - \text{GACTCGATCTCTCTCTTTTATA} - 3' \]
F2-C13A:  \[5'\ - \text{GACTCGATCTCTCTCTTTTTATA} - 3' \]
F2-C13T:  \[5'\ - \text{GACTCGATCTCTCTCTTTTTATA} - 3' \]
F2-T14C:  \[5'\ - \text{GACTCGATCTCTCTCTTTTTATA} - 3' \]
F2-T14A:  \[5'\ - \text{GACTCGATCTCTCTCTTTTTATA} - 3' \]
F2-C21A:  \[5'\ - \text{GACTGATCTCTCTCTTTTTATA} - 3' \]
F2-C21T:  \[5'\ - \text{GACTGATCTCTCTCTTTTTATA} - 3' \]
X-F2:  \[5'\ - \text{ATAATTTCCTCTCTCTTAGCTCA} - 3' \]
"73%":  \[5'\ - \text{ATCTCGCGTCT} - 3' \]
MN-F2:  \[5'\ - \text{TCAGATCGAGAGAGAGGGGCGC} - 3' \]
F3:  \[5'\ - \text{biotin-CGTACCGATCAGAGGAGTCAGTACGC} - 3' \]
F4:  \[5'\ - \text{GCCTCTGATCTCTGATCCCGTGAC} - 3' \]

\(a\) all oligonucleotides were freeze- or vacuum- dried and stored at 4°C until required
\(b\) a thiol modifier is in place of biotin for immobilization on glass slides
\(c\) bold and underlined base indicates base altered from sequence F2
\(d\) italicized and/or underlined bases denote regions complementary to segment(s) of F1
3. MATERIALS AND APPARATUS

Purification Columns. Poly-pak ion exchange columns used for the purification of synthesized nucleic acids were purchased from BioCan Scientific. Centri-Sep purification columns (Princeton Separations Inc.) were used according to the accompanying protocol.

Crystals. The optically polished 9 MHz AT-cut piezoelectric quartz crystals with gold electrodes were purchased from International Crystal Manufacturing Inc.

Glass Slides. Glass slides were purchased from Sigma-Aldrich Canada (product #S 8400).

On-line TSM Instrument. A schematic of the configuration employed for both the introduction of reagents and TSM measurement in a flow-through manner are shown in Figures 3.2 and 3.3, respectively. The devices mentioned above were assembled into a Plexiglas cell where two halves were separated by O-rings. One face of the crystal was in contact with both flowing and static aqueous solutions, while the other face was kept under nitrogen. Temperature was controlled through a Hybaid Micro-4 hybridization oven (HB-MCR4, InterScience, Markham, ON) and measured by a mercury thermometer. Flow-through experiments were performed using a 4-channel EVA-pump Model 1000 peristaltic pump (Eppendorf) which was adapted and combined with an EVA-Valve Model 2000 injector valve (Eppendorf). The PTFE tubing for the sample loop had an inner diameter of 0.5 mm, while all the other tubings had an internal diameter of 0.8 mm. Finally, the TSM responses in liquid were

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measured by an HP 4195A network/spectrum analyzer (Hewlett-Packard). The values of the equivalent circuit were calculated internally by the analyzer from measured data. The calibration of the network analyzer at a centre frequency of 9 MHz was accomplished using a pre-set calibration program. The analyzer scanned 401 points about a centre frequency of 9 MHz (with 120 kHz bandwidth). The system was set, by user input, to record the acquired data every 30 seconds.

Contact angle measurements were obtained using a goniometer, courtesy of Dan Kwok, Dept. of Engineering, University of Toronto.

X-ray photoelectron spectroscopy (XPS) spectral data were obtained with a Leybold MAX 200 XPS (Leybold, Cologne, Germany) instrument using an unmonochromatized Mg Ka source and an analysis area of $2 \times 4 \text{ mm}^2$. Survey and low resolution spectra were obtained using a pass energy of 192 eV; high resolution spectra were acquired with a pass energy of 48 eV. All spectra were satellite-subtracted and normalized using software and elemental sensitivity factors provided by the manufacturer. The binding energy scale was further calibrated to 285.0 eV for the main C (1s) feature in order to compensate for sample charging effects.

Scintillation Counter. Radiochemical counting of solutions containing $^{125}$I-labelled Tat$_{12}$ and $^{32}$P-labelled B-TAR and devices coated with these species was accomplished using a Riastar 103271 $\gamma$-ray detector (Hewlett-Packard) and a 1219 Rackbeta scintillation counter (Fisher Scientific), respectively.

Confocal Microscope. A ScanArray 4000 Microarray Analysis System (Packard BioChip Technologies), including ScanArray Acquisition Software, was used to scan the slides and analyze data, respectively.
Figure 3.1. Diagram of a 9 MHz AT-cut quartz crystal with gold electrodes on each face.
Figure 3.2. Schematic diagram of the TSM sensor flow cell.
Figure 3.3. Instrumental set-up.
4. PROCEDURES

a. Effect of Solution on TSM Response

The TSM sensor devices were placed in the cell holder and connected to the network analyzer. A constant flow of buffer was maintained until a stable frequency was established. At this point, the buffer was changed or 500 μL of various concentrations of avidin or neutravidin were injected. The series resonance frequency was monitored until a new stable frequency value was achieved. The original buffer was then reapplied.

b. Radiochemical Analysis of TAR-Tat12 Interaction

The Tris buffer was initially washed through the flow cell at a flow rate of 0.1 ml/min for 20 min. A 500 μL injection of 1 mg/mL neutravidin was made, followed by additional washing by buffer flow for 30 min. At this point, either 500 μL containing 1 nmol 125I-labelled Tat12 (Tat12*) or 500 μL containing 100 pmol 32P-labelled or cold biotinylated-TAR (B-TAR* or B-TAR, respectively), was injected. For the injection of either Tat12* or B-TAR*, the TSM sensor was subsequently washed with buffer for 40 min and then removed from the cell holder and measured. The injection of B-TAR was followed with 75 min of buffer washing and injection of 500 μL of 1 nmol Tat12*. The TSM sensor surface was then washed with continuous flow of buffer for 40 min, removed and measured. For some experiments, this last procedure was repeated.
c. Network Analysis of Hybridization

The buffer was initially washed through the flow cell at a flow rate of 0.075 mL/min. The flow of the buffer was maintained until a stable frequency reading was attained. A 500 μL volume of a 1 mg/mL solution of neutravidin was then injected. The pump was stopped when the injection was completed for approximately seven minutes before the flow stream was switched back to the buffer. After the frequency was observed to maintain a level reading, a 500 μL aliquot of solution containing biotinylated F1 was injected into the flow system. The pump was then stopped to allow the neutravidin-biotin reaction to equilibrate. The buffer solution was applied after this step. A 500 μL aliquot of non-biotinylated nucleotide was injected subsequent to the observation of a stable frequency. Again, the pump was stopped to allow the interaction between the immobilized oligo and the probe oligo to equilibrate. The buffer was then reapplied to the modified crystal surface.

Variations of this basic procedure were performed to study the hybridization events at higher flow rates. A nominal flow rate of 0.3 mL/min was used for these experiments.

d. Network Analysis of TSM Sensor Regeneration by λ-exonuclease

The experimental protocol was followed as described above with the exception that, following the stabilization of the immobilized biotin-F1, the buffer was switched from Tris buffer to Glycine buffer. All of the reactants used in subsequent steps were also suspended in Glycine buffer. The frequency was monitored and, after
re-stabilization, target oligonucleotide was introduced. Once again, the pump was
stopped to allow complete interaction of probe and target before reintroducing buffer.
Once the frequency was again stable, 80 μL of dilute λ-exonuclease (5 μL
λ-exonuclease + 75 μL buffer) was introduced, followed immediately with 80 μL of
buffer. The pump was then stopped for 15 min before reintroducing constant flow of
buffer. At this point, the experiment may be stopped or cycled through the target
hybridization and λ-exonuclease digestion steps a number of times.

e. 32P Labelling of Nucleic Acids

A solution of 42 to 200 pmol of nucleic acid, T4 Polynucleotide Kinase (PNK),
T4 PNK buffer, and [γ-32P] ATP was incubated for 15 min at 37°C. The reaction
solution was then extracted with d.d. H2O, NaOAc and phenol/chloroform/isoamyl
alcohol after centrifuging for 15 min following vigorous mixing. The aqueous layer
was washed with chloroform to remove traces of phenol. Three volume equivalents of
ethanol were added to the aqueous layer and this solution was kept on dry ice 30 min
to overnight. The supernatant was drawn off subsequent to centrifugation for 30 min.
The nucleic acid pellet was washed with ethanol and centrifuged for 15 min, again
drawing off the supernatant and allowing the pellet to dry.
f. Radiochemical Analysis of Hybridization and Regeneration

The sequence of experimental steps was followed as previously described. However, these experiments were performed without the use of the network analyzer and, thus, the frequency could not be monitored for stabilization prior to carrying out each step. Hence, each step of the protocol was performed according to a predetermined time outline as follows:

<table>
<thead>
<tr>
<th>time</th>
<th>Expt 1</th>
<th>Expt 2</th>
<th>Expt 3</th>
<th>Expt 4</th>
<th>Expt 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Tris buffer</td>
<td>Tris buffer</td>
<td>Tris buffer</td>
<td>Tris buffer</td>
<td>Tris buffer</td>
</tr>
<tr>
<td>60</td>
<td>neutavidin</td>
<td>neutavidin</td>
<td>neutavidin</td>
<td>neutavidin</td>
<td>neutavidin</td>
</tr>
<tr>
<td>90</td>
<td>Gly buffer</td>
<td>biotin-F1</td>
<td>biotin-F1</td>
<td>biotin-F1</td>
<td>biotin-F1</td>
</tr>
<tr>
<td>105</td>
<td>5'-32P-F2</td>
<td>Gly buffer</td>
<td>Gly buffer</td>
<td>Gly buffer</td>
<td>Gly buffer</td>
</tr>
<tr>
<td>120</td>
<td>5'-32P-F2</td>
<td>5'-32P-F2</td>
<td>5'-P-F2</td>
<td>5'-P-F2</td>
<td>5'-P-F2</td>
</tr>
<tr>
<td>135</td>
<td>STOP</td>
<td>5'-exo/stop</td>
<td>5'-exo/stop</td>
<td>5'-32P-F2</td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>STOP</td>
<td>STOP</td>
<td>STOP</td>
<td>STOP</td>
<td>STOP</td>
</tr>
<tr>
<td>165</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>STOP</td>
</tr>
<tr>
<td>225</td>
<td></td>
<td>start pump</td>
<td>start pump</td>
<td></td>
<td>STOP</td>
</tr>
<tr>
<td>285</td>
<td></td>
<td>STOP</td>
<td>5'-32P-F2</td>
<td></td>
<td>STOP</td>
</tr>
<tr>
<td>315</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>STOP</td>
</tr>
</tbody>
</table>

As outlined above, the TSM sensor was exposed to 5'-exo nuclease for 90 min instead of 15 min, as in the network analyzer experiments. An additional series of experiments was performed reducing the exposure time back to 15 min, for comparison. All cpm values were corrected for background cpm by subtracting the counts obtained from pure scintillant. Additionally, all counts were corrected to time=0 for each series. The amount of 32P-F2 was calculated using cpm values obtained from known aliquots of stock diluted by the same volume of scintillant used for each sample.
**g. Fluorophore Labelling of Nucleic Acids**

The F2 and MN-F2 oligonucleotides were 3'-end labelled with Alexa 546 and non-biotinylated F1 was 3'-end labelled with either fluorescein or BODIPY 630. Approximately 100 pmol of oligonucleotide was incubated with NE Reaction buffer (potassium acetate, Tris acetate, magnesium acetate, DTT), CoCl₂, Alexa 546 dUTP, d.d. H₂O, and deoxynucleotidyl transferase for 15 min at 37°C. Subsequent to incubation, the reaction solution was mixed and then incubated for 10 min at 70°C. Purification of the labelled oligos was achieved using the Centri-Sep columns and accompanying protocol.

**h. Preparation and Silanization of Glass Substrate**

The glass slides were cleaned by immersion in 25% aqueous ammonia solution overnight and then rinsing with d.d. H₂O for 10 min followed by anhydrous EtOH. The clean slides were then immersed in a 1% 3-mercaptopropyl trimethoxysilane (MPTS), 95% EtOH, and 16 mM acetic acid solution for 30 min at room temperature. This was followed by rinsing with 95% EtOH/16 mM acetic acid (pH 4.5) and then curing under nitrogen for two hours. The slides were stored in a dessicator until required.
Figure 3.4. Structures of (a) Alexa 546- and (b) BODIPY 630/650- labelled dUTP used for modifying oligonucleotides.
i. Immobilization of Modified Oligonucleotides onto Silanized Glass

The oligonucleotide was diluted with 500 mM NaHCO$_3$/NaH$_2$CO$_3$ buffer (pH 9.0) to a concentration of 10 $\mu$M and spotted onto the silanized glass slide surface. The slides were incubated in a humid chamber for two hours at room temperature. The immobilized slides were then washed with TNTw solution (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20).

j. Confocal Microscopy Analysis of Hybridization and Regeneration

The slides were placed in humid chambers for five minutes to hydrate them. The labelled target (≥1 $\mu$M) was deposited onto the probe-modified areas using a dropper or the arrayer, under dark conditions. The humid chamber was then sealed and incubated for one hour at 37°C. The slides were then briefly rinsed with a 1 M Tris-HCl/5 M NaCl solution, followed by curing under nitrogen for 10 min and then rinsing with d.d. H$_2$O. The slides were then scanned using the confocal microscope.

To study the regeneration of the probe-modified surface by $\lambda$-exonuclease digestion, the slides were first rinsed with the glycine buffer and then dried under nitrogen and then placed in the humid chambers for five min at 37°C. A volume of dilute $\lambda$-exonuclease (45 $\mu$L + 15 $\mu$L Gly buffer) was deposited onto the slide over the hybridized area. The chamber was sealed and incubated at 37°C for 30 min. Following incubation, the slides were rinsed with glycine buffer, dried under nitrogen and scanned. The slides could then be cycled through the hybridization and digestion protocols again.
IV. RESULTS AND DISCUSSION

1. AVIDIN AND NEUTRAVIDIN ADSORPTION

Avidin is used widely in bioanalytical chemistry in order to immobilize biological species, such as nucleic acids, to substrates, through the strong bond it forms with the biotin moiety. It is common practice to synthesize DNA and RNA molecules with biotin placed at the 3' or 5' end of the nucleic acid chain. Avidin is a basic, homotetrameric glycoprotein having a total molecular mass of 67-68 kDa. The protein possesses a disulfide bond in each subunit and is expected to be adsorbed readily onto gold via a metal-sulfur interaction. Finally, each subunit is capable of binding a biotin moiety with an affinity of $10^{15}$ M$^{-1}$. Neutravidin does not contain any carbohydrate residues, has a molecular weight of 60 kDa and binds biotinylated species with approximately the same affinity as the parent molecule.

a. Effect of Buffer on avidin adsorption

In order to ascertain the optimum conditions for the real-time measurement of biotinylated RNA-peptide interactions (next section), we embarked on a study of the necessary precursor for those experiments, viz., the flow-injection analysis (FIA) adsorption of avidin and neutravidin to the TSM surface. Figures 4.1 and 4.2 depict the responses of plasma-cleaned gold surfaces to the introduction of avidin solutions containing buffer with different electrolyte concentrations and for electrodes frequency
Figure 4.1. Response of 9 MHz TSM sensor with a polished gold electrode to avidin (10 mg/ml) in various concentrations of electrolyte. [NaCl]: 0 (●), 70 (■), 650 (▲) mM.
Figure 4.2. Response of 9 MHz TSM sensor with a rough gold electrode to avidin (10 mg/ml) in various concentrations of electrolyte. [NaCl]: 0 (●), 70 (▲), 650 (●) mM.
displaying two different morphologies. Figure 4.1 shows the series resonance as a function of time for the polished gold-coated quartz crystal when a 10 mg/mL avidin solution of various electrolyte concentrations is flowed over the surface of the sensor. The comparative TSM responses using rough, gold-electrode crystals are depicted in Figure 4.2. Avidin adsorbed with the highest shift in series resonance frequency when the buffer had a salt concentration of 0 mM or 70 mM NaCl using the polished surface. The shifts in \( f_s \) were similar for both at approximately \(-120\) Hz. A buffer having 650 mM NaCl resulted in a much lower shift in \( f_s \) of only \(-60\) Hz. Likewise, the TSM responses to avidin adsorption on rough surfaces, using these different buffers, are depicted in Figure 4.2. Using the rough surfaces, the results produced were similar with a minor difference in that the 70 mM NaCl buffer produced a slightly smaller shift than the 0 mM NaCl. The observed frequency shifts were \(-130\) Hz and \(-150\) Hz, respectively. The shift in \( f_s \) for the 650 mM NaCl buffer for the rough surface was \(-70\) Hz. Both circumstances produce similar trends: as the concentration of NaCl is increased from 0 to 70 mM, there is not much difference, however, as it is increased to 650 mM, the shift is significantly diminished. It was observed that, regardless of the surface morphology, a concentration of 650 mM NaCl appeared to interfere with the adsorption of avidin. The results of these experiments confirm that 70 mM NaCl represents an optimum electrolyte medium both in terms of the level of electrode-protein interaction and macromolecular stability. Formation of layers of charged species at the electrode and/or ionic strength changes resulting in protein structural change may be responsible for the diminished response to protein at the high concentration of electrolyte, and for the reversal of behaviour at the 0 and 70 mM
values for the two surfaces. There is marginal increase in protein adsorption for both sets of experiments when a rough electrode morphology is employed; this is clearly connected to the compromise between increased electrode surface area and the ability of the protein to form a packed layer on the surface.

To further study the adsorption of avidin, detergent was added to the buffer used to make up the avidin solution. It was observed that the addition of detergent did not yield an increase in the series resonance frequency for the adsorption of avidin. It might be expected that the time required to reach maximum frequency would decrease due to the fact that detergents are generally used to denature proteins. This was not observed. The denaturing of the quaternary structure would result in the increase of molecules passing over the surface. For example, if the bonds between the subunits were disrupted, there would be four times as many molecules in the solution as original avidin molecules. This would increase the probability of the surface interacting with one of them. In addition, the subunits would become bulkier with a loss of tertiary structure. This expansion of the surface area of the proteins would increase the probability of interactions at the surface of the crystal. As the protein is further denatured, it would be expected that the time required to reach maximum adsorption would decrease due to the subsequent increases in both surface area and number of molecules. As well, the change in series resonance frequency would decrease because the resultant molecules would have lower molecular mass than native avidin. However, denaturation would also affect the disulfide bond of the avidin subunits. This would decrease the adsorption of protein, as this characteristic of secondary structure is essential to surface interaction. Using the rough gold crystals,
the frequency shifts were the same for both the experiment using the original buffer and the experiment using the buffer containing 0.1 % (w/v) of the detergent to make up the 10 µg/mL avidin solution. Both resulted in an approximate frequency shift of -150 Hz (Figure 4.3.a). The results were slightly different using the smooth crystals (Figure 4.3.b). The frequency shifts for the runs using the original buffer and the 0.1 % detergent buffer were -120 Hz and -90 Hz, respectively. A comparison of frequency shifts for the injection of the detergent-containing buffer without avidin and the injection of the 0.1 % detergent buffer with an avidin concentration of 10 µg/mL showed that the detergent adhered to the surface (Figure 4.4). A frequency shift of -34 Hz was observed for the injection of the buffer without avidin and -90 Hz for the buffer with avidin. However, upon re-application of the original buffer to the treated surface, a frequency increase of 20 to 30 Hz was observed for both cases (Figure 4.5). If it is assumed that this is due to detergent removal, this shows that the detergent interferes with avidin adsorption by also adsorbing to the surface of the TSM, but not permanently (i.e. easily removed). Since the detergent does not assist in protein adsorption, it is not advantageous to include it in the buffer. In addition, the detergent denatures the avidin and the change in frequency may be the result of ineffective adsorption of the denatured protein. The effects of the denaturation of avidin at this point in the procedure on the subsequent reactions with B-TAR RNA and Tat-12 are not known. It is possible that the avidin will not renature when the detergent is washed from the flow cell and, thus, affect the immobilization of TAR RNA.
Figure 4.3. Series resonance frequency response for adsorption of 10 μg/ml avidin in 0% and in 0.1% detergent solution on (a) polished and (b) rough, 9 MHz, gold-electrode TSM devices.
Figure 4.4. Comparison of absolute changes in frequency for 10 µg/ml avidin in 0.1% detergent solution and for 0.1% detergent solution alone.
Figure 4.5. Comparison of frequency responses for reintroduction of Tris buffer following treatment with 10 μg/ml avidin in 0.1% detergent and with 0.1% detergent alone.
b. The effect of surface morphology on avidin adsorption

A comparison of surfaces was performed using gold (polished and rough) and silver (polished) crystal surfaces. The kinetics proved to be similar for all three types of crystals. Comparison of the concentration profiles for two of three surfaces - rough gold and polished silver - (Figures 4.6 and 4.7 and 4.8) indicate that the amount of time required to reach maximum protein adsorption is the same regardless of the electrode metal and surface morphology. The shifts on the rough Au crystal surface ranged from $-100$ Hz to $-155$ Hz and on the polished Ag surface from $-95$ Hz to $-155$ Hz (Figures 4.6 and 4.7). The shift observed for $10 \ \mu g/mL$ avidin injection with the polished Au surface was at the midpoint of this range at a value of $-120$ Hz (Figure 4.1).

A comparative study of the effects of changing the parameters of the buffer and crystal surface on the subsequent steps involving B-TAR RNA and Tat-12 interactions was not performed due to an insufficient supply of these molecules. The purpose was simply to study the effect of these changes on the initial step of the experimental procedure.

c. Adsorption of Neutravidin

Neutravidin can be substituted for avidin, as a substrate for biotin. A comparison of the kinetics of adsorption of neutravidin to a gold surface is summarized in Figure 4.8. For concentrations of neutravidin greater than $5 \ \mu g/mL$, maximum adsorption is reached after approximately $1000$ s. However, the equivalent
Figure 4.6. Changes in frequency for a series of avidin concentrations (1, 2.5, 5, 10 and 500 μg/ml) on 9 MHz, rough, gold-electrode TSM devices.
Figure 4.7. Changes in frequency for a series of avidin concentrations (1, 2.5, 5, 10 and 500 μg/mL) on 9 MHz, polished, silver-electrode TSM devices.
Figure 4.8. Changes in frequency for a series of neutravidin concentrations (1, 5, 10, 500 and 1000 μg/ml) on 9 MHz, polished, gold-electrode TSM devices.
adsorption of neutravidin requires an increasingly longer period of time with decreasing concentrations. Although a minor disadvantage when it comes to real-time measurements with biotinylated species, neutravidin yields a significantly larger frequency shift which, in part, may be associated with a higher packing density of the electrode surface. Moreover, we have discovered that hydrophilic adsorptive surfaces appear to compromise the integrity of the biotin binding sites in the parent molecule. This is likely caused by reorientation phenomena related to polar surface-carbohydrate interactions.

d. Surface characterization

To characterize the polished crystals that were subjected to a plasma cleaner and those that were not, stationary contact angle measurements were obtained from both types of surfaces (Table 4.1). The crystals that were not plasma cleaned were found to have an average contact angle of 80° with a standard deviation of ± 5°. The crystals subjected to plasma cleaning and directly measured were found to have an average contact angle of 47° with a standard deviation of ± 6°. Crystals that were subjected to plasma cleaning and kept in a closed container for 12 days were found to have a contact angle of 53° with a standard deviation of ± 9°. These results prove that the cleaning treatment significantly alters the surface free energy of the crystal surface. The crystals that were plasma cleaned were hydrophilic relative to the crystals that were not. This alteration of the surface free energy was also shown to persist over at least a number of days by the observance that the crystals retained their hydrophilic character after almost two weeks. The higher standard deviation, however,

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Table 4.1. Stationary contact angle measurements for plasma-cleaned and non-plasma-cleaned 9 MHz, polished, gold-electrode TSM devices.

<table>
<thead>
<tr>
<th>Non-plasma-cleaned</th>
<th>Plasma-cleaned</th>
<th>Plasma-cleaned - 12 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>79.2</td>
<td>44.5</td>
<td>46.9</td>
</tr>
<tr>
<td>76.7</td>
<td>49.2</td>
<td>52.6</td>
</tr>
<tr>
<td>77.0</td>
<td>38.0</td>
<td>47.3</td>
</tr>
<tr>
<td>81.6</td>
<td>59.0</td>
<td>61.2</td>
</tr>
<tr>
<td>78.8</td>
<td>45.3</td>
<td>39.6</td>
</tr>
<tr>
<td>84.5</td>
<td>54.4</td>
<td>59.5</td>
</tr>
<tr>
<td>92.0</td>
<td>46.0</td>
<td>63.0</td>
</tr>
<tr>
<td>78.5</td>
<td>44.2</td>
<td></td>
</tr>
<tr>
<td>75.9</td>
<td>41.5</td>
<td></td>
</tr>
<tr>
<td>77.8</td>
<td>47.0</td>
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</tr>
<tr>
<td>77.2</td>
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</tr>
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<td>75.8</td>
<td>47.0</td>
<td></td>
</tr>
<tr>
<td>71.6</td>
<td>47.0</td>
<td></td>
</tr>
<tr>
<td>88.8</td>
<td>47.0</td>
<td></td>
</tr>
<tr>
<td>Mean = 79.7</td>
<td>Mean = 46.9</td>
<td>Mean = 52.9</td>
</tr>
<tr>
<td>Std. Dev. = ± 5.4</td>
<td>Std. Dev. = ± 6.1</td>
<td>Std. Dev. = ± 8.7</td>
</tr>
</tbody>
</table>
may indicate that the surface changes over time (i.e. variation in like-treated surfaces increases with time). The high standard deviations with all three surface types may be attributed to the fact that the crystals are unique; they are all manufactured according to the same process, yet no two crystals can be guaranteed to have the same surface properties. Due to differentiation in surfaces, each will respond uniquely to treatment by plasma cleaning.

Further characterization of the hydrophobic and hydrophilic surfaces was carried out by X-ray photoelectron spectroscopy (XPS). Spectra were obtained for both surface types under the conditions of adsorbed avidin, adsorbed neutravidin and no protein adsorption. Protein adsorption was achieved by the methods previously described, up to and including the second buffer wash. This wash was included in order to eliminate any loosely bound protein from the surface that would normally be washed from the surface at this point in a general run. Thus, XPS was performed on the surface that the biotinylated TAR RNA would encounter. Elemental analysis was executed for oxygen, carbon, and gold on the bare surfaces and nitrogen was added to the list of elements for the protein-adsorbed surfaces. The addition of nitrogen analysis was included for the protein-treated surfaces because proteins have peptide bonds and this analysis would give a verification of the presence of protein on the surface. Additionally, there was no noticeable peak for nitrogen in the survey plots for the bare surfaces to justify high resolution analysis of the nitrogen peak range for these surfaces. The results are tabulated in Table 4.2. From the percent atomic composition given, it was observed that there were significant differences in the C:O ratios of the hydrophilic and hydrophobic surfaces for both the avidin-treated and bare surfaces.
Table 4.2. Surface elemental composition data collected from XPS analysis of protein-adsorbed and bare 9 MHz, polished, gold-electrode TSM devices which have undergone plasma-cleaning (hydrophilic) and non-plasma-cleaning (hydrophobic) procedures.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Surface</th>
<th>%C</th>
<th>%N</th>
<th>%O</th>
<th>%Au</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>hydrophobic</td>
<td>44</td>
<td>2</td>
<td>9</td>
<td>60</td>
</tr>
<tr>
<td>none</td>
<td>hydrophilic</td>
<td>31</td>
<td>9</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>avidin</td>
<td>hydrophobic</td>
<td>60</td>
<td>5</td>
<td>19</td>
<td>16</td>
</tr>
<tr>
<td>avidin</td>
<td>hydrophilic</td>
<td>44</td>
<td>9</td>
<td>25</td>
<td>22</td>
</tr>
<tr>
<td>neutravidin</td>
<td>hydrophobic</td>
<td>58</td>
<td>7</td>
<td>24</td>
<td>11</td>
</tr>
<tr>
<td>neutravidin</td>
<td>hydrophilic</td>
<td>57</td>
<td>9</td>
<td>26</td>
<td>8</td>
</tr>
</tbody>
</table>
The implication is that the plasma cleaning treatment interacts with hydrocarbon impurities on the surface to force this alteration in relative atomic composition. This, in turn, affects the interaction of avidin with the gold surfaces. The high resolution spectra of the C1s and O1s regions show significant differences between the hydrophilic and hydrophobic surfaces for both conditions (Figures 4.9, 4.10, 4.12 and 4.13). The C1s spectrum of the hydrophilic, bare crystal shows a smaller peak for C-C and C-H bonds than the corresponding spectrum for the hydrophobic surface. In addition, there is the appearance of a third peak in the O1s range, which indicates the presence of a highly oxidized oxygen species. A comparison of the oxygen peaks on the spectra for the avidin-treated surfaces clearly indicates that there is different behaviour of avidin on the two surfaces. On the hydrophobic surface, there is evidence of only one peak, whereas, there is a distinctive second peak at 532 eV for the avidin-treated hydrophilic surface. Furthermore, there is a difference in the spectra for C1s of avidin on the two surfaces. There is a higher content of carboxylic carbon in the spectra for avidin on the hydrophilic surface than on the hydrophobic surface. These differences may be accounted for by the hydrophilic surface inducing a conformational change in the adsorbed avidin. This change may result in the exposure of internal acidic residues to the exterior of the protein. This would explain the increase in the carboxylic carbon peak of the spectrum for avidin on the hydrophilic surface because the XPS analysis depth is only ~50 Å. Avidin is much greater than 50 Å in diameter and, therefore residues on the interior of the protein would not be detected by XPS analysis. XPS is a dry technique and the conformation of the protein may be compromised from its state in solution. However, both surfaces are studied

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Figure 4.9. High resolution O-1s spectra of bare, 9 MHz, polished, gold-electrode TSM devices by XPS analysis after (a) non-plasma cleaning (hydrophobic) and (b) plasma cleaning (hydrophilic) procedures.
Figure 4.10. High resolution O-1s spectra obtained by XPS of avidin adsorbed onto 9 MHz, polished, gold-electrode TSM devices which have (a) hydrophobic and (b) hydrophilic surfaces.
Figure 4.11. High resolution O-1s spectra obtained by XPS of neutavidin adsorbed onto 9 MHz, polished, gold-electrode TSM devices which have (a) hydrophobic and (b) hydrophilic surfaces.
Figure 4.12. High resolution C-1s spectra of bare, 9 MHz, polished, gold-electrode TSM devices by XPS analysis after (a) non-plasma cleaning (hydrophobic) and (b) plasma cleaning (hydrophilic) procedures.
Figure 4.13. High resolution C-1s spectra obtained by XPS of avidin adsorbed onto 9 MHz, polished, gold-electrode TSM devices which have (a) hydrophobic and (b) hydrophilic surfaces.
Figure 4.14. High resolution C-1s spectra obtained by XPS of neutravidin adsorbed onto 9 MHz, polished, gold-electrode TSM devices which have (a) hydrophobic and (b) hydrophilic surfaces.
under the same conditions, varying only one condition – the surface hydrophobicity. Hence, the alteration of the protein between liquid and air states may be eliminated as factoring into the difference observed between avidin on the two surfaces. The C1s and O1s spectra for neutravidin adsorbed on the hydrophilic and hydrophobic surfaces do not display any significant differences (Figures 4.11 and 4.14). Neutravidin appears to interact equally well with both surfaces. This contrast between neutravidin and avidin interactions at the two surfaces may be linked to the fact that avidin has variable carbohydrate prosthetic groups which also interact with the surface and other avidin molecules, whereas, neutravidin does not. These sugar residues may destabilize the avidin molecule when it is attached to a hydrophilic surface to the point that the protein is structurally compromised. This would further explain the observation that the subsequent reactions of B-TAR RNA and Tat_{12} are affected by a hydrophilic surface. If avidin is structurally compromised, the biotin-binding site(s) may be affected and, thus, the TAR RNA would not be properly immobilized on the surface for interaction with Tat_{12}.
2. INTERACTION OF B-TAR WITH TAT$_{12}$

Using the aforementioned neutravidin-biotin interaction, we have studied the binding of one HIV-1 Tat-derived peptide to TAR RNA in the flow injection analysis (FIA) configuration by both network analysis and radiochemical analysis. The peptide, which consists of 12 amino acids (Tat$_{12}$) corresponding to the RNA binding domain of the Tat protein, and a 31-mer oligonucleotide RNA were used as models for the biological system (Figures 1.5 and 1.6). Following adsorption of avidin (in the non-glycosylated form) to the surface of the TSM gold electrode, a 5'-biotin modified TAR RNA (B-TAR) was allowed to interact with and bind to the neutravidin which, under the conditions employed, produced a stable nucleic acid-bearing surface. The interactions of the peptides with B-TAR could then be examined by injection of dispersions of the peptides over the sensor surface using the FIA system described previously. Experiments using $^{32}$P-labelled B-TAR and $^{125}$I-labelled Tat$_{12}$ were performed and the results are tabulated in Table 4.3. It was observed that, regardless of the presence of B-TAR, the same amount of Tat$_{12}$ would bind to the encountered surface. This may be a result of the protein-protein interactions between neutravidin and the Tat$_{12}$ peptide when B-TAR is absent. It may also be evidence that there is a significant amount of nonspecific binding of the peptide to the crystal surface.

The results of analogous network analysis study have been described in detail in the doctoral thesis of Dr. Hongbo Su$^{169}$, and will be described here only briefly for the purposes of comparison with radiochemical analysis of this phenomenon. The typical sensor responses (vs. time) for sequential introduction (followed by washing)
Table 4.3. Radiochemical experiments performed using $^{32}$P-labelled B-TAR (denoted B-TAR*) and $^{125}$I-labelled Tat$_{12}$ (Tat$_{12}$*). Solution conditions were B-TAR (500 µL containing 100 pmol) and Tat$_{12}$ (500 µL containing 1 nmol).

<table>
<thead>
<tr>
<th>Surface</th>
<th>Treatment (neutravidin +)</th>
<th>$\gamma$ - count (cpm)</th>
<th>Amount Tat$_{12}$ on Surface (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hydrophobic</td>
<td>Tat$_{12}$*</td>
<td>14 670.1 ± 237.4</td>
<td>16 ± 0.5</td>
</tr>
<tr>
<td>hydrophobic</td>
<td>Tat$_{12}$*</td>
<td>11 158.4 ± 207.0</td>
<td>12 ± 0.4</td>
</tr>
<tr>
<td>hydrophobic</td>
<td>Tat$<em>{12}$* + Tat$</em>{12}$*</td>
<td>17 633.5 ± 260.3</td>
<td>19.5 ± 0.6</td>
</tr>
<tr>
<td>hydrophobic</td>
<td>B-TAR + Tat$_{12}$*</td>
<td>10 834.1 ± 204.0</td>
<td>12 ± 0.4</td>
</tr>
<tr>
<td>hydrophilic</td>
<td>Tat$_{12}$*</td>
<td>22 210.9 ± 292.1</td>
<td>25 ± 0.8</td>
</tr>
<tr>
<td>hydrophilic</td>
<td>B-TAR + Tat$_{12}$*</td>
<td>21 295.2 ± 286.0</td>
<td>24 ± 0.8</td>
</tr>
<tr>
<td>hydrophilic</td>
<td>B-TAR*</td>
<td>$\beta$ - count (cpm)</td>
<td>Amount B-TAR on Surface (pmol)</td>
</tr>
<tr>
<td>hydrophilic</td>
<td>B-TAR*</td>
<td>4 484.00 ± 131.2</td>
<td>0.93 ± 0.03</td>
</tr>
<tr>
<td>hydrophilic</td>
<td>B-TAR*</td>
<td>3 979.50 ± 123.6</td>
<td>0.83 ± 0.02</td>
</tr>
<tr>
<td>hydrophilic</td>
<td>B-TAR*</td>
<td>3 347.50 ± 113.4</td>
<td>0.70 ± 0.02</td>
</tr>
<tr>
<td>hydrophilic</td>
<td>B-TAR*</td>
<td>5 507.57 ± 145.5</td>
<td>1.14 ± 0.03</td>
</tr>
<tr>
<td>hydrophilic</td>
<td>B-TAR*</td>
<td>4 891.00 ± 137.1</td>
<td>1.02 ± 0.03</td>
</tr>
</tbody>
</table>
neutravidin, B-TAR or non-biotinylated TAR RNA (TAR), and a number of dispersions of Tat₁₂ to the FIA instrument are shown in Figure 4.15. The adsorption of the neutravidin to the gold electrode surface of the TSM is confirmed by the reduction of \( f_s \) by a value of, typically, 300 Hz. Further passage of buffer solution results in a stabilization of \( f_s \) at a decreased frequency, confirming that the immobilized neutravidin is irreversibly bound under the conditions employed. Typically, \( f_s \) decreases a further 50 Hz on exposure of the immobilized neutravidin to B-TAR. Again, the signal stabilizes at this further decreased frequency confirming the expected strong binding of the biotin moiety to the avidin layer. The injection of a control sample of TAR RNA without the biotin modification does not generate an alteration in \( f_s \). Furthermore, no sensor response is observed in a second control experiment, where the Tat₁₂ peptide is exposed only to the immobilized neutravidin layer. In contrast, sequential injections of dispersions of Tat₁₂ to the system with attached RNA produces transient increases in \( f_s \) to a maximum level of \( \sim 30 \) Hz. This reversible response is expected in view of the transient exposure of the RNA to the peptide and the non-covalent nature of the Tat-TAR interaction.

By way of comparison of analogous research using TSM analysis and the radiochemical results presented here, it was noted that a sensor response was only observed for the specific interaction of Tat₁₂ with B-TAR attached to the surface. This implies that a binding event between Tat₁₂ and B-TAR must occur to produce a signal. The mere addition of mass to the surface through adsorption of the peptide does not alter the resonance frequency. This also displays the inadequacy of the Sauerbrey expression to explain certain phenomena arising when the TSM device is operated in
Figure 4.15. Sensor responses in series resonance frequency for sequential introduction of dispersions of Tat$_{12}$ to TSMs with surface-immobilized neutravidin and B-TAR, or neutravidin alone. Also shown is a control experiment involving injection of TAR RNA (without biotin) to a neutravidin surface. Solution conditions were neutravidin (500 µL at 1.0 mg/mL), B-TAR (500 µL containing 100 pmol RNA), Tat$_{12}$ (400 µL at a concentration of 0.8 × 10^{-6} M repeatedly exposed to neutravidin, and 1.0 ml at concentrations of 2.5, 0.8, 0.7, 0.6, 0.4, 0.2, and 0.1 × 10^{-6} M for exposure to B-TAR), buffer (10 mM Tris, 70 mM NaCl and 0.2 mM EDTA at pH 7.5).
the liquid phase. Experiments using radio-labelled B-TAR also reinforce the findings of XPS for neurtavidin. By comparing the amount of B-TAR on the neurtavidin surface of both hydrophilic and hydrophobic surfaces, it was observed that the amount of neurtavidin adsorbed to the surface was roughly the same for both conditions. This assumes that the amount of B-TAR calculated to be on the surface is representative of the amount of neurtavidin available for interaction.
3. DNA/DNA INTERACTIONS

In the development of a nucleic acid biosensor, it is important to consider the possibility of being able to differentiate various nucleic acid interactions. These include complementary, non-complementary and single-base mismatch interactions, as well as other degrees of non-complementary interactions (i.e. between single-base and fully non-complementary). Thus, research was performed using a series of synthesized oligonucleotides to study the responses of the TSM acoustic wave device to interfacial hybridization of complementary, non-complementary and single-base mismatch sequences.

a. Complementary and non-complementary oligonucleotides

Figure 4.16 shows the series resonance frequency ($f_s$) of a TSM sensor as a function of time during the introduction of neutravidin and then the biotinylated oligonucleotide, F1, to the polished Au-electrode of the device. The adsorption of the protein on the sensor surface is confirmed by the reduction in $f_s$ by a value of, typically, 300 Hz. The frequency decreases a further 50 Hz on exposure of the immobilized protein to the biotinylated oligonucleotide (F1). It should be noted that previous work has demonstrated that there is no resemblance of the experimental change in frequency caused by F1 to that predicted by the mass response theory for the surface population (determined by radiolabelling). In subsequent figures, and discussion of them, the responses for neutravidin and F1 are assumed to have occurred for convenience.
Figure 4.16. Series resonance frequency-time plot for the on-line introduction of neutravidin and biotinylated 25-mer oligonucleotide (F1). Arrows represent points of injection of indicated solutions. Experiments were conducted under ambient temperature conditions.
Typical results for experiments involving addition of the complementary (F2) and non-complementary (X-F2 and "73\%") oligonucleotides to the system described above are compared in Figure 4.17. The times of injection of the nucleic acids, the stoppage of flow and reintroduction of buffer are indicated by arrows. It is noteworthy that when the surface modified with F1 is challenged with buffer containing the fully complementary sequence there is an initial frequency decrease in the range of 31-42 Hz. We attribute this result to hybridization of the complementary oligos even under the condition of ambient temperature employed for the experiments. Series resonant frequency versus time plots for experiments in which the modified oligo surface was challenged by the non-complementary strands X-F2 and "73\%" show a significant difference from those for complementary strands. As shown in Figure 4.18, it was observed that the frequency decreases 10 to 15 Hz for the X-F2 oligomers and approximately 5 Hz for the "73\%" oligomer followed by stabilization while the crystal surface remained exposed to the DNA-containing solution. However, the frequency increases to the value observed prior to the injection once the buffer was reapplied to the crystal surface. This may be explained by the formation of intermediates which are subsequently removed by the flow of buffer. The difference in frequency shifts for the interaction of the probe and the oligonucleotides, X-F2 and "73\%", can be attributed to the relative percentage of complementarity between these oligomers and F1. The sequence X-F2 has one segment of 11 bases and one of 5 bases which are complementary to F1. In contrast, "73\%" has one segment of 4 bases and another of 3 bases that are complementary to F1. Therefore, there is a much higher probability that intermediate hybrids are formed between the probe and X-F2
Figure 4.17. Change in series resonance frequency-time plot for on-line introduction of the complementary oligonucleotide, F2, and non-complementary oligonucleotides, X-F2 and '73%', to surface-attached F1. Experiments were conducted at ambient temperatures.
Figure 4.18. Change in series resonance frequency-time plot for second challenge of F2 to F1:F2, F1:X-F2 and F1:"73%" systems. Note responses for the latter two cases and no response for the former.

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than between the probe and "73%". In addition, the F1:X-F2 hybrids should be stronger than the F1:"73%" hybrids because potentially more bases are involved in the former.

A particularly interesting feature of the plots depicted in Figure 4.17 is the tendency of the value of $f_s$ to increase for the F1:F2 interaction (on the reintroduction of buffer). Although there is a temptation to attribute this effect to "wash-off" of F2 from the device, in the vein of the mass response dogma, this is not the case as shown by the experiments summarized in Figure 4.18. These plots depict further challenges by F2 to both the F1:F2, F1:X-F2 and F1:"73%" systems. Note that in the former case no additional response associated with hybridization occurs implying strongly that no freed F1 sites are available for interaction. With respect to the other systems, where we postulated above that weakly interacting intermediates were involved, the second challenge with F2 does instigate a response. We attribute the observation for the fully complementary case to the role played by the relatively slow annealing of the hybrid under the conditions employed. Since the structure of the hybrid is subject to alteration during the time course of the experiment, we must conclude that the frequency increase is associated with changes in the viscoelastic properties of the oligonucleotide layer. Similar such increases have been seen in other systems and were attributed to either interfacial viscosity or viscoelastic effects.\textsuperscript{170}

Finally, we note that an interesting phenomenon occurred when the flow rate was increased to 0.3 mL min\textsuperscript{-1}. The resonant frequency oscillated around the before-injection baseline before settling at a decreased frequency (approximately -30 Hz shift) (Figure 4.19). This is possibly attributed to the dispersion of the F2 solution
into the flow cell and over the surface. Increasing the flow rate increases the pressure in the flow cell and alters the dispersion or concentration profile of the DNA injection relative to the slow flow rate. Thus, it is reasonable that the increased flow rate may induce different hybridization intermediates than those that occur with the slower flow rate. Conformational shifts between intermediate states could explain the oscillating series resonant frequency observed. It would then follow that the two DNA oligonucleotides form a stable hybrid state when the frequency stabilizes and no longer oscillates.

b. Complementary Hybridization as a Function of Temperature

Experiments were conducted at various temperatures in a closed, controlled environment (rather than laboratory ambient conditions) to determine the effect of this parameter on the signal associated with the interaction of complementary F2 with probe F1 (Figure 4.20). At 23°C a decrease in frequency upon injection of F2 (-38 to -50 Hz) followed by a slow increase was observed, much as described above for the experiments performed under ambient conditions. The results obtained at 25°C and 31°C consisted of frequency shifts of -55 and -60 Hz, respectively. However, the reintroduction of buffer resulted in quite different behaviour at these two temperatures in that the return to baseline signal is much more rapid at the higher temperature. We attribute this result to an increased level of annealing of the two strands. At lower temperatures there is a higher probability of the occurrence of incorrect pairing of bases. Experiments conducted at -45°C and -56°C showed that
Figure 4.19. Series resonance frequency response to F1:F2 interaction at increased flow rate.
Figure 4.20. Change in series resonance frequency versus time plot for F1:F2 interaction as a function of temperature.
there was a lower overall change in frequency (~-30 Hz) when the biotinylated probe was challenged with the complementary strand. The series resonance frequency also displayed sharp increases after the injection of the complementary strand. This is probably caused by a combination of the wash-off of the complementary strand and the stability of the formed duplex. The complementary strand does not interact with the same stability at the higher temperatures because these values are very close to the melting temperature of the double strand, 68°C. Accordingly, we propose that the subsequent increase of the signal at these temperatures is due to removal of F2 from the surface and, subsequently, from the cell. This is supported by the fact that at 45°C the signal resulting from injection of F2 is almost exactly duplicated with a second injection, indicating that the complementary strands present in the second injection are not hindered from interacting with the probe-modified surface. This implies that there is little double-stranded DNA formed to block hybridization. This was not the case at lower temperatures, as discussed above, where it was observed that the first injection of the complementary sequence blocks the subsequent interaction of F2 with F1. Thus, at these temperatures, only unstable hybrids are formed resulting in a rapid decrease of frequency.

The experiments discussed above indicate that the optimum temperature for observing complementary hybridization is in the vicinity of 25°C. However, one of the purposes of the present work is the differentiation of responses for complementary and point mutation sequences and this distinction may be enhanced at a higher temperature. Accordingly, we embarked on a detailed study of the role played by thermal effects for hybridization of F1 with a set of single-base mutated
c. Hybridization with Point Mutated Oligonucleotides

Experiments using the eight DNA sequences synthesized to be one base different from the complementary sequence, F2, were performed under ambient conditions. The results show that these sequences do hybridize with F1. The initial frequency shifts for these point mutation oligomers range from 27 to 38 Hz, which is similar to that observed for hybridization with F2 (Figure 4.21). There appears to be a difference in the general trend observed after the initial frequency shift among these hybridization events and the true hybridization with F2. The frequency of the mutant hybrids tends to drift steadily, approaching the baseline achieved prior to injection, whereas, the F1:F2 hybrids are more apt to drift to a smaller value and then stabilize. However, as with the complementary strand experiments, F2 (not the mutant) is inhibited from attaching to F1. Accordingly, the drift behaviour must reflect different annealing rates with the mutated nucleic acid.

In order to ascertain the effect of hybridization temperature on the ability to distinguish complementary and single-base mutation chemistry we performed experiments for five of the specified eight sequences - F2-T14A, F2-T4C, F2-T4G, F2-C21A and F2-C21T - at 55°C (Figure 4.22). A sixth sequence, F2-T14C was studied at 50°C. As intimated above the greatest change in frequency (~30 Hz) when F1 was challenged with complementary strand F2. Substitution of a cytidine for and a thymidine at position 14 (F2-T14C) produced a profile at 50°C similar to that for the fully complementary sequence at 55°C with the exception that the initial change
Figure 4.21. Change in series resonance frequency versus time plot for the on-line introduction of complementary and single-base mutated oligonucleotides to surface-attached F1. Experiments were conducted at ambient temperatures.
in frequency was smaller (~ -25 Hz). Comparing this with the results obtained for F2-T14A at 55°C shows that there is a smaller change in frequency for the substitution of a purine for a pyrimidine than for the substitution of a pyrimidine for another pyrimidine. In addition, the off-rate for F2-T14A appears to be higher than that for F2-T14C. This can be attributed to the difference in temperature at which the two experiments were performed, although, considering the similarity of the responses observed for F2 at these two temperatures, this factor plays a very small role. More likely, the difference lies in the fact that the hydrogen bonding and stacking interactions between the two strands are more perturbed with the T to A mutation than the T to C. This middle sequence is special in that it has purines on one strand and pyrimidines on the other. Substituting a pyrimidine on the purine strand would greatly disrupt the stacking of this purine:pyrimidine sequence, more so than the loss of hydrogen bonding by substituting a pyrimidine for another pyrimidine. If full hybridization interactions occur, the F2-T14A mutant forces two adenosine bases to interact, whereas, the F2-T14C mutant puts a cytosine opposite an adenosine. However, it must be kept in mind that the response for both of these is still within 10 Hz of the response for F2. This suggests that the mutation of a base in the middle of a 25-mer sequence does not alter the hybridization kinetics to a significant extent.

Mutating the base at the 4-position resulted in two very different responses. Substituting thymine for cytosine (F2-T4C) produced a response similar to that observed for F2-T14A. The initial decrease in frequency was ~20 Hz followed by a sharp rise in series resonance frequency and stabilization of the signal at about 15 Hz.
Figure 4.22. Change in series resonance frequency versus time plots for challenge of F1 with indicated single-base mutated oligonucleotides. Experiments were conducted at 55°C with the exception of F2-T14C, which was performed at 50°C.
above the baseline established prior to injection of the target. The observed response for substitution of the thymine for guanosine at this same position was different in that the initial change in series resonant frequency was only about -13 Hz. This observed discrepancy again shows the importance of the stacking stabilization of the double helix. Substituting a pyrimidine for another pyrimidine destabilizes hybridization interaction to a lesser extent than substituting a purine for a pyrimidine. This also gives evidence to the importance of end effects, particularly for short oligomers. Disrupting the base-pairing towards the end of the double strand has a greater effect on the overall stability of the hybrid than disrupting base-pairing in the middle as evidenced by the smaller change in initial frequency for the T4 mutants relative to the T14 mutants.

The C21 mutants resulted in series resonance frequency profiles similar to F2-T4G. Substituting cytosine for adenosine or thymine did not produce different initial frequency shifts; both resulted in a decrease of approximately 12 Hz. However, the off-rates were markedly different. The subsequent rise in frequency for F2-C21A was much sharper than for F2-C21T. This may be explained again by the fact that this substitution is a purine for a pyrimidine and would be more disruptive than the pyrimidine-pyrimidine substitution.

Double-stranded oligonucleotides in bulk solution have dynamic zippering and unzipping along about 5 base pairs of the double strand at both ends. This is called “end effects”. Thus, in bulk solution, it would be expected that mismatches at the ends affect the hybridization to a lesser extent than mismatches imposed towards the middle of the strand. This does not appear to be the case for surface-attached
oligonucleotides; mismatches at the ends of surface-attached oligonucleotides do affect hybridization interactions. It appears that the TSM sensor is more sensitive to the type of substitution (i.e. pyrimidine to purine or pyrimidine to pyrimidine) made at the surface end of the target (T4) than at the solution end of the target (C21). The disruption of the base pairing at the surface may possibly contribute to changes in the viscoelasticity and slip parameters which influence the TSM response. The C21-mismatch targets do not appear to hybridize as effectively as either the T4 or C14 mismatches, relative to the complementary F2, as observed by the relative initial changes in frequency associated with each interaction. It is suggested that this may be due to interference in the recognition of the probe for the target.
4. REGENERATION OF PROBES BY \( \lambda \)-EXONUCLEASE

One aspect in developing a commercial nucleic acid biosensor is the ability to regenerate the original probe surface. Consideration was made to the fact that the TSM cell is composed of plexiglas and, as such, cannot endure the use of solvents. As well, the TSM responds to temperature fluctuations and changes in electrolyte concentration, as well as solution composition. The probe strand is immobilized via a protein-substrate interaction and the protein would be sensitive to many of these methods for denaturing dsDNA. Thus, it was necessary to devise a method of melting or denaturing the double-stranded nucleic acids without using the common techniques of temperature and/or solvent manipulation. Proteins are used \textit{in vivo} to manipulate DNA for transcription, replication, translation, regulation, repair, etc. Inspection of commercially available nucleic acid enzymes (nucleases) resulted in the choosing of \( \lambda \)-exonuclease for this purpose. Lambda exonuclease is a trimeric toroidal protein, which attaches to dsDNA and digests a single strand processively in the 5' to 3' direction, while leaving the opposing strand intact. This was ideal because our probe was labelled with a biotin at its 5'-end and would, thus, be unavailable for enzyme attachment. Hence, the target strand would be selectively digested and the probe strand would remain intact. This enzyme is most active against blunt-ended dsDNA with an available 5' phosphate. Lambda exonuclease also requires Mg\(^{2+}\) as a co-factor and, thus, a different buffer (Glycine) was incorporated into the experimental procedure. After examination of experimental data, it was decided that it was best to change the buffer system after the attachment of the biotinylated probe and subsequent
Figure 4.23. TSM frequency response for initial F1:F2 interaction, subsequent F2 injection, λ-exonuclease digestion, and final F2 injection. Points of injection are indicated by arrows.
Figure 4.24. Comparison of change of frequency versus time plots for initial F1:F2 interaction and F1:F2 interaction after λ-exonuclease digestion. Note that the plots are almost superimposable.
washing with the Tris buffer. Figure 4.23 show that this method is successful in regenerating sites for F2 hybridization. Initially, the target produces a frequency shift response of -25 Hz but, with a subsequent second injection of F2, no further response was observed. This was followed with the λ-exonuclease digestion protocol. Following the observation of a stable signal at the new frequency, F2 was again injected over the TSM device and a response was observed for F1:F2 interaction by a frequency shift of -25 Hz. As expected, the TSM response indicates that the F2 target was digested and the F1 probe was left whole. A comparison of TSM responses for F1:F2 initial interaction and after λ-exonuclease digestion is shown in Figure 4.24 indicates a good correlation between the two responses. This clearly shows that the probe surface was successfully achieved by λ-exonuclease digestion. This comparison was taken from a separate experiment from Figure 4.23 to show that the response can be regenerated.

In order to establish that this was not unique to the F1:F2 system used, a second set of complementary 25-mer oligonucleotides was synthesized. The above experiments was repeated using the F3 probe and F4 target with similar results. Figure 4.25 is an expanded plot of the initial F3:F4 interaction followed by a second injection of F4. After allowing sufficient buffer washing, λ-exonuclease digestion was carried out, followed by a third injection of F3. As expected, the results are similar to the F1:F2 system: initial interaction between the complementary F3:F4 oligonucleotides results in a decrease of roughly 25 Hz; the second injection produces no appreciable response; whereas, a shift of -22 Hz is observed for F3:F4 interaction following λ-exonuclease digestion. A direct comparison of the TSM frequency
Figure 4.25. TSM frequency response for initial F3:F4 interaction, subsequent F4 injection, λ-exonuclease digestion, and final F4 injection. Points of injection are indicated by arrows.
Figure 4.26. Comparison of change of frequency versus time plots for initial F3:F4 interaction and F3:F4 interaction after \(\lambda\)-exonuclease digestion.
Figure 2.27. TSM series resonance frequency versus time plot for λ-exonuclease interaction with single-stranded 3'-biotinylated F1 followed by interaction with F1:F2 dsDNA.
responses for the initial and after digestion F3:F4 interactions is made in Figure 4.26.

The experiment was also performed using an F1 probe that was 3'-biotinylated and 5'-phosphorylated (Figure 4.27). In this experiment, no initial degradation of the F1 probe was observed upon exposure to λ-exonuclease. However, after the probe was exposed to the complementary target, F2, and λ-exonuclease was again introduced to the surface, a response was observed for the digestion interaction. Upon subsequent exposure to F2, a response was no longer observed. This is in direct contrast to the results using the 5'-biotinylated F1 probe. This verifies the activity of this enzyme in two ways: 1) λ-exonuclease is most active against dsDNA and thus there was no response with λ-exonuclease and the probe-modified surface, even though a 5' phosphate was available for interaction; and 2) λ-exonuclease digests in the 5' to 3' direction, hence the null response observed for F2 injection post digestion, as the probe was selectively digested because it was 5' exposed to the bulk solution containing the enzyme.
5. ANALYSIS OF RADIO-LABELLED OLIGONUCLEOTIDES

The surfaces of the TSM devices were characterized using 5'-^32P-labelled oligonucleotides F1 and F2, as well as 3'-biotinylated F1. The corrected scintillation counts and calculated amounts of ^32P-oligonucleotides are tabulated in Table 4.4. The experimental conditions differ from the TSM network analysis in that the steps were carried out according to a set time and not the re-stabilization of the frequency signal. In addition, the concentration of target F2 was varied between sets of experiments and the time of surface exposure to λ-exonuclease (i.e. time of digestion) was varied, as indicated in Table 4.4. It was observed that both the concentration and digestion time affected the trend of calculated amounts of ^32P-F2.

The first set of experiments (Table 4.4.a) employed dilute concentrations (38 pmol per injection) of ^32P-F2 and increased exposure time to λ-exonuclease (90 min) compared to the time (15 min) used for network analysis. The calculated amounts of ^32P-F2 on the surface for the different protocols indicate that hybridization between F1 and F2 occurs. Lambda exonuclease digestion reduces the amount of F2 target on the surface to less than 1.5 % the amount before digestion. Interestingly, the regeneration of F1 probe is reduced with this increased digestion time as the calculated amount of ^32P-F2 for target injection after λ-exonuclease digestion (1.5 % of the F1:F2 interaction) is not significantly different than that for the amount remaining after digestion alone (1.5 % of F1:F2) and much lower than the amount for initial probe-target interaction of 0.78 pmol. This shows that, although it appears that the probe surface was almost completely regenerated by target digestion, there was some
Table 4.4. Calculated amounts of $^{32}$P-F2 for the conditions: (a) F2 (39 pmol), $\lambda$-exo (90 min); (b) F2 (735 pmol), $\lambda$-exo (90 min); (c) F2 (750 pmol), $\lambda$-exo (15 min); (d) F2 (1490 pmol), $\lambda$-exo (15 min).

<table>
<thead>
<tr>
<th></th>
<th>(a) amount F2* (pmol)</th>
<th>(b) amount F2* (pmol)</th>
<th>(c) amount F2* (pmol)</th>
<th>(d) amount F2* (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutravidin +</td>
<td>0.04</td>
<td>0.06</td>
<td>0.15</td>
<td>-</td>
</tr>
<tr>
<td>F2*</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>F1:F2*</td>
<td>0.79</td>
<td>2.15</td>
<td>1.74</td>
<td>2.5</td>
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<tr>
<td></td>
<td>0.78</td>
<td></td>
<td>1.33</td>
<td></td>
</tr>
<tr>
<td>F1:F2* +</td>
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</tr>
<tr>
<td>$\lambda$-exo</td>
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<td></td>
<td>0.21</td>
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<tr>
<td>F1:F2 + $\lambda$-exo</td>
<td>0.016</td>
<td>0.42</td>
<td>0.37</td>
<td>1.72</td>
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<td>F2*</td>
<td></td>
<td></td>
<td>0.41</td>
<td></td>
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<tr>
<td>F1:F2 + F2*</td>
<td>0.52</td>
<td>0.72</td>
<td>0.38</td>
<td>0.96</td>
</tr>
</tbody>
</table>

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inhibition to the interaction of the renewed probe with additional target. The results also indicate that there is nonspecific binding of F2 to neutravidin at the level of 5 % of the F1:F2 hybridization interaction. There is also evidence of F1 interaction with a second injection of F2 subsequent to the initial F1:F2 hybridization, by an increase of 67 % of the amount of F2 on the surface. Neither of these phenomena is observed by network analysis as previously shown in Figure 4.18. As discussed in section 4.2, the occurrence of a specific binding event appears to be involved in the TSM response and not merely the addition of mass to the surface (as is the case for nonspecific binding).

The second series of experiments (Table 4.4.b) varied the concentration of target injections to 735 pmol per injection, while maintaining the digestion time at 90 min. Increasing the F2 concentration resulted in increased calculated amounts of F2 on the surface for all experiments. The relative amount of nonspecific adsorption decreased to roughly 3 % of the specific F1:F2 interaction. The relative amount of digestion also increased to 7.5 % from 1.5 % compared to the same experiment in (a). The amount of F1 probe available for interaction with F2 also increased by the gain in the relative amount of target interaction post digestion to 20 %, compared with 1.5 % in (a). However, the results show that there is a discrepancy between the amount of target removed and the amount of probe available for hybridization. Theoretically, if 92.5 % of the target was removed, then 92.5 % of the original amount of probe should be available for re-hybridization. It appears that there is an inhibiting factor to this interaction. Again, there was a significant amount of F2 on the surface from a second injection of F2, 35 % relative to an initial F1:F2 interaction.

The third series of experiments (Table 4.4.c) differed from the (b) series in the
time allowed for digestion; the time was reduced to 15 min (in accord with the original TSM network analysis experiments). The data from this series provided greater calculated amounts of F2 on the surfaces encountered for each experiment relative to the amounts calculated for the (b) series, with the exception of the last posted experiment, which decreased. The amount of nonspecific adsorption increased to 10 % of the F1:F2 interaction. The amount of \( \lambda \)-exonuclease digestion brought the amount of F2 on the surface down to 14 % of the amount prior to digestion. In addition, the amount of probe available for re-hybridization correspondingly increased to between 20 and 50 % of the original amount. Even so, the amount available for re-hybridization does not equal the amount of target removed by digestion. As well, the amount of F2 interacting from the second injection post initial F1:F2 interaction, decreased to 16 % relative to the (b) series.

Noting the increase of signals with the increase of concentration between series (a) and series (b), the concentration was increased between (c) and (d), keeping the digestion time at 15 min. This provided increased overall amounts of F2 for each surface, as expected. The amount of initial hybridization increased to 2.50 pmol from an average of 1.53 pmol. The amount of target removed by \( \lambda \)-exonuclease digestion was 67 % relative to the calculated amount for initial F1:F2 hybridization. This coincides with the amount of available probe for the re-hybridization, regaining 69 % of the initial amount of F2 on the surface. The small difference between these two values is considered insignificant considering the variable of using different surfaces for each experimental datum. Again, it should be noted that there is a significant response for the second injection of F2 after initial F1:F2, but this is not observed by
This series of experiments show that both the concentration of target and the
time for digestion by \( \lambda \)-exonuclease are important to the regeneration of the probe
surface. As the concentration of F2 is increased, the relative amount of target
digestion also increases. At lower concentrations, the target is more completely
removed. However, at lower concentrations, the amount of probe available for
re-hybridization also decreases. When the digestion time was increased to 90 min, the
amount of target digestion was, indeed, more complete, as might be expected.
However, this increased time also allowed for increased interaction between the
enzyme and the protein (neutravidin) layer to which the probe was attached. Thus, the
amount of probe available for hybridization decreased, though, theoretically, more
should be available based on the increased amount of target digested. The (d) series
incorporated both high concentrations of target and short digestion times. This
resulted in an equivalent amount of target removal and probe availability. Even so, the
amount of target removed was lower than the amounts for series (a) through (c).
There is, however, a tradeoff in the increased availability of probe. It would be
beneficial to consider increasing the concentration to further test this trend.

Comparison of the set of data suggests that, with increased exposure time, the
enzyme interacts with the neutravidin, forming a new protein layer, incorporating at
least part of the probe layer and inhibiting interaction with target oligonucleotides. In
effect, the probe layer is shielded from subsequent interaction with target molecules by
adsorbed \( \lambda \)-exonuclease. However, high concentrations of target and short digestion
times improve the effective regeneration of the probe surface.
6. ANALYSIS OF FLUOROPHORE-LABELLED OLGONUCLEOTIDES

Initial work with oligonucleotide probes on glass slides was performed in "macroarrays" to test the viability of the immobilization and hybridization protocols before proceeding to robotic printing of microarrays. For these experiments, the F1 probe was 5'-labelled with fluorescein and the F2 and MN-F2 targets were labelled with Alexa 546 at the 5'-end. A confocal microscope was used to scan the slides for fluorescence. On the colour scale, white represents high intensity fluorescence, then red, yellow, green and blue representing consecutively lower intensities of fluorescence. The probe-attached slides were scanned with an excitation wavelength of 554 nm and monitored at a wavelength of 572 nm for Alexa. Because of the limitations of the confocal microscope used, the slides were scanned for fluorescein at a wavelength of 532 nm and detected at 552 nm. The appropriate wavelengths of 492 nm and 512 nm, respectively, could not be achieved. Even so, fluorescence of the fluorescein label is detectable and distinguishable from the Alexa 546 fluorescence. Figure 4.28 shows that the probe immobilization was not consistently successful. Each of the three slides should display three spots where the silanized glass was exposed to the thiol- and fluorescein-modified probes. However, as noted, the apparently low intensity of fluorescence is due to the inadequacy of the particular system used. Subsequent steps with fluorescent targets indicate that there is sufficient probe immobilized to the surface. Smudging of the spots can be seen from these scans.
Figure 4.28. Analysis of three slides covalently modified with fluorophore-labelled probe Fl by confocal microscopy.
Figure 4.29. Analysis of F1-modified slides exposed to fluorophore-labelled complementary F2 by confocal microscopy. The slides (a, b, c) correspond with those in the previous figure.
and, thus, cover wells were applied to subsequent slide work. Once immobilization was verified, the fluorophore-labelled target, F2, was applied over the areas of immobilization. Figure 4.29 clearly indicates that there is some interaction between the probe and target. This is presumed to be hybridization, however, it was checked by comparing the response of complementary F2 and non-complementary MN-F2 (Figure 4.30). Two slides, which had immobilized probe were exposed to MN-F2 and F2, separately. The slide exposed to the complementary target had a significant increase in fluorescence, whereas no appreciable change in fluorescence was observed for the non-complementary interaction with the probe.

The next step was to test the activity of λ-exonuclease against the F1:F2. Thus, a slide was modified with probe F1 as per the protocol and exposed to complementary F2. At the same time, chemisorption of the target to the silanized slide was tested. The top spot indicates the area which does not have any probe and the bottom spot indicates the area which does have probe immobilized to it (Figure 4.31). All scans were performed using the protocol for the Alexa 546 dye, which was attached to the targets only. The first scan, (a), represents the fluorescence of the slide before interaction with F2. As observed, both areas appear the same prior to exposure to F2. However, as shown in (b), the amount of fluorescence for the interaction of complementary probe and target is much greater than that for mere adsorption of F2 to the derivatized glass slide. Upon subsequent exposure to λ-exonuclease digestion, there is a significant decrease in fluorescence intensities for the F1:F2 hybrids and less of a significant change for the adsorbed F2. The change in fluorescence intensity for the F1:F2 surface is expected because of target digestion and, thus, effective removal
Figure 4.30. Comparison of complementary, F2, and non-complementary, MN-F2, with immobilized F1. Confocal microscopy was performed for (a) the F1-modified slide and (b) the subsequent interaction with non-complementary MN-F2 and (c) a second F1-modified slide and (d) the subsequent interaction with complementary F2.
Figure 4.31. λ-exonuclease activity against chemisorbed F2 (top spots) and F2 exposed to immobilized F1 (bottom spots). (a) before F2 exposure; (b) after F2 exposure; (c) after λ-exonuclease digestion; and (d) after re-exposure to F2.
of the fluorophore. No change in fluorescence is expected for the area with adsorbed F2 (no F1) because as described in section 1.1.f., λ-exonuclease is only active against dsDNA. The observed change may be explained by the use of buffers and washing with copious amounts of solution. It may simply be that some of the adsorbed oligonucleotide was washed from the surface during the digestion procedure. In the final step, F2 was again applied to the two areas (d). This resulted in the increase in fluorescence of both areas to close to the same intensity as for initial exposure to F2. As observed by radiochemical experiments and, to a lesser extent TSM analysis, it is difficult to achieve 100 % digestion and re-hybridization signals.
CONCLUSIONS

The results of the preliminary work on the TSM sensor response to different solution conditions provide evidence that the TSM sensor is sensitive to a number of factors, not just added mass. The concentration of electrolyte and the presence of detergents affect the sensor response and must be taken into account when preparing experiments employing biofilms with the TSM sensor. The type of electrode and surface morphology are other factors which should be considered; different surfaces may be useful for different purposes. For our work, it was decided that polished, gold electrode crystals were the best choice. However, one should be careful concerning the treatment of these surfaces prior to use as it is possible to significantly alter the properties of the surface as observed by contact angle measurements for two different cleaning procedures.

Research concerning the HIV-1 TAR RNA and Tat protein interaction provide support for the use of the TSM sensor for detecting biomolecular chemistry at the solid-liquid interface. Radiochemical data showed that nonspecific adsorption of Tat12 occurred, however, this was not observed by TSM acoustic wave network analysis. Hence, there is a strong indication that the acoustic wave technique is capable of detecting selective binding events even when significant nonspecific adsorption occurs in concurrent fashion. Accordingly, the observations made herein indicate that a re-evaluation of the restrictive mass-response concept for a TSM structure incorporating a biofilm immersed in liquids is necessary.
The results of the TSM analysis of complementary, non-complementary and single-base mismatch oligonucleotide interactions confirm that acoustic wave devices of the transverse shear wave type act as label-free, sensitive detectors of interfacial nucleic acid chemistry. In this regard, it is particularly important to appreciate that these sensors not only respond to the presence of added or lost material, but also to changes in duplex structure. This factor is obvious in the experiments involving fully complementary hybridization where the sensor response is governed by viscoelastic and acoustic coupling changes associated with annealing effects. This opens up interesting possibilities for the application of acoustic physics to the study of macromolecular structure at interfaces and to the understanding of kinetic processes involving biochemical moieties. These observations stand in sharp contrast to the misleading mass response dogma which is being perpetuated continuously in connection with the behaviour of various types of acoustic wave devices in liquid media.

The ease of operation of the on-line TSM system in terms of flow, stop-flow and variable temperature conditions offers significant potential for the characterization of biochemical on- and off-rates. Such measurements have been delineated as being of growing importance in biochemistry in recent times, particularly off-rates.173 From this research, it was noted that the acoustic wave sensor is capable of distinguishing relatively subtle changes in the type and location of single-base mismatches with respect to oligonucleotide hybridization.

Finally, regeneration of oligonucleotide probes attached to surfaces was verified by three techniques: TSM acoustic wave analysis, radiochemical analysis, and
confocal microscopy. Lambda exonuclease digestion provided a surface which allowed complete replication of the initial response for probe-target interaction. This would indicate that the target from the initial probe-target hybrids was completely digested. Replication of these experiments using 32P-labelled target showed that the target was not completely removed from the surface by enzyme digestion. This implies that the TSM acoustic wave network analyzer must be sensitive to the interfacial binding event and not merely the addition or loss of mass. This also supports the results of the study of TAR-Tat12 interactions, as mentioned previously. Even so, radiochemical analysis showed that at high target concentrations and short digestion times, the amount of target digested is equivalent to the amount of probe available for further hybridization. Loss of probe availability at low target concentrations and long digestion times may be explained by the increased possibility for protein-protein interaction at the surface between λ-exonuclease and neutravidin. When the target concentration is lowered, there are more free probe sites and less surface area coverage, as well as fewer sites of dsDNA for the enzyme to act upon. This allows for increased area of proteins interaction. By also increasing the digestion time, the proteins are even more likely to interact. In effect, it appears that the enzyme is forming a new protein layer over the surface, thus, making it improbable that targets will have any interaction with the free probes. Since the TSM sensor is housed in a plexiglas cell, the options for removing this protein layer are limited.

Covalent immobilization of the probe onto glass slides provided a means of eliminating the underlying neutravidin layer. The results of confocal microscopy show a marked decrease in fluorescence after enzyme digestion followed by a return to
high-intensity fluorescence upon re-hybridization. Though the results were not quantifiable, relative comparison indicates that there is some residual enzyme on the surface. It is possible that, once the enzyme reaches the surface, it does not easily detach from the probe strand and remains “stuck” on the surface. This might be removed by conditions which would denature the quaternary and/or tertiary structure of the enzyme. These initial results are promising, however, more research is required on the removal of the regenerating enzyme.

It is apparent that there is still much work to do in the area of interfacial nucleic acid chemistry. The study of interactions of complementary oligonucleotides having single mismatches would be enhanced by the investigation of systems having more than one mismatch. It is also important to apply this technology to a real example of genetic defect (for example, cystic fibrosis). In order to increase the usefulness of the proposed method of probe regeneration, it would be beneficial to study other nucleases for digestion and removal of targets. Two suggestions for investigation are Mung Bean Nuclease which is a single-strand nuclease and digests ssRNA or ssDNA leaving duplex regions intact and Exonuclease VII which is an exodeoxyribonuclease that digests single-stranded DNA from both the 3’ and 5’ ends. It is also necessary to elucidate a means of enzyme removal in order to eliminate subsequent interference of any adsorbed protein(s). Finally, it is essential to investigate nucleic acid chemistry in nanolitre volumes on surfaces further to improve the regeneration of high density microarrays.
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