SURFACE-ATTACHED BIOMOLECULES AND CELLS
STUDIED BY THICKNESS SHEAR MODE ACOUSTIC WAVE
SENSOR

by

Xiaomeng Wang

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

© Copyright by Xiaomeng Wang 2008
Surface-Attached Biomolecules and Cells Studied by

Thickness Shear Mode Acoustic Wave Sensor


Xiaomeng WANG

Department of Chemistry, University of Toronto

ABSTRACT

The thickness shear mode acoustic wave (TSM) sensor, operated in a flow-through format, has been widely used in bioanalytical research. My research is mainly focused on the study of surface-attached biomolecules and cells using the TSM sensor, including lesions in DNA, conformational change of calmodulin, as well as the properties and attachment of rat aortic smooth muscle cells.

Aldehydic apurinic or apyrimidinic sites (AP sites) that lack a nucleobase moiety are one of the most common forms of toxic lesions in DNA. In this work, synthesized oligodeoxiribonucleotides containing one, two, or three abasic sites were hybridized to complementary sequences immobilized on the gold electrode of the TSM device by affinity binding. The influence of AP sites on local base stacking energy and geometry caused a dramatic destabilization of the DNA duplex structure, which was detected by the TSM sensor. The signals detected by TSM correlated well with the thermostability of DNA duplexes in solution. The results indicate that both the number of sites and their localization in the double-stranded structure influence the stability of a 19 b.p. duplex.

TSM was also used to detect the binding of ions or peptides to surface-attached calmodulin. The interaction between calmodulin and ions induced an increase in resonant
frequency and a decrease in motional resistance. In addition, these signal changes were reversible upon washing with buffer. The response was interpreted as a decrease in surface coupling induced by exposure of hydrophobic domains on the protein, and an increase in the length of calmodulin by approximately 3 Å. In addition, the interaction of the protein with peptide together with calcium ions was detected successfully, despite the relatively low molecular mass of the 2-kDa peptide.

In addition, the attachment of smooth muscle cells to various surfaces has been monitored by TSM. These surfaces include laminin, fibronectin and bare gold. The results of these experiments in terms of changes of frequency ($f_s$) and resistance ($R_m$) were analyzed. The responses of the surface-bound cells to the introduction of various ions, depolarisation events and damage subsequent to exposure to hydrogen peroxide were also observed. Morphological changes in the cells, as confirmed by atomic force microscopy and scanning electron microscopy, are correlated with results from the TSM sensor.
ACKNOWLEDGEMENTS

First and foremost, I would like to give my deep thanks to my supervisor, Prof. Michael Thompson, for his endless encouragements and guidance in the lifetime of my graduate study in Toronto. I want to thank him for providing me the opportunity to study and work in his biosensor group at the University of Toronto and for the financial support to help me complete this thesis.

Furthermore, remarkable thanks to Prof. Tibor Hianik from Comenius University, Slovak Republic, for his instruction and help for the DNA experiments. In addition, thanks for his approval for using the DNA data in my thesis. And great thanks to S. Andreev, N. Dolinnaya and T. Oretskaya from M.V. Lomonosov Moscow State University, Russia, for measuring DNA UV melting curves, which made the TSM results for DNA experiments more creditable.

Great thanks to Prof. Ren-Ke Li at University Health Network, one of the largest cancer research institutes in North America, for his generous support to let me carry out the cell culture in his research group. His warm-heartedness and profound knowledge of cell mechanisms impressed me most. I would also like to thank the whole members of his group, especially Dr. Chung-Dann Kan, Ms. Shu-Hong Li, Ms. Linda Li, Ms. Brenda Su and Ms. Yuemei Zhang for training me to do cell culture and providing me valuable advice regarding my cell experiments.

Special thanks to Prof. Ulrich Krull in the Department of Chemistry, University of Toronto, for his amazing teaching and discussion over the years, as well as his valuable suggestions to my thesis. His sincere attitude to science will always set a great lifetime example to me.

Notable thanks to Mr. Jon Ellis for the help regarding some of the calculations through my thesis. This helps me better explain the surface phenomena on TSM sensor. Valuable thanks to other members in Mike’s group, Dr. David Stone, Dr. Larissa Cheran, Dr. Scott Ballantyne, Dr. Blaszykowski Christophe, Mr. Mingquan Zhang, Ms. Hong Huo, Mr. Saman Sadeghi, Ms. Elaine Chak, Ms. Shilin Cheung, Ms. Sonia Sheikh and other excellent students. Their valuable help and friendship companies me to accomplish this thesis. I really appreciate it.

I would also like to thank Prof. Gilbert Walker for letting me use the AFM instrument in his group. And thanks to the training and help for AFM experiment provided by Dr. Shan
Zou. Furthermore, I would like to thank Mr. Doug Holmyard (Mount Sinai Hospital, Toronto) and Dr. Neil Coombs (University of Toronto) for assistance with SEM measurements.

Last but not least, I owe my loving thanks to my parents, Sufen Yang and Zhongge Wang, and my husband, Shu Pan, for their encouragements, understanding and unreserved love to me. I could not finish my study without their support. I dedicate this thesis to them for their endless love.
Table of contents

ABSTRACT .................................................................................................................................. II

ACKNOWLEDGEMENTS ........................................................................................................ IV

Table of contents ......................................................................................................................... VI

List of tables .................................................................................................................................. X

List of figures .............................................................................................................................. XI

1. Introduction ............................................................................................................................... 1

1.1 Biosensor technology .............................................................................................................. 1

1.2 Acoustic wave sensor .............................................................................................................. 1

1.2.1 Network analysis method ................................................................................................... 3

1.2.2 Influence of working environment on the operation of the thickness shear mode acoustic wave sensor (TSM) ................................................................................................. 10

1.2.3 Interfacial acoustic wave propagation and the physics of surface-attached biochemical macromolecules in liquid ............................................................................................. 11

1.3 Immobilization of biomolecules onto surfaces ..................................................................... 19

1.3.1 Immobilization by physical adsorption .............................................................................. 21

1.3.2 Covalent immobilization ................................................................................................ 21

1.3.3 Bioaffinity immobilization ............................................................................................. 22

1.3.4 Protein cross-linking and DNA conjugation ................................................................... 25

1.3.5 Entrapment of biomolecules ........................................................................................... 25
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.4 Characterization of surfaces</td>
<td>26</td>
</tr>
<tr>
<td>1.4.1 Scanning electron microscopy</td>
<td>26</td>
</tr>
<tr>
<td>1.4.2 Atomic force microscopy</td>
<td>27</td>
</tr>
<tr>
<td>1.4.3 X-ray photoelectron spectroscopy</td>
<td>28</td>
</tr>
<tr>
<td>1.4.4 Secondary ion mass spectroscopy</td>
<td>29</td>
</tr>
<tr>
<td>1.4.5 Contact angle measurement</td>
<td>30</td>
</tr>
<tr>
<td>1.5 Surfactants</td>
<td>31</td>
</tr>
<tr>
<td>1.6 DNA</td>
<td>32</td>
</tr>
<tr>
<td>1.7 Calmodulin</td>
<td>36</td>
</tr>
<tr>
<td>1.8 Smooth muscle cells</td>
<td>39</td>
</tr>
<tr>
<td>1.9 Layout of this thesis</td>
<td>43</td>
</tr>
<tr>
<td>2. Experimental section</td>
<td>44</td>
</tr>
<tr>
<td>2.1 Reagents and materials</td>
<td>44</td>
</tr>
<tr>
<td>2.1.1 Detergent</td>
<td>44</td>
</tr>
<tr>
<td>2.1.2 DNA</td>
<td>44</td>
</tr>
<tr>
<td>2.1.3 Calmodulin</td>
<td>46</td>
</tr>
<tr>
<td>2.1.4 Cells</td>
<td>46</td>
</tr>
<tr>
<td>2.2 Instruments and methods</td>
<td>47</td>
</tr>
<tr>
<td>2.2.1 Thickness shear mode acoustic wave sensor</td>
<td>47</td>
</tr>
<tr>
<td>2.2.2 UV-VIS spectrophotometer</td>
<td>50</td>
</tr>
</tbody>
</table>
2.2.3 X-ray photoelectron spectroscopy.................................................................................. 50
2.2.4 Optical microscope......................................................................................................... 50
2.2.5 Scanning electron microscopy ....................................................................................... 50
2.2.6 Atomic force microscopy ............................................................................................... 51
2.3 Procedures............................................................................................................................. 51
2.3.1 Detergent ........................................................................................................................ 51
2.3.2 DNA ............................................................................................................................... 51
    2.3.2.1 Preparation of DNA layers on the TSM electrode................................................... 51
    2.3.2.2 UV-monitored thermal denaturation of DNA duplexes........................................... 52
2.3.3 Calmodulin..................................................................................................................... 52
    2.3.3.1 Biotinylated calmodulin........................................................................................... 52
    2.3.3.2 SAM linked calmodulin........................................................................................... 55
2.3.4 Cells................................................................................................................................ 57
    2.3.4.1 Cell culture............................................................................................................... 57
    2.3.4.2 Cell introduction and cell detachment from surface................................................ 57
    2.3.4.3 Ionic influence and cell depolarization.................................................................... 58
    2.3.4.4 Cell interactions with hydrogen peroxide................................................................ 58
        2.3.4.4.1 TSM................................................................................................................... 58
        2.3.4.4.2 Optical microscope............................................................................................ 58
        2.3.4.4.3 Scanning electron microscope........................................................................... 58
        2.3.4.4.4 Atomic force microscope .................................................................................. 59
3. Results and discussion ............................................................................................................ 60
List of tables

Table 1. Oligonucleotides used in this thesis................................................................. 45

Table 2. XPS data for four surfaces in terms of major elemental ratios. Sample 1 is the new crystal cleaned by normal procedure; Sample 2 is the new crystal cleaned by SDS solution; Sample 3 is neutravidin-coated crystal cleaned by normal procedure; Sample 4 is neutravidin-coated crystal cleaned by SDS solution............................................. 61

Table 3. Changes of frequency and motional resistance caused by addition to the ODN-coated surface of the TSM with complementary (APC), non complementary ODNs (BASE) or those that contained 1 (AP1), 2 (AP2) or 3 (AP3) abasic sites. Results represent mean ±S.D from the independent experiments in each series. ....................... 69

Table 4. UV-monitored melting of 19 b.p. DNA duplexes containing AP sites (R), where *T_m – melting temperature, **ΔT_m – the difference in melting temperature for perfect control and modified duplexes........................................................................................................... 71

Table 5. Acoustic wave sensor responses for injecting different concentrations of CaCl_2 to biotinylated CaM surface................................................................................ 87

Table 6. Acoustic response on addition of the indicated sample to the sensor surface. In the first two rows, calcium ions are introduced to the surfaces indicated. The third row shows the response of calcium binding to calmodulin after the effect of the electrolyte has been removed by subtraction........................................................................ 93

Table 7. Summary of known conformational shifts of calmodulin on binding calcium, and their likely acoustic effect and device response...................................................... 94

Table 8. XPS data for three surfaces in terms of major elemental ratios....................... 108

Table 9. Comparison of cell attachment to different surfaces. .......................................116
List of figures

Figure 1. Thickness shear mode acoustic wave sensor.......................................................... 4
Figure 2. Butterworth-Van Dyke equivalent circuit of the TSM sensor: (a) the circuit parameters; (b) the impedance of the circuit. ................................................................. 5
Figure 3. (a) Typical Z-θ plots in the resonance region of an AT-cut quartz resonator. (b) Typical B-G plots in the resonance region of an AT-cut quartz resonator. ................. 9
Figure 4. Schematic diagram of interfacial factors that govern the behaviour of the TSM sensor in the liquid phase......................................................................................... 14
Figure 5. Diagrammatic representations of TSM quartz acoustic wave biosensor with (A) adsorbed rigid biolayer, showing the thickness wavelength extension and (B) the same situation in liquid, with density ρ_L and viscosity η_L. In (A), the standing wave is confined within the substrate and adsorbed layer, with reflection of the shear wave occurring at the upper surface of the adsorbate. For the ideal case, the adsorbate is assumed perfectly rigid, and there is no dissipation in the film. However, a biolayer will behave viscoelastically, and dissipate some of the acoustic energy. In (B), the liquid cannot support a shear wave, so the wave is dissipated viscously, within 177 - 250 nm from the surface for a 9 MHz crystal........................................................................ 18
Figure 6. Commonly used methods of immobilization....................................................... 20
Figure 7. Biotin-bound avidin. Biotin is shown as a stick model, and avidin is shown as a combination of both a cartoon model and a surface model. The image is produced from PDB 1AVD using PyMol................................................................. 24
Figure 8. Damaged DNA with one AP site. The AP site is shown in the form of a stick model. (Produced from PDB 1A9I.)................................................................. 34

XI
Figure 9. N-terminal domain of Ca\textsuperscript{2+}-bound calmodulin molecule, produced from PDB 1J7O........................................................................................................................................................................................................................................................................................................... 37

Figure 10. Fibronectin (top) and laminin (bottom), produced from PDB 1TTG and 1NPE. The binding site (arginine-glycine-aspartate, RGD) of fibronectin is shown in a stick model of molecular structure................................................................................................................................................................................................. 42

Figure 11. TSM instrument within 37°C incubator........................................................................................................................................................................................................................................................................................................... 49

Figure 12. Experimental setup for biotinylated CaM interacting with ions or peptide on TSM surface........................................................................................................................................................................................................................................................................................................... 54

Figure 13. Protein linked to 11-MUA SAM layer........................................................................................................................................................................................................................................................................................................... 56

Figure 14. Experimental setup for SAM-linked CaM interacting ions or peptide on TSM surface........................................................................................................................................................................................................................................................................................................... 56

Figure 15. Changes of series resonance frequency $\Delta f_s$ (full line) and motional resistance $R_m$ (dashed line) following addition of neutravidin (1), biotinylated DNA (X-BASE, see Table 1) (2) and complementary ODN (APC, see Table 1) (3). (B) represents flow of buffer A. The points of addition are indicated by arrows. ........................................................................................................................................................................................................................................................................................................... 63

Figure 16. Changes of series resonance frequency $\Delta f_s$ (full line) and motional resistance $R_m$ (dashed line) following addition of neutravidin (1), non-complementary ODN (BASE, see Table 1) (2), biotinylated DNA (X-BASE, see Table 1) (3), non-complementary ODN (BASE) (4) and ODN contained one abasic site (AP1, see Table 1) (5). (B) represents flow of buffer A. The points of addition are indicated by arrows. The general procedure and average response times for signal stabilization after addition of reagents are: Firstly, inject (B) to get stable baseline, the time was usually around 40 min. Secondly, inject (1) and wash the surface with (B) for 15 min. Thirdly, inject (2) and wash the surface with (B) for 15 min. Fourthly, inject (3), stop for 10 min and
wash the surface with (B). Fifthly, inject (4), stop for 30 min and wash the surface with (B) for 15 min. Lastly, inject (5), stop for 30 min and wash the surface with (B) for 15 min or towards the end of the experiment.  

Figure 17. Changes of series resonance frequency $\Delta f_s$ (full line) and motional resistance $R_m$ (dashed line) following addition of neutravidin (1), biotinylated DNA (X-BASE) (2) and ODN contained two abasic sites (AP2, see Table 1) (3). (B) represents flow of buffer A. The points of addition are indicated by arrows.  

Figure 18. Changes of series resonance frequency $\Delta f_s$ (full line) and motional resistance $R_m$ (dashed line) following addition of neutravidin (1), biotinylated DNA (X-BASE) (2) and ODN contained three abasic sites (AP3, see Table 1) (3). (B) represents flow of buffer A. The points of addition are indicated by arrows.  

Figure 19. UV melting curves measured at 260 nm (in integral (1) and differential (2) forms) for 19 b.p. DNA duplexes with zero, one and two abasic sites: BASE-APC (A), BASE-AP1 (B) and BASE-AP2 (C), respectively. The superposition of the melting curves for undamaged duplex BASE-APC (○) and duplex containing three abasic sites BASE-AP3 (●) (D). All measurements were performed in buffer B at a single strand concentration of 4 $\mu$M.  

Figure 20. Crystal structures of (a) apo- and (b) holo-calmodulin. The apo form is closed, with the Ca$^{2+}$-binding domain rotated to block the central helix region. The holo form shows a more open structure, with the central helix in a rigid, elongated form. The four black spheres in (b) are four calcium ions. The crystal structures were obtained from the Protein Databank$^{169,170}$, with PDB ID 1CFD for apo-calmodulin$^{171}$ and PDB ID 4CLN for the holo form$^{172}$. Both images were generated from the PDB coordinates using PyMol (DeLano Scientific, San Francisco, USA).
Figure 21. Biotinylated CaM (3.00 μM) first interacting with 1 mM MgCl₂ and 1 mM CaCl₂ in DPBS buffer containing larger than 100mM NaCl. This is followed by interaction with 1 mM MgCl₂ and 1 mM CaCl₂ in 10 mM NaCl water solution. Solid line shows frequency change and dashed line shows resistance change. The arrow shows the injection time, and the data in the parentheses represent the concentration ratio of Ca²⁺ or Mg²⁺ to Na⁺ in the solution. The first additions of CaCl₂ and MgCl₂ are in DPBS buffer, and the second additions are in NaCl solution......................... 78

Figure 22. Neutravidin interacting with 1 mM CaCl₂ and 1 mM MgCl₂. Solid line shows frequency change and dashed line shows resistance change. The arrow shows the injection time and the data in the parentheses represent the concentration ratio of Ca²⁺ or Mg²⁺ to Na⁺ in the solution. .................................................................................... 81

Figure 23. The relevant part of Figure 22 has been blown up to clearly show the frequency change and the resistance change induced by ions..................................................... 82

Figure 24. Bare gold interacting with 1 mM MgCl₂ and 1 mM CaCl₂. Solid line shows frequency change and dashed line shows resistance change. The arrow shows the injection time and the data in the parentheses represent the concentration ratio of Ca²⁺ or Mg²⁺ to Na⁺ in the solution. .................................................................................... 83

Figure 25. The reversible interaction between biotinylated CaM (2.45 μM) and 1 mM Ca²⁺/Mg²⁺ using buffer to wash away ions. Solid line shows frequency change and dashed line shows resistance change. The arrow shows the injection time................. 84

Figure 26. Biotinylated CaM interacting with different concentrations of CaCl₂. Solid line shows frequency change and dashed line shows resistance change. The arrow shows the injection time and the data in the parentheses represent the concentration ratio of Ca²⁺ to Na⁺ in the solution............................................................... 86
Figure 27. Injecting different concentrations of CaCl\textsubscript{2} and MgCl\textsubscript{2} to gold surface. Solid line shows frequency change and dashed line shows resistance change. The arrow shows the injection time and the data in the parentheses represent the concentration ratio of Ca\textsuperscript{2+} or Mg\textsuperscript{2+} to Na\textsuperscript{+} in the solution. ................................................................. 88

Figure 28. Injecting different concentrations of CaCl\textsubscript{2} and MgCl\textsubscript{2} to neutravidin surface. Solid line shows frequency change and dashed line shows resistance change. The arrow shows the injection time and the data in the parentheses represent the concentration ratio of Ca\textsuperscript{2+} or Mg\textsuperscript{2+} to Na\textsuperscript{+} in the solution........................................... 89

Figure 29. Control experiment of BSA with calcium ions. Solid line shows frequency change and dashed line shows resistance change. The arrow shows the injection time. .................................................................................................. 91

Figure 30. Biotinylated CaM interacting with Ca\textsuperscript{2+} followed by binding with peptide. Solid line shows frequency change and dashed line shows resistance change. The arrow shows the injection time. ............................................................................................................. 100

Figure 31. Injecting smMLCK peptide to gold surface. Solid line shows frequency change and dashed line shows resistance change. The arrow shows the injection time. ....... 100

Figure 32. Injecting smMLCK peptide to neutravidin surface. Solid line shows frequency change and dashed line shows resistance change. The arrow shows the injection time. ............................................................................................................. 101

Figure 33. SAM linked CaM interacting with ions and peptide. Solid line shows frequency change and dashed line shows resistance change. The arrow shows the injection time. ............................................................................................................. 103

Figure 34. XPS spectra for 11-MUA modified surface. ................................................................. 105

Figure 35. XPS spectra for EDC/NHS linked 11-MUA modified surface. ..................... 106

Figure 36. XPS spectra for calmodulin-coated surface. ......................................................... 107
Figure 37. TSM graphs for cells immobilization at different surfaces. Black line represents frequency change. Grey line represents resistance change. L, F, C, D represent laminin, fibronectin, cells, and DPBS respectively.................................................................111

Figure 38. Kinetics associated with attachment of cells. Black line represents differential frequency change. Grey line represents differential resistance change. L, F, C, D represent laminin, fibronectin, cells, and DPBS respectively.................................115

Figure 39. Detachment kinetics of the surface-attached cells when washed by trypsin. Black line represents differential frequency change. Grey line represents differential resistance change. F, C, D represent fibronectin, cells, and DPBS respectively. ........117

Figure 40. Schematic representation of the shear wave penetration depth into a muscle cell on a TSM surface. Of interest is that the shear wave does not penetrate through the entire cell, but instead dissipates within the cytoplasm. Cell sizes and shear wave dissipation into the cytoplasm are not to scale. δ is the viscous decay length into the fluid. This representation shows that when traveling through cells, the acoustic wave could not penetrate the whole cell and therefore does not reach the bulk buffer, and instead is dissipated with the cytoplasm. In reality, the measured signals are an average of acoustic wave propagation over a heterogeneous surface. ..................... 121

Figure 41. Ion effect on the attachment of cells and on cell depolarization. Black line represents frequency change. Grey line represents resistance change. .................... 125

Figure 42. The effect of RGDS peptide to the cells attached onto fibronectin surface. Black line represents frequency change. Grey line represents resistance change. The arrow shows the injection time. ......................................................................................... 127

Figure 43. (a), (b) are the microscope images of aortic smooth muscle cells on TSM crystal surface. Circles in (b) show the shrunken cells. (c) is the TSM graph. H, M represent H₂O₂ and cell culture medium respectively. Black line represents frequency change.
Grey line represents resistance change. ................................................................. 132

Figure 44. The images of surface-attached cells under the SEM. (a) shows the structure of
the cells before interacting with H$_2$O$_2$. (b) and (c) are the structures seen after
interaction. ................................................................................................................. 134

Figure 45. AFM images of the healthy cells before H$_2$O$_2$ interaction. Right: height image,
Left: phase image ........................................................................................................ 136

Figure 46. AFM images of cells after H$_2$O$_2$ interaction. Right: height image, Left: phase
image .......................................................................................................................... 137

Figure 47. AFM images for cells after both H$_2$O$_2$ interaction and medium solution washing.
Right: height image, Left: phase image ..................................................................... 138
1. Introduction

1.1 Biosensor technology

A key issue in detection science for analytical chemistry is to achieve portability and continuous on-site monitoring of a wide range of analytes\(^1\). One common strategy to reach this goal is to utilize chemical sensors. Ideally, these sensors are miniaturised measuring devices optimised to interact with a specified analyte\(^2\). Chemical sensors are comprised of three essential components: the detector, the transducer and the output system. The detector recognizes the physical stimulus which is converted to a useful, invariably electronic output by the transducer. The output system itself involves amplification, display, etc. in an appropriate format\(^3\). The term ‘biosensor’ is now generally applied to those sensors with a biological/biochemical detection system\(^3\). The critical features of the biosensor are the selectivity and sensitivity for the detection of specific targets and the reliability in the presence of other potentially interfering species. The combination of these features with miniaturization, low cost and essentially real-time measurements in a variety of applications has generated intense commercial interest from medical, clinical, industrial, agricultural, food, process monitoring and environmental areas. Among them, medical and clinical applications are foreseen as the most lucrative and important avenues for biosensors and, accordingly, most research and development has been devoted to this area.

Based on the physics of the selected transduction processes, biosensors can be classified as electrochemical, thermal, magnetic, electrical, optical and piezoelectric sensors. This thesis is devoted to one type of piezoelectric biosensor, i.e. the thickness shear mode acoustic wave sensor (TSM).

1.2 Acoustic wave sensor
The piezoelectric effect occurs in crystals without a center of symmetry\(^4\). These crystals possess a polar axis due to dipoles associated with the orientation of atoms in the crystalline lattice\(^5\). When pressure is applied to these crystals, charges are generated in the crystals due to the shift of dipoles resulting from the displacement of its atoms. If a stress is applied across an appropriate direction, the resulting atomic displacement will induce a corresponding change in the net dipole moment. This action produces a net change in electrical charge on the faces of the crystals, the degree and direction of this polarization change depending upon the relative orientation of the dipoles and the crystal faces\(^6\). In addition, crystals that exhibit this direct piezoelectric effect always exhibit the converse effect as well. Many types of crystals exhibit the direct and converse piezoelectric effect, but the electrical, mechanical, and chemical properties of quartz make it the most commonly used crystal as transducer materials in analytical applications\(^7\).

The piezoelectric effect of the transducer material allows one to generate acoustic waves at ultrasonic frequencies by applying periodically varying electrical fields\(^8\). As a result, the biosensors based on this principle have been named as acoustic wave sensors. Currently, two general classes of acoustic wave sensors have been studied and some of them have been commercialized. These sensors include bulk acoustic wave sensors (BAW, also as TSM) and surface acoustic wave devices (SAW). In a SAW device, electrodes (named interdigital transducers) are on the same side of the quartz crystal. And the interdigital transducers act as a transmitter and receiver to excite a wave that travels across the crystal face\(^6\). The physical deformation of the wave is confined to the surface of the crystal. SAW devices were first used in chemical or biosensing applications in 1970s\(^9,10\). These devices are more mass sensitive than TSM. However, because biological solutions can severely attenuate surface acoustic waves, this sensor has not been further developed and commercialized\(^6,11\). On the other hand, TSM sensing systems have shown a practical application in bioanalytical areas and thus are the main focus of
this work.

In TSM sensors, AT-cut crystals, referring to quartz wafers cut at +35°15’ angle from the z-axis, have been used as transducer material. AT-cut crystals have a temperature coefficient of nearly zero, indicating the resonant frequencies are stable over a wide range of temperatures\textsuperscript{4, 12}. Two gold electrodes sandwich the crystal and generate the acoustic wave through the entire crystal by applying a voltage (shown in Figure 1). The particle movement in the crystal is perpendicular to the direction of wave propagation and parallel to the surface of the crystal. In order for resonance to occur a standing wave must be generated between opposite faces of the crystal and this will be achieved by matching the integer fraction of the crystal thickness with the wavelength. The relation is shown in the following equation:

\[ \lambda = \frac{2b}{n} \]  

where \( b \) is the thickness, \( \lambda \) is the wavelength and \( n \) is the resonance mode.

1.2.1 Network analysis method

Network analysis method has been used to completely characterize the TSM sensor device\textsuperscript{13-17}. This method was first developed by Kipling and Thompson\textsuperscript{18} and is also the characterization technique used in this research. As shown in Figure 2, TSM can be described by an electrical model, which is a circuit consisting of inductive, capacitive, and resistive components in series. The circuit is called the equivalent circuit of the crystal and responds to an applied voltage or current in the same way as the crystal itself. This circuit is comprised of a series branch and a parallel capacitance. The components of the series branch correspond to the mechanical model in the following manner: \( R_m \) in \( \Omega \) is the energy dissipation during oscillation due to internal friction, mechanical losses in the mounting system and acoustical losses to the surrounding environment; \( L_m \) in H is the inertial component related to the displaced mass (m)
Figure 1. Thickness shear mode acoustic wave sensor.
Figure 2. Butterworth-Van Dyke equivalent circuit of the TSM sensor: (a) the circuit parameters; (b) the impedance of the circuit.
during oscillation; and $C_m$ in F is the elasticity property of the quartz. Since the series branch is associated with the motion of the quartz plate, it is commonly referred as the motional branch. The capacitance parallel to the series branch is named as $C_0$, which is simply the static capacitance of the quartz resonator with the electrodes at frequencies far from resonance. The equivalence circuit parameters are related to physical characteristics of the crystal as shown below:

$$R_m = \frac{e^3 r}{8A\epsilon^2}$$  \hspace{1cm} (2)

$$L_m = \frac{e^3 \rho}{8A\epsilon^2}$$  \hspace{1cm} (3)

$$C_m = \frac{8A\epsilon^2}{\pi^2ec}$$  \hspace{1cm} (4)

$$C_0 = \frac{k\epsilon_0 A}{e}$$  \hspace{1cm} (5)

where $e$ and $A$ are the thickness and area of the quartz plate; $k$, $r$, $\rho$, $\epsilon$, and $c$ are dielectric constant, damping coefficient, density, piezoelectric stress constant and the elastic constant of the quartz crystal respectively; $\epsilon_0$ is the permittivity of free space.

In this research, impedance (or admittance) analysis method was used for the equivalent circuit to elucidate the properties of the TSM resonator as well as the interaction of the crystal with the contacting medium. The definition of electrical admittance is the ratio of current flow to applied voltage. Admittance and impedance are reciprocally related to each other. The total admittance of the TSM resonator is given by:

$$Y(\omega) = j\omega C_0 + \frac{1}{Z_m} + \frac{1}{Z_0} + \frac{1}{Z} = \frac{I}{V}$$  \hspace{1cm} (6)

Here $I$ is the current and $V$ is the voltage. $Z_0$ is the impedance of $C_0$ and $Z$ is the total impedance of the circuit shown in Figure 2. $Z_m$ is called motional impedance, which is the impedance of $R_m$. 
\( C_m \) and \( L_m \). \( Z_0, Z_m \) and \( Z \) can be further written as:

\[
Z_0 = -\frac{j}{\omega C_0} = jX_0
\]

(7)

\[
Z_m = R_m + j\omega L_m - \frac{j}{\omega C_m} = R_m + jX_m
\]

(8)

\[
Z = \frac{Z_0 Z_m}{Z_0 + Z_m} = \frac{R_m X_0^2 + j\left[\frac{R_m^2 X_0^2 + X_0 X_m (X_0 + X_m)}{R_m^2 + (X_0 + X_m)^2}\right]}{R_m + (X_0 + X_m)^2} = R + jX
\]

(9)

where \( X_0 \) is the reactance of \( C_0 \), \( X_m \) is the reactance of \( L_m \) and \( R_m \) in series. \( R \) and \( X \) are complicated expressions that are functions of the four parameters \( R_m, L_m, C_m \), and \( C_0 \) and the angular frequency, \( \omega \).

\( \theta \) is the phase angle of \( Z \) shown as:

\[
\theta = \tan^{-1}(X/R)
\]

(10)

The phase angle, \( \theta \), is usually simply called the phase (given in unit of radians).

When the device is in resonance in the air, \( R_m=0 \). Under this condition, if phase angle is zero, which is:

\[
X = \frac{R_m^2 X_0 + X_0 X_m (X_0 + X_m)}{R_m^2 + (X_0 + X_m)^2} = 0
\]

(11)

by calculation, one can find two different frequencies corresponding to this zero phase point. The lower frequency is approximately equal to the series resonant frequency \( f_s \). As \( \omega_s = 2\pi f_s \), the \( f_s \) expression for the unperturbed device can be calculated from the above equation, as shown in the equation below:

\[
f_s \approx \frac{1}{2\pi \left(\frac{1}{L_m C_m}\right)^{\frac{1}{2}}}
\]

(12)

The resonant frequency of TSM sensor is measured by \( f_s \). On the other hand, when \( f > f_s \), a net inductance is produced at the motional branch. As this net inductance resonates with the parallel
capacitance \( C_0 \), a “parallel resonance” is produced and therefore the higher frequency at zero phase is defined as the parallel resonant frequency, \( f_p \), given by:

\[
f_p \approx \frac{1}{2\pi} \left[ \frac{1}{L_m} \left( \frac{1}{C_m} + \frac{1}{C_0} \right) \right]^{\frac{1}{2}}
\]  

(13)

Normally, the determination of the impedance magnitude and phase angle at each frequency near the fundamental resonance of the quartz crystal can be obtained by the impedance analysis method with the measured voltage and current. Figure 3 (a) shows the magnitude and phase angle of impedance of the 9MHz crystal used in the TSM sensor for this research. The phase angle of impedance is also proportional to the difference in time between the point of maximum voltage across the crystal and the point of maximum current flowing through the crystal. The impedance analysis method fits the recorded data to an equivalent electrical network circuit and the parameters in this circuit are calculated from the impedance frequency curves. The equivalent electrical network will respond to an applied voltage and current in the same way as TSM sensor.

Since admittance and impedance are related to each other by the reciprocal relationship (shown in Equation 6), \( Y \) (admittance) has also been measured by using network analysis method. The equation for \( Y \) is shown below:

\[
Y = G + jB
\]  

(14)

where \( G \) is the conductance, and \( B \) is the susceptance. B-G plots are shown in Figure 3 (b). These admittance plots are the inverse of the impedance plots. The magnitude of the admittance \(|Y|\) is near maximum at \( f_s \) and near minimum at \( f_p \). \( \Gamma \) is the width of the resonant envelope in the admittance plots and it has direct relationship with \( R_m \). The following equation is given for \( \Gamma \):

\[
\Gamma = \frac{1}{2} \left( f_B^{\text{min}} - f_B^{\text{max}} \right)
\]  

(15)

The quality factor, \( Q \), of any resonant system is defined as the ratio between the energy stored
Figure 3. (a) Typical $Z$-$\theta$ plots in the resonance region of an AT-cut quartz resonator. (b) Typical $B$-$G$ plots in the resonance region of an AT-cut quartz resonator.
and the energy dissipated per resonant cycle. Q is given by the following equation \(^5, 19^\):

\[
Q = \frac{\omega}{2\Gamma} = \frac{2\pi f_m L_m}{R_m} = \frac{1}{R_m \sqrt{C_m}} = \frac{1}{D}
\]

where D is the dissipation. Therefore, \(\Gamma\) and \(R_m\) are the two important parameters for energy dissipation in the TSM system.

1.2.2 Influence of working environment on the operation of the thickness shear mode acoustic wave sensor (TSM)

In 1959, Sauerbrey\(^1^9\) discovered that the change in the TSM resonance frequency is related to the mass deposited onto the surface of the sensor, which is expressed below:

\[
\Delta f = -2f_0^2 \frac{\Delta m_f}{A(\mu_s \rho_s)^{1/2}}
\]

where \(f_0\) is the resonance frequency of the sensor, \(m_f\) is mass of film deposited onto the surface causing the frequency change, \(\Delta f\) is frequency change associated with the deposited mass, \(A\) is film area (cm\(^2\)), \(\mu_s\) is piezoelectric substrate shear modulus and \(\rho_s\) is piezoelectric substrate density.

However this assumption is only valid for an ideal thin layer of mass that is strongly coupled to the sensor and oscillated synchronously with the surface when operated in vacuum. Furthermore, it does not take into account viscoelastic properties of the deposited mass or the difference in acoustic impedance between the quartz and deposited layer. Therefore, when TSM is used in a liquid environment, it is advisable to develop and utilize a new model.

In a liquid environment, besides mass change from the surface, a number of different factors have to be taken into consideration for the sensor operation, such as density, viscosity, viscoelasticity, conductivity, surface free energy, surface roughness, molecular slippage, viscous energy losses and dielectric effects. Historically, many theoretical models have been developed.
to explain the process of coupling the oscillating surface to a liquid medium\textsuperscript{20-31}. However, many of these earlier models lack consideration of the interface properties between the crystal and liquid, such as surface roughness, interfacial viscosity and slippage and surface free energy, which affect the ultimate acoustic signal.

In this research, suitable models have been selectively used. In 1985, Kanazawa and Gordon developed a simple physical model which couples the shear wave in the quartz to a damped shear wave in the liquid\textsuperscript{22}. This model considers the changes of viscosity and density of the interface and is given below:

$$
\Delta f = -f_0^\frac{1}{2}\left(\frac{\eta_L \rho_L}{\pi \mu_Q \rho_Q}\right)^\frac{1}{2},
$$

where $f_0$ is the oscillation frequency of the quartz crystal, $\eta_L$ and $\rho_L$ are the absolute viscosity and density of the liquid, respectively, and $\mu_Q$ and $\rho_Q$ are the elastic modulus and density of the quartz. In this theory, the quartz is a lossless elastic solid and the liquid is a purely viscous fluid. In this system, the oscillation of the quartz crystal generates a standing wave across the thickness of quartz crystal. The frequency shift is induced by the coupling of this oscillation with a damped propagating shear wave in the adjacent liquid.

The above two models are valid under limited conditions. Sauerbrey equation can be used in the air when thin layer material is attached; and Kanazawa and Gordon equation can be used in the liquid without material attached. In addition, these two models have not considered any slippage effect due to the coupling change between surface-attached thin layer and liquid environment. This slippage effect will be discussed in the next section 1.2.3. In my experiments, suitable models are appropriately selected to include as many important effects as possible.

1.2.3 Interfacial acoustic wave propagation and the physics of surface-attached biochemical macromolecules in liquid
As mentioned above, the behavior of the resonator in liquids is governed by a number of factors, which include density, viscosity, viscoelasticity, conductivity, surface free energy, surface roughness, molecular slippage, viscous energy losses and dielectric effects of the surface-bound film and surrounding fluid. A few of these factors important to this research are introduced here. One is surface roughness. As suggested by Thompson et al.\textsuperscript{32}, surface roughness affects the response of the TSM sensor both by the increased mass loading due to liquid trapping and by the increased energy dissipation from the crystal into the liquid. Besides surface roughness, interfacial slippage also concerns acoustic wave researchers. Many theories describe TSM responses in a “no-slip” boundary condition as mentioned in both of Sauerbrey and Kanazawa/Gordon equations. This condition assumes both that liquid layer at the interface is tightly bound to the TSM metal electrode surface and that during shear motion of the device this layer does not slip relative to the metal surface. As a result, the oscillating TSM electrode and the adjacent molecular layer of the liquid will move at the same velocity. However, if there is a slip between the electrode and the adjacent fluid layer, the decay length of the shear wave propagating into the fluid and the effective thickness of the layer will be affected. The decay length, $\delta$ is given by:

$$\delta = \left( \frac{2\eta}{\omega \rho} \right)^{1/2}$$

(16)

where $\rho$ and $\eta$ are the liquid density and shear viscosity, respectively\textsuperscript{33}. The acoustic wave decays exponentially with a decay length. Ferrante et al. reported the first detailed study of molecular slip at the solid-liquid interface of an AT-cut TSM sensor\textsuperscript{34}. They pointed out that in low-viscosity liquids the slip is less than that in liquids of high viscosity, such as glycerol. Another important factor affecting TSM system is viscoelasticity. A viscoelastic film deposited on the TSM sensor surface is subjected to an oscillatory driving force at the interface of resonator. The base of film travels synchronously with the resonator surface provided the film is
bonded to the surface tightly. However, the upper portions of the films may lag behind the driving surface. As reported by Reed et al. 35, if the film is sufficiently thin and rigid, the entire film will move synchronously with the resonator surface; otherwise, the upper regions of the film will lag behind the driven resonator/film interface. Significant shear deformation is induced in the film causing elastic energy to be stored and dissipated. As a result, when the results from TSM are going to analyzed, these boundary conditions should be considered. The other factors influencing the behavior of the resonator in liquids are also shown in Figure 4.

Prior to a discussion of the physicochemical aspects of biomolecules present at the liquid-device interface, I will briefly review the physics behind the propagation of high-frequency acoustic waves through such a biomolecule monolayer. The type of transverse-shear mode acoustic wave biosensor employed in the present work functions by the generation of a standing shear wave in a piezoelectric substrate, usually electroded quartz, with a surface-adhered biologically-active layer. Biochemical events at such a layer can cause changes in wave properties that are monitored over time. The acoustic wave travels through the substrate with very little dissipation, and is reflected at the solid interface to maintain the standing wave. Changes in the location of the solid interface (the thickness of the substrate and attached layer) can be monitored as changes in the resonant frequency \( f_r \) of the standing wave. The resonant frequency is proportional to the reciprocal of the wavelength of the resonant wave, which corresponds to twice the thickness of the substrate. Changes in the resonant frequency, measured over time, can be linked to adsorption of a molecular layer on the substrate surface. Basically, this causes the thickness of the compound device to change. In the case of a purely adsorptive condition occurring in air, a decrease in the resonant frequency (measured in Hertz) corresponds to an increase in the mass or thickness of the attached layer as shown in the Sauerbery equation.
Figure 4. Schematic diagram of interfacial factors that govern the behaviour of the TSM sensor in the liquid phase.
When this device is operated in solution, a component of the energy of the standing wave is not reflected at the solid-liquid interface, and is dissipated in the liquid, since a purely viscous liquid does not support shear motion. The detector ‘feels’ the effect of the density and viscosity of the solution, which are measured as a decrease in $f_s$ and an increase in dissipation. The dissipation normally arises as a viscous damping force, and the most common measure for this is a change in motional resistance ($R_m$, in units of $\Omega$), which represents the energy dissipation in the electrical circuit approximation of the resonator device.

Another measure of dissipation is the width of the resonant envelope ($\Gamma$), as mentioned in the network analysis method. The unit of $\Gamma$ is Hertz. If the solid boundary is sharp, then the acoustic wave will reflect ideally at a single interface, with very little dispersion, and the resonant peak will be very thin. As the solid-liquid interface becomes more diffuse, and reflections occur through a range of thickness boundaries, the resonant peak widens, corresponding to greater energy dissipation. The resonance width is a useful parameter, since, under no slip condition, any change in $\Gamma$ due to surface contact with a purely viscous liquid will be of the same value and opposite to the change in resonant frequency, $f_s$, with both values being proportional to the root of the density-viscosity product of the liquid, $(\rho \eta)^{1/2}$. In fact, many authors refer to this as the ‘complex’ frequency, $f^* = f' + i\Delta\Gamma$, where $i = (-1)^{1/2}$. In this formulation, an increase in the width of resonance corresponds to an increase in dissipation, and is generally considered as a similar measurement as $R_m$.

However, if there is a slip between the solid surface and the adjacent fluid layer, the absolute value of change in $\Gamma$ will not be equal to the absolute value of change in $f_s$ induced from energy dissipation, even though the relationship between $\Gamma$ and $R_m$ is still valid. The following two equations below show how to calculate $\Delta f_s$ and $\Delta \Gamma$ separately under slip conditions$^{36,37}$:
\begin{align}
\Delta f_s &= \Delta f_a + \Delta f_{l+sl} = -\frac{2f_0^2 \Delta m_a}{(\rho_q \mu_q)^{3/2} A} - \frac{f_0^{3/2} (\rho \eta)^{3/2}}{(\pi \rho_q \mu_q)^{3/2}} \left[ \frac{1}{(1 + a)^2 + a^2} \right] \tag{17} \\
\Gamma &= \frac{f_0^{3/2} (\rho \eta)^{3/2}}{(\pi \rho_q \mu_q)^{3/2}} \left[ \frac{1 + 2a}{(1 + a)^2 + a^2} \right] \tag{18}
\end{align}

where $\Delta f_a$ is the frequency change induced by the weight of adsorbate according to the Sauerbrey equation, $\Delta f_{l+sl}$ is the frequency change induced by viscosity of the liquid environment and the interfacial slippage, and $a$ is the ratio of the slip length ($\lambda$) and the velocity decay length ($\delta$) in the liquid. Accordingly, $a$ can be written as:

\[ a = \frac{\eta}{\chi \delta} = \frac{\lambda}{\delta}, \tag{19} \]

where $\delta$ is the velocity decay length of the shear wave as shown in Equation (19) ($\delta = 177\text{nm-250nm}$ for dilute aqueous solutions at room temperature), $\chi$ is the sliding friction coefficient ($g/cm^2s$). The slip length $\lambda$ can be written as$^{38,39}$:

\[ \lambda = f \left[ \frac{\epsilon_{la}}{\epsilon_{ll}} \right] \frac{\Gamma_a}{\Gamma_m} + (1 - \frac{\Gamma_a}{\Gamma_m}) \approx f \left( \frac{\epsilon_{la}}{\epsilon_{ll}} \right) \frac{\Gamma_a}{\Gamma_m}, \tag{20} \]

where $\epsilon_{la}$ is the characteristic energy between the adsorbate and liquid interaction, $\epsilon_{ll}$ is the characteristic energy between liquid and liquid interaction, $\Gamma_a$ is the surface excess of adsorbate and $\Gamma_m$ is the maximum surface excess of adsorbate.

Because of the complex situations already mentioned, when operating TSM in liquid, it is very important to analyze the properties of biolayer-coated TSM surface in order to determine the actual situation. It is possible to select a good model to analyze the surface phenomena reported in this thesis. However, even though the above mathematical models have been semi-quantitatively applied to explain my experimental results, they are not fully accurate. Therefore, it is necessary to consider the effects of all possible interfacial properties in order to create a new comprehensive model in the future to explain the operation of the sensor in liquid.
Use of this device in a biosensor configuration always involves the interaction of surface-bound biomolecules with a ligand known to instigate structural changes in the biomolecule monolayer. These changes can be detected acoustically. However, the interpretation of such signals is complex, since the device is sensitive to changes in both energy storage and dissipation, arising from perturbations at both the substrate-layer and liquid-layer interfaces. These changes may appear as shifts in both $f_s$ and $R_m$, with separation between the two parameters presenting something of a challenge. Three main situations can affect one or both of $f_s$ and $R_m$: (a) addition or alteration of a rigid layer, (b) addition or alteration of a biochemically-based layer with viscoelastic properties, and (c) changes in interfacial properties at the liquid-solid interface, including electrolytic effects. In (a), the change of rigid layer will result in a simple shift in $f_s$. This shift can be modelled as a Sauerbrey wavelength extension,\(^{30}\) whereby an increase in the acoustic thickness of the layer results in an extension of the effective wavelength and, therefore, a decrease in the resonant frequency. This classic case is shown Figure 5 (A), and is the basis of the so-called Quartz Crystal Microbalance. Provided that the adsorbed layer is sufficiently thin and that its acoustic properties are similar to the quartz substrate, there will be no change in the dissipation.

In (b), the acoustic properties of the bound viscoelastic layer cannot be assumed to be identical to the quartz substrate and, accordingly, the extension of wavelength approximation is invalid. In this case, the film will still behave as an added mass layer, yielding a decrease in $f_s$. However, importantly, dissipative effects may occur as well and could cause an increase in $R_m$ along with the decrease in $f_s$. With respect to (c), changes in interaction at the layer-liquid interface can decouple the liquid from the device. This decoupling effectively changes the density-viscosity product mentioned above, and would appear as a decrease in $(\rho \eta)^{1/2}$. It is believed that (b) and (c) are precisely the situations that can arise from conformational or structural changes generated in a surface-attached biomolecule monolayer, which is shown in
Figure 5. Diagrammatic representations of TSM quartz acoustic wave biosensor with (A) adsorbed rigid biolayer, showing the thickness wavelength extension and (B) the same situation in liquid, with density $\rho_L$ and viscosity $\eta_L$. In (A), the standing wave is confined within the substrate and adsorbed layer, with reflection of the shear wave occurring at the upper surface of the adsorbate. For the ideal case, the adsorbate is assumed perfectly rigid, and there is no dissipation in the film. However, a biolayer will behave viscoelastically, and dissipate some of the acoustic energy. In (B), the liquid cannot support a shear wave, so the wave is dissipated viscously, within 177 - 250 nm from the surface for a 9 MHz crystal.
When TSM is used as a cellular biosensor, the situation becomes even more complicated. The average height of normal cells (around 500nm) is bigger than the decay length ($\delta$) of acoustic wave in liquid (177nm-250nm). Therefore, it is assumed that TSM can not detect the upper part of the cells. When using TSM as a cellular biosensor, the assumption is that the detection objects of TSM are extracellular matrix (ECM), cell membrane and cytoplasm. In that case, situations (b) and (c) are still valid when applied to explain the results for cellular experiments.

In the following sections, TSM is applied in the liquid phase to study surface-attached biomolecules. Three major applications are: to detect lesions in DNA, to study conformational change of calmodulin, and to analyze the attachment and properties of cells. But, before getting to the details of that work, some of the surface immobilization and surface characterization techniques will be selectively reviewed.

1.3 Immobilization of biomolecules onto surfaces

Immobilization techniques are very important for the practical use of biosensors. Ideally, after immobilization, the biological component still retains substantial biological activity; the association between biological film and the sensor surface is tight; and the immobilized biological film has long-term stability, durability and good specificity\(^3\). However, it is difficult for many of the immobilization methods to meet all these requirements. The commonly used immobilization techniques are based on the following mechanisms: (1) physical adsorption, (2) covalent binding, (3) bioaffinity immobilization, (4) cross-linking between molecules, and (5) membrane or gel entrapment (shown in Figure 6). Generally, the choice of a suitable immobilization strategy is determined by the physical and chemical properties of both surface and biomolecules\(^40\). In this section, some of these immobilization methods will be reviewed.
Figure 6. Commonly used methods of immobilization
1.3.1 Immobilization by physical adsorption

Physical immobilization is realized by a non-covalent attachment of molecules onto solid supports that is based on electrostatic, Van der Waals, and hydrophobic interactions. Belosludtsev et al. and Lemeshko et al. have shown that the negatively charged phosphate backbone of DNA favored physical immobilization onto charged gels, polymers and membranes. This simplest and straightforward method has, however, been limited by the immobilized heterogeneous and randomly oriented biomolecule layer and some degree of reversible adsorption. The reason for these limitations is that the biomolecules form many contacts in different orientations to minimize repulsive interactions with the substrate and the previously adsorbed biological layer. Hence, physical immobilized biosensors are not very sensitive and their specificity is also not satisfying. In addition, some biomolecules lose their biospecific activity during the process of physical immobilization and become denatured. As a result, the right orientation and conformation of biomolecules during the process of physical adsorption can not be guaranteed during the following binding events in the biosensor operation. Since the orientation and conformation of biomolecules are particularly important to pharmaceutical and biosensor research, other more suitable approaches are required to immobilize biomolecules.

1.3.2 Covalent immobilization

Covalent immobilization is conducted by forming covalent bonds between side-chain-exposed functional groups of proteins with suitably modified surfaces. The covalent bond is the strongest intermolecular interaction in biochemistry and its dissociation energy is about 100 kcal/mol. As a result, this immobilization technique results in a minimal loss of biomolecule activity, irreversible binding and a high surface coverage. Biomolecules such as proteins and peptides have many functional groups available for covalent bonding onto various modified surfaces. These bondings include amino group from lysine and hydroxyl-lysine onto surfaces of...
carboxylic acid / active ester (N-hydroxyl succinimide, NHS) / epoxy / aldehyde, thiol group from cysteine onto surfaces of maleimide / pyridyl disulfide / vinyl sulfone, carboxyl group from aspartate and glutamate onto surfaces of amine, and hydroxyl group from serine and threonine onto surfaces of epoxy. Similar methods, including carbodiimide-method, reactive anhydrides method and activated ester method, can also be applied to DNA immobilization provided that phosphate and sulfonate groups share similar properties with the carboxyl group from protein. However, the attachment may occur simultaneously through many residues, enhancing heterogeneity in the population of immobilized proteins.

Self-assembled monolayers (SAM), i.e., the ordered molecular assemblies of different organic materials, are always involved in biomolecule covalent immobilization. SAM of organosilicon are currently used for coating glass slides utilized in the area of biochips. In addition, SAM of organosulfur are also widely used for biosensor and imaging techniques. Generally, biomolecules such as proteins attach onto the SAM coated surface by covalent bonding, this SAM layer enhancing the surface biocompatibility and protecting proteins from denaturation. The main drawback of covalent immobilization during nonspecific protein adsorption, which causes background noise interfering with the detection signal, has been partially avoided by using a protein repulsion SAM layer, such as poly(ethylene glycol) and poly(carboxybetaine methacrylate). Even though many publications have been devoted to SAM layer related covalent immobilization, this area is still an evolving field.

1.3.3 Bioaffinity immobilization

Biochemical affinity reactions control the orientation of immobilized biomolecules and limit their heterogeneous attachment, which is an important advantage over other immobilization techniques. In addition, it is also possible to detach biomolecules and make the same surface reusable. This immobilization method has been mainly studied on two well-known interactions: Avidin-Biotin and Histidine(His)-Tag systems.
The interaction between avidin and biotin is one of the strongest noncovalent bonds known\textsuperscript{40}, with an association constant of about $10^{15}$ M\textsuperscript{-1} (shown in Figure 7). Avidin is a glycoprotein consisting of four polypeptides that are connected with carbohydrates by glycosidic bonds\textsuperscript{41}. It is stable over wide pH and temperature range and it can bind up to four molecules of biotin\textsuperscript{40}. Streptavidin and neutravidin, which are similar to avidin, have similar or higher binding affinity to biotin. Therefore, these two proteins have been widely used as substitutes to avidin. Biotin, also referred as vitamin H, is commonly used as a conjugated reagent for attaching biomolecules onto the avidin modified surface. This usage is due to the relatively smaller size of biotin. The conjugation does not affect the conformation, size, or functionality of the bonded biomolecules\textsuperscript{40}; on the other hand, avidin is comparably large thus it is better to be attached onto a substrate surface by physical/covalent immobilizations before linking to biotin. In this thesis, biotinylated calmodulin has been immobilized onto the neutravidin-coated gold surface and detected by acoustic wave sensor\textsuperscript{51}.

Placing affinity tags at defined positions of biomolecules has also been widely used as an affinity immobilization method. The most popular tag is poly(His), which is also used in affinity chromatography for protein purification\textsuperscript{52}. Poly(His) is small in size and has good compatibility with organic solvents. In addition, it can be effectively purified under native and denaturing conditions\textsuperscript{40}. During the purification experiment, biomolecules such as proteins with a $(\text{His})_6$ tag at the C- or N-terminus are attached to a Ni-chelated complex (such as Nitriloacetic acid, NTA) which is covalently immobilized to the substrate surface\textsuperscript{53}. The specific chelating interaction between the Ni-chelated complex and His-tagged biomolecules involves the octahedral coordination of the Ni$^{2+}$ with two valences occupied by two imidazole groups from the His-tag and four ligands donated by the Ni-chelated molecule\textsuperscript{40}. Schmid et al. also show that the binding of His-tagged green fluorescent protein to the NTA is highly specific with reasonable affinities and in addition is fully reversible upon addition of a competitive ligand.
Figure 7. Biotin-bound avidin. Biotin is shown as a stick model, and avidin is shown as a combination of both a cartoon model and a surface model. The image is produced from PDB 1AVD using PyMol.
(histidine, imidazole), reprotonation of the histidine residue, or removal of the metal ion via EDTA complexation\textsuperscript{53}.

### 1.3.4 Protein cross-linking and DNA conjugation

Intermolecular cross-linking of biomolecules such as protein is achieved by attaching to either other protein molecules or functional groups on an insoluble support matrix by a functional reagent. This method offers enhancement of stability of the absorbed biomolecules that are covalently bound onto the surface. Previously, bovine serum albumin (BSA) was employed to link an enzyme with the aid of glutaraldehyde to produce a thin layer on the sensor surface\textsuperscript{54}. Currently, protein A, which is a cell wall component of \textit{Staphylococcus aureus} and can bind with the Fc portion of the antibody, has been widely used as a linking material to immobilize immuno-proteins\textsuperscript{55}. However, the disadvantage of this method includes the uncontrollable orientation of surface-attached protein, high consumption of biological materials, low activity multilayers of biomolecules, and large diffusional barriers to reach the reaction zone.

Oligonucleotide-directed immobilization has also been used to attach biomolecules onto surfaces\textsuperscript{40}. Biomolecules of interest are conjugated with ssDNA through covalent or affinity attachment\textsuperscript{56-58}. The ssDNA hybridizes with the surface-attached complementary oligonucleotides. Hence, biomolecules are immobilized onto the surface. The requirement for using this method is that the surface-attached oligonucleotides need to have high hybridization efficiency and no cross reaction with the biomolecules of interest. Research also shows that the immobilized biomolecules can be completely removed by alkaline denaturation of the DNA double helix and therefore a complete regeneration of the surfaces can be achieved\textsuperscript{40, 59, 60}.

### 1.3.5 Entrapment of biomolecules

The procedure of entrapping biological components in polymer gels, membranes, or surfactant matrices is based on a simple sample preparation procedure and thus has been
successfully used in the past few years. Using this method, the structure and functionality of entrapped biomolecules are preserved, because there is no chemical alteration happening to these molecules. The entrapment medium could be a conducting or non-conducting polymer or some kind of gel. For example, a negatively charged perfluorinated sulfonate polymer, Nafion™, has been widely used for the entrapment of proteins because of its inherent property of reducing interference through charge repulsion. However, the entrapment method needs the size of the entrapped molecules to be small enough to pass through the matrix and thus to be detected. In addition, the leakage of the biological species results in a loss of activity, further limiting the application of this method.

1.4 Characterization of surfaces

Biosensors, especially acoustic wave sensors, need to have adequate characterization of surfaces with attached biomolecules. Surface properties may determine the success of a biosensor and it is essential to understand surfaces in order to correlate any surface modifications with changes in biological performance. Choice of the surface characterization method can be affected by a large number of considerations including the needed measurement, the analyzed surface area, the required precision and accuracy, the impact of the technique on the surface, limitations imposed by the surface and the influence of the sample on the instrument. In addition, the availability of the equipment, its ease of use and the cost of analyzing the surfaces are also factors to be considered. Because there are quite a few surface characterization methods, selected techniques that are commonly used for analyzing biosensor surfaces will be briefly introduced in this chapter. These techniques include both microscopy and spectroscopy methods: scanning electron microscopy (SEM), atomic force microscopy (AFM), X-ray photoelectron spectroscopy (XPS), secondary ion mass spectroscopy (SIMS) and contact angle measurement.

1.4.1 Scanning electron microscopy
In a typical SEM, the primary beam of electrons is either emitted from a cathode and then accelerated to an anode, or is emitted via field emission. The electron beam is focused by condenser lenses, passes through objective lens and scans in a raster fashion over the sample surface. The primary electron beam usually interacts with the sample in a vacuum environment, which results in the emission of electrons and electromagnetic radiation. Finally, secondary electrons ejected from the surface are detected to produce a high-resolution image.

SEM sample preparation includes several steps. Firstly, biomolecules should be fixed onto the surfaces by certain chemicals, such as glutaraldehyde and paraformaldehyde. Secondly, the surface needs to be dried using a critical point dryer. Thirdly, attachment to a metallic stub is required before coating with a layer of conductive materials (usually gold, sometimes carbon). Lastly, the surface is inserted into the SEM and data are collected. However, this preparation procedure has some drawbacks. For example, fixation sometimes can shrink any attached cells; whereas, drying and coating processes might also alter surface morphology.

The technique of SEM has been used to assess the morphology of endothelialization. Reichelt et al. reported that protein inclusions (so-called cores) found in higher plant peroxisomes have been characterized by high resolution scanning electron microscopy. In my thesis, SEM was used to differentiate the morphology change of smooth muscle cells before and after interaction with hydrogen peroxide.

1.4.2 Atomic force microscopy

AFM has very high-resolution, i.e. fractions of a nanometer. This instrument consists of a cantilever with a sharp tip at its end. This tip scans across the surface and creates a three-dimensional image. The force of interaction between the sample surface and the tip is monitored through the cantilever deflection, which is normally measured by a laser spot reflected from the top of the cantilever into an array of photodiodes. Based on different interacting forces between the sample surface and the AFM tip, different modes of AFM
operation have been employed. Generally, there is the contact mode measuring repulsive force between the surface and the tip, the non-contact mode measuring attractive force, and the tapping mode where the cantilever lightly taps the sample surface with an amplitude typically ranging from 20 to 100 nm. In addition, in the tapping mode, the morphology and surface composition of a sample can be analyzed together by obtaining height and phase images simultaneously.

AFM and SEM have their own advantages and disadvantages, and they are used as complementary methods in biomolecule imaging studies. Firstly, AFM provides three-dimensional surface profiles, whereas SEM can only give two-dimensional images. Secondly, sample preparation of AFM does not require any coating treatments which may damage the sample and therefore give distorted results. Thirdly, AFM can work in a mild environment such as air and liquid phase rather than only operating in a vacuum condition. However, AFM’s smaller scan size, slow scan rate, and the quality of the image are limited by the radius of curvature of the scanning tip.

AFM methods have been used to study surface ligand and intermolecular bond strengths. With coated scanning tips, fundamental interactions between biomolecules have also been studied. For example, mapping the positions of polysaccharides on a living microbial cell surface and detection of extracellular ATP on living cells have been achieved using AFM.

### 1.4.3 X-ray photoelectron spectroscopy

X-ray photoelectron spectroscopy (XPS), also known as ESCA (electron spectroscopy for chemical analysis), is a quantitative spectroscopic technique for surface chemical analysis. It is widely used in biomaterial applications to determine the elemental composition of surfaces to a depth of approximately 100 angstroms. The process of XPS involves irradiation of a surface by a beam of monochromatic X-rays, causing emission of photoelectrons from the core
shell orbitals of atoms\textsuperscript{77}. Measurement of both the kinetic energy (KE) and the number of released electrons from the surface gives the XPS spectra. KE can be related to the binding energy (BE) of each of the emitted electrons, which is shown in the XPS spectra instead of KE, via the following equation:

\[ BE = h\nu - KE - w \]  \hspace{1cm} (21)

where \( h\nu \) is the energy of the X-ray photons being used and \( w \) is the work function of the spectrometer. Since binding energy of the emitted electrons is unique for different elements as well as being sensitive to the chemical state of the atoms, each element produces a characteristic set of XPS peaks at characteristic binding energy values\textsuperscript{78, 79}.

Although special sample preparation is generally not required for XPS, one needs to pay particular attention to avoid sample contamination, which may affect the results\textsuperscript{63}. The limitations of XPS include sample degradation during analysis and the inability to detect lighter atoms (H and He) because of restricting electron shifting from one orbital to another provided by only a single inner electronic orbital\textsuperscript{72}.

XPS has often been applied to the investigations of biomolecules such as proteins at interfaces\textsuperscript{65, 80}. For example, it has been used to detect the presence of adsorbed immobilized proteins and to estimate the amount of protein present\textsuperscript{81}. Recent studies by Wagner \textit{et al.} have showed that XPS detection limits for proteins could be as low as about 10 ng/cm\textsuperscript{2} if the substrate did not contain nitrogen\textsuperscript{82}.

\textbf{1.4.4 Secondary ion mass spectroscopy}

Secondary ion mass spectrometry (SIMS) is a technique for surface and thin film characterization. It uses the process of ion formation by bombarding the tested surface with a highly collimated beam of primary ions or atoms\textsuperscript{78}. The surface then emits secondary particles through a sputtering process, where only a fraction of these emitted particles are ionized. The emitted ions are separated based on the mass-to-charge ratio by a mass analyzer. Both
positively and negatively charged ions are detected to determine the quantitative elemental, isotopic, or molecular composition of the surface. In the field of surface analysis, it is usual to distinguish static SIMS and dynamic SIMS based on the primary particle flux. When the primary beam flux is low, the erosion rate of the sample surface becomes negligible. This kind of SIMS is a technique designed to sample only the uppermost layer of a solid sample. This process is called static SIMS, and involves only a monolayer analysis of a surface. Static SIMS experiments usually combine a pulsed ion beam and a time-of-flight mass spectrometer.

When the primary beam flux is high, the process is called dynamic SIMS. It results in rapid etching of the surface during bulk analysis closely related to the sputtering process and can be used to monitor changes in the elemental composition with depth. Dynamic SIMS usually requires a continuous primary ion beam and a magnetic sector or quadrupole mass spectrometer.

Like XPS, there is no requirement for any special sample preparation for SIMS experiments, provided there is no contamination overcoating the surface. Superior to XPS, SIMS can be used for analyzing all elements. SIMS spectra provide characteristic ‘fingerprints’ for biomaterial surfaces; enable the study of surface adhesion, biocompatibility, membrane dynamics, cellular processes, tissue pathology, as well as drug interactions; and facilitate the monitoring of degradation kinetics of biodegradable polymers.

1.4.5 Contact angle measurement

Contact angle measurement is based on the assumption of thermodynamic equilibrium and a smooth/homogeneous surface which does not transform in the test liquid. A drop of fluid is placed on the surface of interest, an equilibrium position is achieved, and the contact angle determined from the tangent is associated with the shape of the drop as it experiences the properties of the interface.
Usually, the relative hydrophilicity or hydrophobicity of the surface can be determined by this method. Theoretically, a surface with contact angle larger than 90° is hydrophobic, whereas a surface with contact angle lower than 90° is hydrophilic. On extremely hydrophilic surfaces with an effective contact angle of 0°, water droplets completely spread because of the large affinity of the surface for water. On extremely hydrophobic surfaces with contact angle of 180°, water droplets simply rest on the surface, without actually wetting to any significant extent. However, in reality, we need to pay attention to the choice and purity of test liquid, droplet size, and time required to obtain readings, since these factors may affect the obtained contact angle results.

After reviewing the techniques of surface immobilization and surface characterization, use of the TSM instrument in the field of bioanalysis will now be presented. In this thesis, four main research areas are the effect of detergent on biomolecules, the study of lesions in DNA, the analysis of calmodulin conformational change, as well as the detection of the attachment and properties of smooth muscle cells. These four research areas are introduced subsequently in the following sections.

1.5 Surfactants

The elution of adsorbed biomolecules by surfactants is a commonly observed detergency process which is of great practical importance for all cleaning procedures in a variety of applications, such as food processing, pharmaceutical production and dentistry. A reusable surface could be a very promising research area, especially for biosensor studies. Surfactants are usually organic compounds containing both hydrophobic groups (their "tails") and hydrophilic groups (their "heads"). Because of their amphiphilic property, surfactants are soluble in both organic solvents and water. Surfactants are usually classified according to their head group as anionic, cationic, zwitterionic and nonionic. The sodium dodecyl sulfate (SDS)
used in this research is an anionic surfactant. The process of elution of biomolecules from surfaces is related to several factors: surfactant adsorption at solid surfaces; interactions between immobilized biomolecules; and environmental influence on the properties of biomolecules, surfactants and surface\textsuperscript{90, 91}. The two main elution mechanisms are either the solubilization of the surface-attached biomolecules due to their stronger interactions with surfactants than with the surface, or replacement of the adsorbed biomolecules by surfactants that have a strong affinity for the surface\textsuperscript{92}.

In this research, SDS is occasionally used to clean the surfaces of the acoustic wave sensor. Zembala \textit{et al} have shown that as long as the concentration of SDS is high enough, the protein elutability when using this surfactant is equal to one\textsuperscript{90}. The interaction of SDS with globular proteins seems to occur at the cationic sites of the protein molecule, such as arginyl, histidyl and lysyl amino acid side chains\textsuperscript{93}. In addition, near the cationic sites, the alkyl chains of the surfactant may interact with the hydrophobic regions of the protein, causing protein denaturation. Some researchers have shown that when the concentration of the SDS is low, such interaction between SDS and protein may assist adsorption between biomolecules and the surface\textsuperscript{90}, and the length of the alkyl chain may also affect interaction\textsuperscript{93}. The process of competition between elution and protein fixation by surfactants is still under investigation. In this research, SDS has only been used at very high concentration (1\% w/v) to clean the sensor surface.

\section*{1.6 DNA}

DNA damage can arise from various routes, including oxidative stress, the action of environmental pollutants (e.g. nitrates or organophosphate pesticides), and attack from the high-energy radioactive processes\textsuperscript{94}. Base damage, such as the spontaneous deamination of cytosine to uracil, the oxidation of thymine to thymine glycol, or the oxidation of guanine, can be
repaired via abasic site intermediates. The first step in base excision repair of DNA containing damaged bases in vivo is often the hydrolytic cleavage of the C-N bond between the sugar and damaged or abnormal base to generate an aldehydic apurinic or apyrimidinic site (AP), as shown in Figure 8. Such sites constitute one of the most common forms of toxic lesions. They are generated both enzymatically during the repair of DNA damage and spontaneously, as a result of chemical modification of the bases (by carcinogens, alkylating agents, or by ionization radiation) that destabilizes N-glycosidic bonds. The unpaired aldehydic abasic site changes the structural and dynamic properties of DNA that affect the recognition and misreading of lesions by the cellular machinery involved in DNA repair and replication. The presence of these lesions has been shown to slow down, but not block, RNA polymerases and virus reverse transcriptase, and can cause mis-incorporation of nucleoside triphosphates by a DNA polymerase during replication. Thus, when damaged DNA are replicated and transmitted to future cell generations, the changes in nucleotide sequences of DNA become the source of permanent mutations. Accumulation of mutations is connected directly with aging and cancer. In view of the biological significance of AP sites, there is considerable interest in the detection of such lesions.

Three-dimensional structures of DNA duplexes containing native and synthetic abasic sites have been determined by NMR spectroscopy. In these studies, the duplex has retained the B-form geometry and the AP site has induced only local perturbations limited to the lesion site and flanking base pairs. In duplexes containing purine residues opposite the lesion, both the AP site and opposing purine are stacked inside the helix. However, when pyrimidine bases are opposite to the AP site, the duplex structure shows increased conformational flexibility. Depending on the temperature and the nature of nucleobases flanking AP sites, the abasic sugar residue and occasionally the opposite pyrimidine bases are extrahelical. Since AP sites change base stacking and hydrogen bonding, they can also affect the thermodynamic properties
Figure 8. Damaged DNA with one AP site. The AP site is shown in the form of a stick model. (Produced from PDB 1A9I.)
of the DNA duplex\textsuperscript{102}. Accordingly, the presence of AP sites can also affect DNA hybridization. Thus, the study of DNA hybridization between single stranded oligonucleotides (probe) and damaged oligonucleotides (target) can serve as a test of AP site damage.

For this purpose, the model probes oligodeoxyribonucleotides (ODNs) are chemically modified by thiol groups through a short linker or by biotinylation in order to effect attachment to the device by means of chemisorption or by avidin-biotin technology\textsuperscript{103}. Hybridization with damaged target ODN can be detected at the electrode surface by a number of electrochemical methods\textsuperscript{104, 105}, by means of scanning Kelvin nanoprobe\textsuperscript{106}, acoustic wave\textsuperscript{107-109}, or optical methods\textsuperscript{110, 111}. The study of the mechanisms of DNA hybridization using naturally occurring AP sites is difficult, because they are unstable which is due to the fact that the aldehyde (open) form is subject to $\beta$-elimination and strand scission\textsuperscript{112}. This limitation can be overcome by using the 2-(hydroxymethyl)-tetrahydrofuran-3-ol residue, a close structural analog of a natural abasic site. In my experiment, a series of 19-mer synthesized ODNs containing one, two or three tetrahydrofuran AP analogs at the desired positions (AP1, AP2 and AP3 respectively) were utilized, where hybridization with a complementary strand (BASE) incorporates abasic sites opposite T residues. This allows the creation of a model where the type of damage and the distance separating the lesions are known. Unmodified complementary ODNs (APC) was used as a control. In order to immobilize the DNA on a gold electrode, biotinylated 19-mer BASE probes were used. These probes were immobilized to a gold support by means of neutravidin\textsuperscript{113}. In this work, the detection of DNA hybridization has been performed by means of the acoustic wave propagation technique using the thickness shear mode device (TSM). The TSM method has been shown to be a powerful tool for the study of binding events involving biochemical macromolecules or cells at the liquid-device interface\textsuperscript{114}.

Additionally, a solution-based study of the thermal stability of the identical DNA duplexes containing AP sites was performed in order to conduct a correlation between acoustic
wave properties and the UV-monitored melting behavior of DNA duplexes.

1.7 Calmodulin

Calmodulin (CaM), as shown in Figure 9, is a 16.5 kDa protein that is a major receptor for intracellular calcium signals in all eukaryotic membrane-bound organisms\textsuperscript{115-117}. The protein has 148 residues and mediates the activity of a number of calcium-regulating proteins, including protein kinases, phosphatases, nitric oxide synthase, inositol triphosphate kinase, NAD kinase, and phosphodiesterase\textsuperscript{118}. The crystal structure of CaM shows it to be a dumbbell-shaped molecule with two globular domains at the amino and carboxyl termini that are connected by a long central helix. Binding of Ca\textsuperscript{2+} induces a conformational change from a closed conformation in the Ca\textsuperscript{2+} free-state to one that is open\textsuperscript{119}. In the latter conformation, a hydrophobic site is exposed on the surface of each domain\textsuperscript{120, 121}. At the centre of each hydrophobic site, there is a cavity that fits aromatic or long alkyl amino side chains of target proteins\textsuperscript{121, 122}. As a result of these domains, CaM is able to bind to target proteins and activate them with high affinity. In addition, association of CaM to such proteins produces further major structural changes with respect to the Ca\textsuperscript{2+} complex\textsuperscript{122}.

Since calmodulin activity depends critically on its binding with Ca\textsuperscript{2+}, it is necessary to gain an understanding of the characteristics of binding with this ion and the nature of interference effects originating from other ions in the cell. Several other divalent metal ions appear to induce a conformational change in the protein in addition to Ca\textsuperscript{2+}, with Mg\textsuperscript{2+} being the most commonly studied\textsuperscript{123}. Generally, there is agreement that there is a total of four binding sites between CaM and various metal ions\textsuperscript{124, 125}. Coincidental interaction of Ca\textsuperscript{2+} and Mg\textsuperscript{2+} with the protein is expected to result in a direct competition between the ions for these four main sites. However, Gilli \textit{et al.}\textsuperscript{126} recently found that the Mg\textsuperscript{2+} binding sites are different from those for the calcium ion. This Mg\textsuperscript{2+} ion does not act as a direct competitor for Ca\textsuperscript{2+} binding but
Figure 9. N-terminal domain of Ca\textsuperscript{2+}-bound calmodulin molecule, produced from PDB 1J7O
it does prevent the formation of the CaM-Ca$_4$ complex by acting as an allosteric effector. Accordingly, this study confirms earlier results from two groups$^{127-130}$, who suggested that the interaction between divalent cations and CaM involves coupling between four main Ca$^{2+}$ binding sites and several auxiliary cationic sites.

Detection of protein conformational changes in solution has been achieved by mass spectrometry$^{131}$, NMR$^{132}$, gel electrophoresis$^{133}$, fluorescence resonance energy transfer$^{134}$, small-angle X-ray scattering$^{135}$, and circular dichroism$^{136}$. However, the analogous characterisation of such conformational change for proteins attached to a surface, which is important in a number of applications, has constituted a significant challenge. One strategy has been to employ label-free detection technology, although explicit linking of signals from this approach to structural perturbations of macromolecules attached to the detector surface has not figured prominently in the literature. Two techniques that have offered promise in this direction are surface plasmon resonance and acoustic shear wave propagation. An example of the former method is a study of the conformational chemistry of a genetically altered *E. coli* dihydrofolate reductase attached to a carboxymethyl dextran surface$^{137}$. With respect to acoustic wave technology with the thickness shear mode device (TSM), Thompson *et al.*$^{13}$ indicated as early as 1993 that this technique could not only measure the kinetics of the adsorption of proteins to a surface, but also could yield an indication of interfacially-instigated conformational changes. In recent work by Carmon *et al.*,$^{138}$ there is a suggestion that the response of an acoustic wave sensor is influenced by the conformational change of a genetically engineered glucose receptor attached to the device by sulfur-gold linkage. Three recent studies have also observed possible conformational shifts in surface-adhered albumin, detected by acoustic wave biosensors$^{139-141}$. Despite these positive indications, there has been a prevailing view that the acoustic wave device only responds to added mass, whereas I believe the device is also governed by the properties of the interface such as viscoelasticity, coupling to a contacting liquid, and interfacial
slippage. In the present work, the calmodulin system is used to evaluate the potential of acoustic wave technology for the study of surface-attached protein dynamics, in view of the extensive literature on the conformational chemistry of this molecule.

1.8 Smooth muscle cells

Smooth muscle fibers are spindle-shaped, and, like all muscle, can contract and relax. In the relaxed state, each smooth muscle cell is 20-500 micrometers long and a few micrometers wide. The cells are arranged in sheets or bundles and are connected by gap junctions. During contraction, while the cells utilize the action of actin and myosin cross-bridge cycling like any other muscles, calmodulin takes on the regulatory role in smooth muscle cells as opposed to troponin in skeletal muscle cells. In smooth muscle, calmodulin activates the cross-bridge cycling and the development of force in response to a $[\text{Ca}^{2+}]$, transient via the activation of myosin light-chain kinase and phosphorylation of myosin. The modulation of smooth-muscle contraction is realized by a distinct calmodulin-dependent kinase, $\text{Ca}^{2+}$/calmodulin-dependent protein kinase II.

Vascular smooth muscle cells refer to the particular type of smooth muscle cells found within, and composing the majority of the wall of, blood vessels. This kind of smooth muscle cells are one of the main research objects of this work.

In this thesis, the attachment and properties of aortic vascular smooth muscle cells will be studied by TSM. The interaction of cells with surfaces is an important area from the perspectives of both fundamental science and medicine. With regard to the latter, there is an obvious interest in the role of cell-substrate chemistry in terms of implants and other devices subject to issues of biocompatibility. From the pharmaceutical standpoint, there is increasing interest in the behaviour of single cells and populations of communicating cells following exposure to small molecules in particular drugs. The immobilisation of cells onto a substrate
surface is often a precursor to studies of morphological and other changes. An additional related application is that the cell-device combination can act as a sensing structure in its own right. An example of this approach is the so-called neural biosensor which has been employed as a detector for various species\textsuperscript{145, 146}.

Subsequent to an initial cell-surface interaction, morphological changes such as cell spreading can occur\textsuperscript{147}. With respect to cell growth, it has been found that this desired phenomenon is anchorage dependant. Many factors are expected to play a role in cell growth, one example being the influence of the substrate-extracellular matrix (ECM) interaction. In view of the above it is not surprising that there has been a significant effort to develop techniques for the study of cell-surface interactions, and fluorescence and electrochemical methods have figured prominently in recent times. However, it is clear that a limited number of techniques exist that are capable of label-free, real time monitoring of such interactions\textsuperscript{144}.

The thickness shear mode acoustic wave device (TSM) offers sensitive and label-free detection, and can be employed with success in a flow-injection system. It has been widely used in the field of bioanalytical chemistry in recent years, largely because of these properties\textsuperscript{51}. The main applications of the TSM have resided in the detection of nucleic acid hybridisation\textsuperscript{148}, protein conformational changes\textsuperscript{51}, immunochemistry\textsuperscript{149} and other areas\textsuperscript{150, 151}. With respect to cells, studies have been performed on endothelial\textsuperscript{152, 153}, cancer\textsuperscript{150, 154}, bacterial\textsuperscript{155} and cardiac muscle cells\textsuperscript{156, 157}, as well as on blood platelets\textsuperscript{158}. A recent paper described TSM detection of both interneuron communication and response to drugs for surface-attached populations of hypothalamic neurons displaying different levels of confluency\textsuperscript{159}. Finally, single cell detection has been achieved by fabricating microfluidic channels on the surface of a TSM device\textsuperscript{160}.

In the present work, an on-line TSM system has been used to compare the acoustic signals in a semi-quantitative fashion of rat aortic smooth muscle cells attached onto different surfaces, including bare gold and extracellular matrix species, such as fibronectin and laminin
(as shown in Figure 10). Additionally, the responses of these cells to depolarization and oxidative stress were analyzed. Ions (Ca\(^{2+}\) or Mg\(^{2+}\)) were used to affect the attachment of cells and a depolarization event instigated by KCl was detected. The oxidant H\(_2\)O\(_2\) is well known to cause cell morphological changes, so this entity was used as a probe to ascertain if the acoustic technique is capable of detecting such structural alterations. Correlation of the acoustic observations with surface science techniques was also conducted.
Figure 10. Fibronectin (top) and laminin (bottom), produced from PDB 1TTG and 1NPE. The binding site (arginine-glycine-aspartate, RGD) of fibronectin is shown in a stick model of molecular structure.
1.9 Layout of this thesis

The research area presented in this thesis mainly focuses on the application of TSM sensors to pharmaceutical and environmental research development. In addition, based on the experimental results, further theoretical analysis has been provided. In the current chapter, the background knowledge to the TSM system was reviewed and the four main research areas of this work were introduced. These main research areas are the effect of detergent on immobilized biomolecules, the study of DNA base pair mismatch, protein conformational change, and cell behavior.

In this thesis, the research work will be introduced in the following sequence. Firstly, since biosensors are famous for their economical properties, a reusable sensor has always attracted people’s attention. Here in this study, SDS will be examined for its ability to cleanse the TSM sensor. Secondly, the effect of environmental pollutants on DNA structure will be analyzed by TSM. Comparison between normal DNA and damaged DNA will be provided. Thirdly, the study of protein conformational change by TSM will be introduced. It is commonly known that the interaction between calmodulin and ions/peptide will induce a conformational change of calmodulin; however, most studies are based on the fluorescence techniques in solution. In this study, I will use a TSM sensor, a label free method, to look at the interaction between calmodulin and ions/peptide and conformational change of the calmodulin. Lastly, the kinetics of attachment/detachment of cells to a surface will be studied by TSM, while small molecules, such as ions and peptide, will be used to affect the behavior of the cells. Chapter 2 will look at the experimental procedure and techniques in detail. These techniques include surface immobilization (affinity and physicochemical adsorption), surface characterization (SEM, AFM, XPS) and cell culture methods. The results and discussion will be provided in Chapter 3, and is followed by the conclusion and outlook sections introduced in Chapter 4 and Chapter 5, respectively.
2. Experimental section

2.1 Reagents and materials

2.1.1 Detergent

Sodium dodecyl sulfate (SDS) was purchased from Sigma and used to clean surfaces with the attached biological materials. The concentration of SDS used in the cleaning procedure was 1% (w/v).

2.1.2 DNA

19-mer ODNs with one, two and three AP-sites (Table 1) were synthesized by the phosphoramidite method on an automated DNA synthesizer (Applied Biosystems 380B). The modified residues were introduced by replacement of standard nucleoside phosphoramidite with 2-(dimethoxytrityloxymethyl)tetrahydrofuran-3-ol (N,N-diisopropylamido)-β-cyanoethylphosphite under the standard conditions of oligonucleotide synthesis. The ODN modified by biotin (X-BASE, Table 1) was purchased from Thermo Electron Corporation (Germany). The oligonucleotide concentration was determined spectrophotometrically with UV-VIS Hitachi 150-20 (Japan) or UV1700 Shimadzu (Japan) spectrophotometers.

Neutravidin was purchased from Pierce, Rockford, IL, USA and dissolved in Dulbecco’s phosphate buffered saline (DPBS, Sigma, St. Louis, USA) to a concentration of 1 mg/ml. Buffer A solution with pH 7.6 was made up of 0.5 M NaCl, 10 mM Tris-HCl and 1 mM EDTA, and it was used for TSM experiments. Buffer B solution with pH 7.6 was made up of 10 mM Tris-HCl, 10 mM MgCl₂, 100 μg/ml bovine serum albumin (BSA) and 1 mM dithiothreitol (DTT), and it was used for UV melting experiments.
Table 1. Oligonucleotides used in this thesis

<table>
<thead>
<tr>
<th>ODN</th>
<th>5’→3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>GGAAAATTTTCAGCAAGGTG</td>
</tr>
<tr>
<td>AP1</td>
<td>GGAAAATTTTCRGCAGGTG</td>
</tr>
<tr>
<td>AP2</td>
<td>GGAAAATTTTCRGCRAGGTG</td>
</tr>
<tr>
<td>AP3</td>
<td>GGAAARATTTCRGCRAGGTG</td>
</tr>
<tr>
<td>BASE</td>
<td>CACCTGCTGAAATTTC</td>
</tr>
<tr>
<td>X-BASE</td>
<td>X-CACCTGCTGAAATTTC</td>
</tr>
</tbody>
</table>

\(X = \) Biotin  \(\text{R} = \) (abasic site)
2.1.3 Calmodulin

Biotinylated calmodulin (solution, 0.5 mg/ml in DPBS buffer) and smooth muscle myosin light chain kinase (smMLCK) peptide (M_w= 2072 Da) were purchased from VWR Canada (EMD Chemicals Inc, CA) and used after dilution. DPBS buffer, sodium chloride, calcium chloride dihydrate, magnesium chloride hexahydrate and water were all purchased from Sigma (St. Louis, USA). Neutravidin (M_w=60 kDa), obtained from Pierce (Rockford, IL, USA), was used in diluted DPBS buffer (1 mg/ml). Solutions of CaCl_2 at concentrations of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 5.0 mM, respectively, and MgCl_2 at 1.0 mM were employed in the same buffer. Biotinylated calmodulin (CaM) was used at concentrations of 3.00, 2.63 and 2.45 μM, respectively and that of the smMLCK peptide, at 0.10 mg/ml all in diluted DPBS buffer. Calmodulin from Sigma was used as 2.5 μM solution in dilute DPBS buffer. For the surface immobilization of calmodulin, 11-mercaptoundecanoic acid (11-MUA) was purchased from Aldrich and dissolved in ethanol as a 10 mM solution. And N-hydroxyl succinimide (NHS) and N-ethyl-N-(dimethyl amino propyl) carbodiimide hydrochloride (EDC) were purchased from Sigma. 15 mM NHS and 75 mM EDC were dissolved in water and used as a 1:1 solution. BSA (1 mg/ml) in DPBS, dilute using water. The dilute method is the same as used for calmodulin.

2.1.4 Cells

DPBS buffer was purchased from Sigma and used as buffer throughout the whole study. Calcium chloride dihydrate and magnesium chloride hexahydrate were also received from Sigma and the concentration used in this study was 1 mM for CaCl_2 and MgCl_2 separately in DPBS buffer. And potassium chloride crystal (J. T. Baker) was used as 60 mM in DPBS buffer. Laminin and fibronectin were purchased from VWR and the final concentration used was 50 μg/ml separately in DPBS buffer. Trypsin was ordered from Sigma and dissolved in DBPS buffer to final concentration of 0.05% (w/v). Arg-Gly-Asp-Ser peptide was purchased from
Sigma and used as 1 mg/ml solution. Iscove’s modified Dulbecco’s medium (IMDM, Invitrogen Corp) containing 10% fetal bovine serum (FBS, Invitrogen Corp) was used in the cell culture experiment. H₂O₂ (Sigma) was dilute to 200 µM in DPBS buffer. To fix the cells onto the surface for SEM and AFM experiments, 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) and 0.1 M sodium cacodylate buffer with 0.2 M sucrose (pH 7.3) were used as fixing and rinsing solutions, which were gifts from Mount Sinai Hospital. In addition, various concentrations of ethanol (commercial alcohols) were used to help the dehydration process.

2.2 Instruments and methods

2.2.1 Thickness shear mode acoustic wave sensor

AT-cut 9 MHz piezoelectric quartz crystals with gold electrodes were obtained from LapTech Corp. (Bowmanville, Canada). The devices were incorporated into a flow through system as described previously. This system affords the capability to perform the real-time attachment of biomolecules, buffer washing and the continuous monitoring of ligand binding processes. One side of the crystal was exposed to liquid flow in the cell with the other face being flushed with nitrogen gas. A network analyzer (HP4195A Network/Spectrum Analyser, Hewlett Packard, Colorado Springs, USA) was employed to measure the impedance properties of the TSM detector. In addition, the values for the elements of the equivalent circuit were calibrated internally by the analyser. The characteristic parameters were collected continuously within a scanned frequency range. In this work, changes of the series resonance frequency (fₛ) and motional resistance (Rₘ) were recorded during the entire process of binding molecules to the surface.

For DNA and protein experiments, the TSM operation was conducted at room temperature. For the cell experiments, the entire instrument was kept in a 37°C incubator to
keep cells alive (shown in Figure 11).
Figure 11. TSM instrument within 37°C incubator.
2.2.2 UV-VIS spectrophotometer

Absorption spectra and thermal denaturation profiles of DNA with AP sites were measured using a Varian-Cary 50 Bio UV-VIS spectrophotometer (USA) equipped with a thermoelectrically controlled cell holder. For melting experiments, the absorbance of samples at 260 nm was measured every 2 degrees between 20 and 80 °C.

2.2.3 X-ray photoelectron spectroscopy

The samples were run on the Thermo Scientific K-Alpha XPS spectrometer (ThermoFisher, E. Grinstead, UK) located at the University of Toronto. The samples were run at a take-off angle (relative to the surface) of 20°. A monochromatic Al Kα X-ray source was used, with a spot area (on a 90° sample) of 400 μm. Where necessary, charge compensation was provided. Position of the energy scale was adjusted to place the main Au 4f7/2 feature at 84.0 eV. The instrument and all data processing was performed using the software (Avantage) provided with the instrument.

2.2.4 Optical microscope

To prove the results obtained from TSM measurement for the biochemical changes of hydrogen peroxide damaged cells, cell-coated crystal surfaces were periodically observed under an inverted microscope (Nikon Eclipse TE 300, Melville, NY). The images were taken by optical microscopy using a 40x objective and an attached Nikon D70 camera (Nikon).

2.2.5 Scanning electron microscopy

A surface electron microscope (FEI XL30 ESEM) was utilized for detailed study of the morphology change of the cells after interaction with hydrogen peroxide. The SEM was operated in high-vacuum mode at 20kV accelerating voltage. A critical point dryer (CPD Baltec 030) and a gold sputter coater (Denton Desk II) were used after dehydration of the surfaces and before collection of SEM images.
2.2.6 Atomic force microscopy

A Dimension™ 3100 Tapping Mode AFM was used to study the morphology changes of the surface-attached cells. NSC36 Silicon probes from MikroMasch were used with resonance frequencies in the range of 50-105 kHz and with force constant range between 0.15-1.5 N/m.

2.3 Procedures

2.3.1 Detergent

Cleaned used sensors were utilized in some of the experiments in this research. Sodium dodecyl sulfate (SDS) was used to clean the attached biological materials from surfaces. After application of the detergent, the surfaces were washed with acetone, methanol, ethanol and water individually. Finally, they were dried under a stream of nitrogen. The effectiveness of this detergent was tested by XPS measurement. Four surfaces were prepared for these XPS experiments. The first surface was new bare gold crystal cleaned by a usual procedure (sonicated in acetone, ethanol, methanol separately for 5 min, washed by deionized water and dried in the steam of nitrogen). The second surface was new bare gold crystal cleaned by SDS solution and the usual procedure, which began with the surface being sonicated in SDS solution for 5 min and left in this solution for additional half an hour, then cleaned by acetone, ethanol, methanol, and lastly, this surface was washed by deionized water and dried by nitrogen gas. The third surface was protein-coated surface, which was cleaned by the procedure as used for the first surface. The fourth surface was also protein-coated surface, which was cleaned by SDS solution and the usual procedure as employed for the second surface.

2.3.2 DNA

2.3.2.1 Preparation of DNA layers on the TSM electrode

TSM devices were cleaned with acetone, ethanol, methanol, water and then dried using
nitrogen gas before being placed into the flow-cell. The gold electrode of the sensor was continuously flushed with buffer A solution (as shown in section 2.1.1) for 30 min. and then treated by neutravidin as follows. Neutravidin dissolved in DPBS buffer to a concentration of 1 mg/ml was allowed to flow into the cell at a flow rate 62.5 μl/min. After approximately 10 min the signal reached a steady-state value. Then buffer A was allowed to flow at the same rate again for 10 min. Subsequently, X-BASE (shown in Table 1) at a concentration of 1 μM dissolved in buffer A was pumped over the device surface until a steady-state signal was obtained. Additionally, buffer A was flowed for a further 10 min to wash away any non-specific bound probes. Finally, ODNs (as shown in Table 1) with or without abasic sites dissolved in buffer A to a concentration of 1 μM were swept over the device for the same time period, and buffer A was allowed to wash away the non-specific binding of ODNs.

2.3.2.2 UV-monitored thermal denaturation of DNA duplexes

The samples containing 2 μM DNA duplexes BASE-AP1, BASE-AP2, BASE-AP3 were prepared by mixing in 1:1 molar ratio of AP-containing strands with a complementary undamaged oligonucleotide (BASE, as shown in Table 1) in a buffer B (shown in section 2.1.1). Fully matched duplex BASE-APC was used as a control. The samples were heated to 80 °C, cooled slowly and incubated at room temperature 10 min prior melting studies.

2.3.3 Calmodulin

2.3.3.1 Biotinylated calmodulin

Before placing the TSM crystal in the flow cell, the same procedure as employed in the DNA experiments was used to clean the crystal surface (acetone, ethanol, methanol, DI-water and nitrogen gas dried). Diluted DPBS buffer solution was then flushed continuously at a flow rate of 36 μL/min for 30 min prior to sample introduction. Neutravidin solution (500 μL) was then introduced to the flow system, followed by on-line washing with buffer solution to achieve
a stable baseline reading. Biotinylated CaM was then immobilized onto the neutravidin monolayer using the flow-through system. The pump was turned off for a period of time to promote stabilisation of the biotin-avidin interaction, followed by on-line washing with buffer to remove non-specifically bound protein. Using similar protocols, the on-line prepared surfaces were exposed to varying concentrations of CaCl₂ or MgCl₂ (500 μL) and, in some experiments, to a subsequent volume of smMLCK solution. A schematic of the surface modifications used in these experiments is shown in Figure 12.
Figure 12. Experimental setup for biotinylated CaM interacting with ions or peptide on TSM surface.
2.3.3.2 SAM linked calmodulin

For purposes of comparison, a SAM layer of 11-MUA was also prepared to immobilize calmodulin without biotinylation. The cleaned TSM sensor was immersed in a solution of 10 mM 11-MUA in ethanol for about 24 to 36 hours at room temperature. The sensor was then washed with ethanol and immersed in a 1:1 solution of 15 mM NHS and 75 mM EDC in water for about 1 hour. After washing with water and drying under a stream of nitrogen, the sensor was placed into calmodulin solution for 1 hour. This was followed by washing with DPBS and drying under a stream of nitrogen before placing the sensor in the flow cell. Figure 13 shows the mechanism of the interaction between 11-MUA SAM layer and calmodulin molecules. Similar TSM experiments were operated as for the biotinylated calmodulin. The on-line prepared surfaces were exposed to CaCl\(_2\) solution (1 mM) and MgCl\(_2\) solution (1 mM) of 500 μL separately and, in some experiments, to a subsequent volume of smMLCK solution. Figure 14 shows the experimental setup. The homogeneity of SAM layer was further investigated using XPS. Three types of surfaces have been prepared in the XPS experiments: one with only 11-MUA SAM layer; one with 11-MUA and NHS/EDC linker; and one with calmodulin attached onto the modified surface.
Figure 13. Protein linked to 11-MUA SAM layer

Figure 14. Experimental setup for SAM-linked CaM interacting ions or peptide on TSM surface
2.3.4 Cells

2.3.4.1 Cell culture

The aortic smooth muscle cells used in this experiment were taken from a three-month old male Lewis rat. The animal protocol for the study was approved by the Animal Care Committee of the University Health Network, and all animal procedures were performed according to the “Guide for the Care and Use of Laboratory Animals” (the National Academy Press, revised 1996). The cell culture procedure was similar to the previous descriptions in the literature. The rat was euthanized, and the thoracic aorta was harvested by the aseptic technique. Briefly, the rat was excised and the aorta was removed. After removing the surrounding connective tissues and inner endothelial layer, the aorta was minced and digested by a mixture of collagenase and trypsin. The harvested cells were cultured in IMDM containing 10% FBS and then incubated at 37°C in a 5% CO₂ humidified incubator. In this study, the cells were subcultured and second to fourth passaged cells were utilized as experimental cells. For the cell-attachment and detachment experiments, the subcultured cells were kept in DPBS immediately before injecting into the TSM flow cell. For the ion effect and cell membrane damage experiments, the cells were cultured directly on the surface of the TSM crystal. On the fully covered surfaces, there were approximately 10,000 cells on the gold electrode per crystal.

2.3.4.2 Cell introduction and cell detachment from surface

Different extracellular matrices were used to investigate the attachment preference of the aortic smooth muscle cells. Using the total injection analysis method, laminin and fibronectin were immobilised on the TSM surface by chemisorption. Cells in the DPBS buffer were injected subsequent to binding of the protein. Additionally, a bare electrode surface was also used for evaluation of the possibility of the cell attachment. Trypsin was employed in order to effect the detachment of cells from the various surfaces.
Arg-Gly-Asp-Ser peptide was used to examine the mechanism of cell attachment to fibronectin surface. In the TSM flow cell, a similar procedure as mentioned above was applied for attaching cells onto fibronectin surface. After cell attachment, 300 μl peptide at 1mg/ml was injected to affect the binding between cells and fibronectin. DPBS buffer was allowed to flow over the surface for 15 min and cells were injected again onto the surface. This buffer washing and cell attachment procedure was repeated once again before final washing with DPBS buffer.

2.3.4.3 Ionic influence and cell depolarization

Ca$^{2+}$ (1 mM) and Mg$^{2+}$ (1 mM) were used to affect the attachment of the cells onto the surface. K$^+$ (60 mM) were used to depolarize the surface-attached cells. Briefly, a TSM crystal with directly cultured cells was placed in the flow-through cell and the DPBS was injected until a stable baseline was achieved. Ions (Ca$^{2+}$, Mg$^{2+}$ and K$^+$) in DPBS buffer were pumped over this cell-coated crystal surface by flow injection for 15 min. DPBS buffer was finally used again to wash the crystal surface.

2.3.4.4 Cell interactions with hydrogen peroxide

2.3.4.4.1 TSM

In a similar protocol to that used for cell depolarisation, the device with attached cells was placed in the flow-through cell. Instead of using DPBS, the cell culture medium (IMDM) was injected until a stable baseline was achieved. Hydrogen peroxide was diluted to 200 mM by IMDM and pumped over the TSM surface. The pump was stopped for 15 min and the cell culture medium was then used to wash the device in a final step.

2.3.4.4.2 Optical microscope

A preliminary study of the nature of the changes caused by hydrogen peroxide was done by periodically observing cells on surfaces under an inverted optical microscope. The reagent, H$_2$O$_2$, was added directly onto the surface by pipette.

2.3.4.4.3 Scanning electron microscope
SEM was used to observe the morphology change of the cells after interaction with hydrogen peroxide. Two samples were compared. One was the crystal with freshly cultured cells; the other was the crystal with cultured cells after interaction with hydrogen peroxide. For SEM experiments, the cells had to be fixed onto the crystal surface after the attachment using the following steps. Firstly, the cell-coated surface was kept in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) for 1.5 hours. The crystal was then kept in 0.1 M sodium cacodylate buffer with 0.2 M sucrose (pH 7.3) for 10 min. Subsequently, the crystal was kept in 70%, and 90% ethanol, separately, for 10 min. 100% ethanol was also used to wash the crystal surface three times. Finally, the crystal was maintained in 100% ethanol. After fixation, a critical point dryer was used before the SEM experiment to evaporate the ethanol. In addition, gold sputter coating was utilized after the critical point drying to aid the SEM experiment.

### 2.3.4.4 Atomic force microscope

AFM was also used for a confirmation of the morphology change of cells. The cells were fixed onto the TSM crystal surface as done for SEM experiments, except that the critical point dryer and gold sputter coating were not used. A third crystal was also observed, representing the surface interacted with hydrogen peroxide but after subsequent washing by IMDM medium.
3. Results and discussion

3.1 Detergent

In this research, the initial trials of the TSM experiments were conducted mostly by reused but cleaned crystals and confirmations of the results were accomplished by new crystals. The TSM results obtained from reused and new crystals are similar. Since reused TSM crystals were utilized by some of the experiments in this research, it is necessary to make sure that biomaterials did not remain on the surface before running any new experiments. Therefore, the cleaning procedure is crucial for the experiments. A specially designed cleaning method was used to wash the used crystals. As mentioned in the Experimental procedure section, SDS was used to detach the biological adsorbate. The cleaned crystal samples were compared under XPS. Results are shown in Table 2 below. Comparable cleaning results can be achieved by using normal cleaning procedure and SDS cleaning procedure separately to clean unused new crystals as shown in Table 2 (for sample 1 and sample 2). However, when neutravidin was attached onto the crystals, SDS cleaning procedure gave much better results than normal cleaning procedure. As shown for sample 3 and sample 4 in Table 2, since N is the characteristic element for attached protein in XPS spectra, it is notable that sample 3 has more nitrogen signal than sample 4. Therefore, by using SDS cleaning procedure, most of the surface-attached protein would be washed away from the TSM crystal. It has to be pointed out that before cleaning, sample 3 and sample 4 had a similar amount of protein attached on the surface.
Table 2. XPS data for four surfaces in terms of major elemental ratios. Sample 1 is the new crystal cleaned by the usual procedure; Sample 2 is the new crystal cleaned by SDS solution; Sample 3 is neutravidin-coated crystal cleaned by the usual procedure; Sample 4 is neutravidin-coated crystal cleaned by SDS solution.

<table>
<thead>
<tr>
<th></th>
<th>sample 1 (%)</th>
<th>sample 2 (%)</th>
<th>sample 3 (%)</th>
<th>sample 4 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>46.4</td>
<td>48.1</td>
<td>66.2</td>
<td>49.8</td>
</tr>
<tr>
<td>N</td>
<td>0.9</td>
<td>1.2</td>
<td>9.6</td>
<td>1.1</td>
</tr>
<tr>
<td>O</td>
<td>49.1</td>
<td>47.8</td>
<td>23.5</td>
<td>46.4</td>
</tr>
<tr>
<td>S</td>
<td>0.1</td>
<td>0.0</td>
<td>0.2</td>
<td>0.0</td>
</tr>
<tr>
<td>Cr</td>
<td>3.3</td>
<td>2.7</td>
<td>0.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Au</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>
3.2 DNA

3.2.1 Study of duplex formation by acoustic wave propagation

The acoustic wave technique was employed to study the mechanism of hybridization of oligodeoxynucleotides containing 1, 2 or 3 abasic sites by a comparison with undamaged complementary and non-complementary ODNs (shown in Table 1). Figure 15 shows the changes in $f_s$ and $R_m$, following attachment to the device surface of the various compounds. The addition of neutravidin in DPBS resulted in a sharp decrease of the resonance frequency and increase of the $R_m$ value. The motional resistance is characterized by complex behavior. After an initial increase immediately following addition of neutravidin, the $R_m$ value reached a maximum value and then decreased. Addition of buffer A resulted in a further decrease of frequency and increase of $R_m$. This change is caused by the different ionic strength and electrolyte composition of buffer A and DPBS for neutravidin solution. After approximate 30 minutes, the changes of both $f_s$ and $R_m$ were stabilized. The changes of frequency due to addition of neutravidin were approximately 200 Hz, while $R_m$ only slightly decreased in comparison with the initial value prior to addition of neutravidin. The decrease of series resonance frequency following addition of neutravidin is a well-known phenomenon\textsuperscript{164}, and is connected with attachment to a gold surface through hydrophobic interactions. According to the Sauerbrey\textsuperscript{19} expression (shown in section 1.2.2), from changes in frequency ($\Delta f_s$) it is possible to produce a rough idea of the change of mass at the device surface, $\Delta m$, via the known molecular mass of neutravidin (60 kD). Accordingly, it is potentially feasible to arrive at an estimate of the number of neutravidin molecules adsorbed at the gold surface of the electrode.
Figure 15. Changes of series resonance frequency $\Delta f_s$ (full line) and motional resistance $R_m$ (dashed line) following addition of neutravidin (1), biotinylated DNA (X-BASE, see Table 1) (2) and complementary ODN (APC, see Table 1) (3). (B) represents flow of buffer A. The points of addition are indicated by arrows.
According to Sauerbrey, the resonance frequency of the crystal linearly depends on its mass:

$$\Delta f_s = -2.26 \times 10^{-6} f_0^2 \left( \frac{\Delta m}{A} \right)$$

(22)

where \(A\) is the area of working electrode (in my case \(A = 0.28 \text{ cm}^2\)) and \(f_0\) is the fundamental frequency of the quartz crystal (i.e. 9 MHz in my case). Thus, a change of \(\Delta f_s = 200\) Hz corresponds to a change of mass \(\Delta m = 3.06 \times 10^{-7}\) g and the number of neutravidin molecules at the surface is \(3.07 \times 10^{12}\). The results represent the usual invocation of the Sauerbrey effect. However, since the Sauerbrey equation cannot be used with confidence in a liquid environment, it is much more likely that there is a significant contribution from the viscoelasticity of the protein film which affects coupling between the layer adsorbed at the crystal and surrounding electrolyte. The effect of viscoelasticity certainly exists as it follows complex changes of motional resistance \(R_m\) (Figure 15). It has been suggested that a correction factor of approx. 2 could be applied to correct changes of frequency connected with viscoelasticity \(^{108}\). Thus, considering this correction, the number of neutravidin molecules at the surface would be approx. \(1.5 \times 10^{12}\).

From Figure 15, it is seen that after the frequency and \(R_m\) values were stabilized, the biotinylated 19-mer DNA (X-BASE, biotin is placed at the 5’ terminal of ODN) dissolved in buffer A in a concentration of 1 \(\mu\text{M}\) was allowed to flow into the cell. Due to the strong affinity of biotin to neutravidin, a strong binding of X-BASE to the neutravidin molecules took place. This resulted in a decrease of frequency and increase of \(R_m\) value. The following flow of buffer resulted in a slight decrease of the frequency and in a considerable decrease of \(R_m\). The steady-state value of \(R_m\) is, however, similar to that of the crystal covered by the neutravidin layer prior to addition of the biotinylated DNA. Given the results, it is expected that the 19-mer single strand DNA does not contribute substantially to the change of surface viscosity of the layer. The
reason for this is unclear. However, it may be because the length of single stranded DNA is short and at some point it could be described as a rigid monolayer. From the change of the frequency it is possible to estimate the number of ODN molecules adsorbed at a neutravidin layer. As is evident from Figure 15, the binding of biotinylated ODN to a neutravidin layer is accompanied by a decrease of the frequency of 60 Hz. Since the molecular weight of ODN is 6300 D, the number of ODN molecules at the surface would be $4.4 \times 10^{12}$. Thus, according to the Sauerbrey treatment, the number of attached ODNs is about 2.9 times larger than the number of neutravidin molecules chemisorbed at the crystal surface. Radiochemical labeling experiments have previously shown that this ratio of nucleic acid to protein is actually around 1.2$^{164}$. This result again serves notice that the Sauerbrey mass-response model can at best be regarded to yield only approximate values. Other factors in liquid are controlling acoustic wave propagation.

Injection of complementary ODN (APC, concentration 1 $\mu$M) resulted in a further decrease of the resonance frequency (Figure 15). The changes of frequency were approx. 52 Hz, which was slightly lower than the changes of the frequency that are typical for adsorption of X-BASE. The differences between these values are, however, not significant. The changes of frequency following addition of APC are obviously connected with hybridization at the crystal surface. In contrast, the motional resistance increased following addition of complementary ODN. Even after flow of the buffer, the $R_m$ value was higher in comparison with that prior addition of complementary ODN. This considerable change of $R_m$ following binding of APC and a slow relaxation of $R_m$ to a steady state value may offer an indication of a substantial structural change in the DNA layer. These changes are evidence that the double stranded DNA array at the surface contributes considerably to the increase of surface viscosity. This phenomenon can be explained in term of flexibility and electric charges of double stranded (dsDNA) in comparison with single stranded DNA. Certainly, the persistent length of dsDNA is
approximately 50 nm\textsuperscript{165}. The length of 19 b.p. DNA used in this experiment is approximately 6 nm, which is smaller than the persistent length. Therefore ODNs are in an extended conformation. Since the negative charges on dsDNA is higher than on ssDNA, dsDNA has the ability to drag more water molecule to move together with TSM system and cause more coupling or viscous forces between the DNA layer and surrounding electrolyte\textsuperscript{109}. This could be one of the reasons for increased resistance after hybridization.

Figure 16 shows a control experiment after addition of neutravidin and the non complementary ODN (BASE, shown in Table 1 ). The result is that there were no changes of frequency and $R_m$ caused by this addition. The non complementary ODN also did not change the frequency or $R_m$ after addition to the surface with immobilized biotinylated probe (X-BASE), which indicates that there was no interaction between non complementary ODN and X-BASE. Further buffer flow removed all of the BASE probes from the surface. Then the ODN containing one abasic site (AP1) was added at a concentration of 1 $\mu$M. This addition resulted in a decrease of frequency and increase of $R_m$. These changes are, however, lower in comparison with that caused by hybridization of APC, which are the complementary probes to X-BASE. Addition of ODN containing 2 abasic sites (AP2) caused similar changes to that of AP1, which is shown in Figure 17. Presence of three abasic sites in ODN (AP3), however, changed the situation dramatically (Figure 18). As seen from Figure 18, addition of AP3 resulted in a decrease of frequency, with the flow of buffer A yielding an increase of frequency. The resulting frequency changes are considerably lower in comparison than that for ODNs with one or two abasic sites (See Table 3). This result presents evidence regarding the weak interaction of AP3 with the X-BASE probes at the surface of the device. The changes of frequency and resistance for ODN containing different numbers of abasic sites are summarized in Table 3.
Figure 16. Changes of series resonance frequency $\Delta f_s$ (full line) and motional resistance $R_m$ (dashed line) following addition of neutravidin (1), non-complementary ODN (BASE, see Table 1) (2), biotinylated DNA (X-BASE, see Table 1) (3), non-complementary ODN (BASE) (4) and ODN contained one abasic site (AP1, see Table 1) (5). (B) represents flow of buffer A. The points of addition are indicated by arrows. The general procedure and average response times for signal stabilization after addition of reagents are: Firstly, inject (B) to get stable baseline, the time was usually around 40 min. Secondly, inject (1) and wash the surface with (B) for 15 min. Thirdly, inject (2) and wash the surface with (B) for 15 min. Fourthly, inject (3), stop for 10 min and wash the surface with (B). Fifthly, inject (4), stop for 30 min and wash the surface with (B) for 15 min. Lastly, inject (5), stop for 30 min and wash the surface with (B) for 15 min or towards the end of the experiment.
Figure 17. Changes of series resonance frequency $\Delta f_s$ (full line) and motional resistance $R_m$ (dashed line) following addition of neutravidin (1), biotinylated DNA (X-BASE) (2) and ODN contained two abasic sites (AP2, see Table 1) (3). (B) represents flow of buffer A. The points of addition are indicated by arrows.

Figure 18. Changes of series resonance frequency $\Delta f_s$ (full line) and motional resistance $R_m$ (dashed line) following addition of neutravidin (1), biotinylated DNA (X-BASE) (2) and ODN contained three abasic sites (AP3, see Table 1) (3). (B) represents flow of buffer A. The points of addition are indicated by arrows.
Table 3. Changes of frequency and motional resistance caused by addition to the ODN-coated surface of the TSM with complementary (APC), non complementary ODNs (BASE) or those that contained 1 (AP1), 2 (AP2) or 3 (AP3) abasic sites. The experiments were run in triplicate. Results represent mean ±S.D from the independent experiments in each series.

<table>
<thead>
<tr>
<th>ODN</th>
<th>$\Delta f_s$ (Hz)</th>
<th>$R_m$ (Ohm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>49.0 ± 2.9</td>
<td>1.7 ± 0.5</td>
</tr>
<tr>
<td>AP1</td>
<td>39.6 ± 4.5</td>
<td>1.3 ± 0.5</td>
</tr>
<tr>
<td>AP2</td>
<td>42.0 ± 7.4</td>
<td>0.7 ± 0.5</td>
</tr>
<tr>
<td>AP3</td>
<td>13.7 ± 7.0</td>
<td>0 ± 0.3</td>
</tr>
<tr>
<td>BASE</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
3.2.2 Thermal stability of double-stranded oligonucleotides containing abasic sites from UV-detection

19-mer DNA duplexes differing in the number of AP sites (0-3) were characterized by their melting behavior in buffer B (Table 4 and Figure 19). Inspection of UV-melting data reveals that the presence of AP sites leads to significant duplex destabilization. This effect depends dramatically on the quantity of AP sites and their location in the double helix. In the case of the fully paired duplex BASE-APC, the UV-monitored absorption as a function of temperature showed a single reversible transition with a melting temperature \( T_m = 64 \pm 1 \) °C; Figure 19A demonstrates the melting curves presented in integral and differential forms. In contrast to the non-modified double helix, BASE-AP1 containing one abasic residue demonstrates the biphasic melting profile reflecting the superposition of two helix-coil transitions as shown in Figure 19B. This result corresponds to denaturation of two duplex domains, the boundary being at AP site, which induces DNA-bending and increased conformational flexibility. Because the AP site is not located in the middle of double helix, separate transitions can be observed corresponding to short and long duplex domains (with \( T_m = 41 \) and 55 °C, respectively, Table 4). As we know, the incorporation of an abasic site opposite to the deoxyadenosine residue stacked inside the helix does not necessarily alter the melting cooperativity of the short DNA duplex relative to the fully paired Watson-Crick structure\(^{165}\). The new results show that the abasic site opposite to the deoxythymidine residue may alter the melting behavior. Additionally, the second AP site placed in the short domain (BASE-AP2) decreases the \( T_m \) of the corresponding transition (Figure 19C). Therefore, a conversion of the biphasic melting profile to the monophasic type takes place. It should be noted that, the \( T_m \) of BASE-AP2 (45 °C) containing two AP sites is not equal to the dissociation temperature (55 °C) for a long domain of BASE-AP1. The reduced \( T_m \) can be attributed to a destabilizing effect of the unpaired 9-nt "tail". A third AP site placed at the center of the long domain prevents stable
Table 4. UV-monitored melting of 19 b.p. DNA duplexes containing AP sites (\(R\)), where *\(T_m\) – melting temperature, **\(\Delta T_m\) – the difference in melting temperature for perfect control and modified duplexes.

<table>
<thead>
<tr>
<th>DNA duplex</th>
<th>Designation</th>
<th>Type of helix-coil transition</th>
<th>(T_m)*(\pm) 1°C</th>
<th>(\Delta T_m)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'→3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3'→5'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GGAAAATTTTCAGCAAGGTG</td>
<td>BASE-APC</td>
<td>monophasic</td>
<td>64</td>
<td>-</td>
</tr>
<tr>
<td>CCTTTTTAAAGTCGTTCCAC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GGAAAATTTTC(R)GCAAGGTG</td>
<td>BASE -AP1</td>
<td>biphasic</td>
<td>55</td>
<td>9</td>
</tr>
<tr>
<td>CCTTTTTAAAGTCGTTCCAC</td>
<td></td>
<td></td>
<td>41</td>
<td>23</td>
</tr>
<tr>
<td>CGAAAATTTTC(R)C(R)AGGTG</td>
<td>BASE -AP2</td>
<td>monophasic</td>
<td>45</td>
<td>19</td>
</tr>
<tr>
<td>CCTTTTTAAAGTCGTTCCAC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CGAA(R)ATTTC(R)G(R)AGGTG</td>
<td>BASE -AP3</td>
<td>no cooperative transition</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CCTTTTTAAAGTCGTTCCAC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 19. UV melting curves measured at 260 nm (in integral (1) and differential (2) forms) for 19 b.p. DNA duplexes with zero, one and two abasic sites: BASE-APC (A), BASE-AP1 (B) and BASE-AP2 (C), respectively. The superposition of the melting curves for undamaged duplex BASE-APC (○) and duplex containing three abasic sites BASE-AP3 (●) (D). All measurements were performed in buffer B at a single strand concentration of 4 μM.
duplex formation. The absence of a cooperative S-shaped melting curve points to an unstable structure (Figure 19D).

These data indicate the significant role of local structural perturbations at AP sites on the thermostability of DNA duplex. Furthermore, this work confirms that at the temperature conventionally used in electrochemical experiments ($20 \pm 1^\circ C$), the regular 19-mer ODNs and DNA molecules with one or two abasic sites are in duplex form with complementary strand present. In contrast, ODN with three AP residues is predominantly in the single-stranded state.

After reviewing these results of DNA experiments, we have mastered some basic idea of how to apply TSM sensor in bioanalytical field and the limitation of employing Sauerbrey equation in liquid phase. Now I will move on to a more complicated situation with respect to protein attached on TSM surface.

3.3 Calmodulin

3.3.1 Structure and dynamics of calmodulin in solution

To interpret the sensor response when calcium binds to calmodulin on the device surface, it is required to have an understanding of the structural and conformational changes in the protein itself. As mentioned in the introduction chapter, calmodulin is a dumbbell-shaped protein with globular calcium-binding domains on each end, connected by a 28-residue central helix region. In the unbound state, the protein is in a closed form, where the C- and N-terminal domains rotate towards each other and block the hydrophobic regions on the binding-domain surfaces. In this conformation, the central helix is in a bent configuration. The unbound closed state characterised by the dumbbell shape is shown in Figure 20 (a). When calmodulin binds calcium, it reverts to the open state, shown in Figure 20 (b). In total, calmodulin can bind to four calcium ions. In a bound state, the central helix region becomes straight and rigid, and binding domains rotate outwards, exposing the hydrophobic domain.
As a result, the calmodulin molecule is more rigid in the calcium-bound state than in the calcium-free state. As well, calmodulin undergoes a five-percent increase in size on binding of calcium. Small-angle X-ray scattering experiments show an increase in overall length from 58 Å in the unbound state, to 62 Å in the bound state, and a corresponding increase of the radius of gyration ($R_g$) from 20.6 to 21.5 Å. In total, binding of calcium to calmodulin has the effect of stabilizing the central helix region of the protein and reducing the conformational entropy.
Figure 20. Crystal structures of (a) apo- and (b) holo-calmodulin. The apo form is closed, with the Ca$^{2+}$-binding domain rotated to block the central helix region. The holo form shows a more open structure, with the central helix in a rigid, elongated form. The four black spheres in (b) are four calcium ions. The crystal structures were obtained from the Protein Databank$^{170,171}$, with PDB ID 1CFD for apo-calmodulin$^{172}$ and PDB ID 4CLN for the holo form$^{173}$. Both images were generated from the PDB coordinates using PyMol (DeLano Scientific, San Francisco, USA).
3.3.2 Binding of biotinylated calmodulin with ions

We now turn to the responses observed for interaction of calmodulin with ions and peptide, conducted in the flow-through format. This work includes a number of control experiments designed to establish the validity of signals produced by genuine binding events. Figure 21 shows a typical plot of the responses obtained for the complete sequence of additions to the flow-injection system of the linking reagent neutravidin, biotinylated calmodulin, and DPBS buffer solution, followed by $\text{Ca}^{2+}$ and $\text{Mg}^{2+}$ ions introduced under various conditions. Attachment of neutravidin produced the expected reduction in frequency of around 200 Hz, accompanied by an increase in $R_m$ due to the viscoelastic character of the neutravidin layer. From previous work\textsuperscript{141, 174}, as also mentioned in DNA experiments, the frequency decrease of 200 Hz is shown to arise from a surface population of protein of approximately 1 pmol·cm$^{-2}$. Although attachment of this protein to gold has been attributed to metal-sulfur interaction, it is also possible that it arises through hydrophobic forces\textsuperscript{51, 175, 176} This might be due to the hydrophobic domains present in the protein structure interacting with the metal surface\textsuperscript{176}.

Confirmation of the on-line formation of the neutravidin-protein complex is evident from the 100 Hz decrease in $f_s$ and concomitant change in $R_m$. There are 7-8 biotin binding sites per CaM molecule, since biotin normally binds to surface-available lysine groups\textsuperscript{177} However, biotin-protein binding normally follows a Gaussian distribution, so there will be an average of 3-4 biotin linkages per CaM. Neutravidin has four biotin binding sites\textsuperscript{178} However, no more than two calmodulin molecules will bind per neutravidin, due to the relative sizes of the molecules. Assuming two calmodulin molecules per neutravidin, and a ratio of molecular weights of 1:4, the frequency decrease of 100 Hz on immobilization of biotinylated calmodulin is reasonable. As well, biotinylation is reported to have no effect on the general activity of calmodulin in solution and on surfaces\textsuperscript{179}. 

77
Figure 21. Biotinylated CaM (3.00 μM) first interacting with 1 mM MgCl₂ and 1 mM CaCl₂ in DPBS buffer containing larger than 100mM NaCl. This is followed by interaction with 1 mM MgCl₂ and 1 mM CaCl₂ in 10 mM NaCl water solution. Solid line shows frequency change and dashed line shows resistance change. The arrow shows the injection time, and the data in the parentheses represent the concentration ratio of Ca²⁺ or Mg²⁺ to Na⁺ in the solution. The first additions of CaCl₂ and MgCl₂ are in DPBS buffer, and the second additions are in NaCl solution.
Introduction of buffer solution itself caused significant responses with virtually no signal being produced by calcium or magnesium ions in the same solution. The latter result is clearly connected with the ratio of Na$^+$ to divalent ion present in this solution (100:1). Under these conditions, the monovalent ion is expected to interfere with Ca$^{2+}$ or Mg$^{2+}$ binding in view of the protein association constant of $10^2$ M$^{-1}$ for Na$^+$. Subsequent introduction of divalent ions at a much lower concentration ratio of Na$^+$:M$^{2+}$ (10:1) caused the change of frequency and resistance, which suggests that signals are found for CaM binding of these ions. This was the condition used for all following experiments.

Given the strong indication of genuine protein-divalent ion attachment, it is necessary to perform careful control experiments conducted in the absence of CaM. (The underlying substrate is likely to consist of partially CaM-covered neutravidin or gold.) Figure 22 and Figure 24 depict introduction of various ions to neutravidin previously introduced to the device electrode and to a bare gold electrode. It is noteworthy in these experiments that little or no change in $f_s$ obtained for interaction of the ions with either surface, although this is not the case with respect to the responses in $R_m$. In sharp contrast to the above, Figure 25 shows that both Ca$^{2+}$ and Mg$^{2+}$, interacting with the CaM surface, cause increases in $f_s$, with the largest rise occurring for the former ion. These results are consistent with the binding of the ions to the protein where the association constants are $10^6$ to $10^7$ M$^{-1}$ for calcium and $10^2$ to $10^4$ M$^{-1}$ for magnesium. From the point of view of acoustic detection, the expected additional mass associated with the ions is obviously minimal in terms of an anticipated shift in frequency, but more importantly the change in $f_s$ is positive, which is in the opposite direction to that predicted from the Sauerbrey response mentioned previously. According to the Sauerbrey equation, added mass should cause a frequency decrease; whereas in Figure 25 the addition of ions onto the surface caused frequency increases. Furthermore, return to buffer flow in the system confirms that the attachment of both divalent ions is completely reversible as would be expected from the
values of the association constants.
Figure 22. Neutravidin interacting with 1 mM CaCl₂ and 1 mM MgCl₂. Solid line shows frequency change and dashed line shows resistance change. The arrow shows the injection time and the data in the parentheses represent the concentration ratio of Ca²⁺ or Mg²⁺ to Na⁺ in the solution.

No change in frequency and a decrease in Rm were observed with 1 mM CaCl₂ or 1 mM MgCl₂ when employing a dilute DPBS buffer containing 10 mM NaCl and in the presence of neutravidin only.
Figure 23. The relevant part of Figure 22 has been blown up to clearly show the frequency change and the resistance change induced by ions.
Figure 24. Bare gold interacting with 1 mM MgCl$_2$ and 1 mM CaCl$_2$. Solid line shows frequency change and dashed line shows resistance change. The arrow shows the injection time and the data in the parentheses represent the concentration ratio of Ca$^{2+}$ or Mg$^{2+}$ to Na$^+$ in the solution.

No change in frequency and a decrease in Rm were observed with 1 mM CaCl$_2$ or 1 mM MgCl$_2$ when employing a dilute DPBS buffer containing 10 mM NaCl using a bare gold crystal.
Figure 25. The reversible interaction between biotinylated CaM (2.45 μM) and 1 mM Ca\textsuperscript{2+}/Mg\textsuperscript{2+} using buffer to wash away ions. Solid line shows frequency change and dashed line shows resistance change. The arrow shows the injection time.
Figure 26 shows the results of experiments similar to the one given in Figure 25, but this time various concentrations of calcium ions were used to interact with CaM on surface. The changes of frequency and resistance are shown in Table 5. Clearly, for the calcium concentrations used, a saturation of the $f_s$ response is found. As noted above, this is not the case for changes in $R_m$, where a gradual increase for increasing ion concentration was observed. The control experiments were similar to those described previously, and thus excluded the non-specific adsorption conditions. As shown in Figure 27 and Figure 28, the results for the control experiments indicated that the change of frequency for both of gold and neutravidin surfaces were nearly unaffected by the different concentrations of calcium ions.
Figure 26. Biotinylated CaM interacting with different concentrations of CaCl₂. Solid line shows frequency change and dashed line shows resistance change. The arrow shows the injection time and the data in the parentheses represent the concentration ratio of Ca²⁺ to Na⁺ in the solution.
Table 5. Acoustic wave sensor responses for injecting different concentrations of CaCl₂ to biotinylated CaM surface.

<table>
<thead>
<tr>
<th>Concentration/mM</th>
<th>$\Delta f$/Hz</th>
<th>$\Delta R_m$/Ω</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>+10.0</td>
<td>+1.0</td>
</tr>
<tr>
<td>1.0</td>
<td>+9.6</td>
<td>+1.3</td>
</tr>
<tr>
<td>1.5</td>
<td>+10.0</td>
<td>+1.8</td>
</tr>
<tr>
<td>2.0</td>
<td>+9.2</td>
<td>+2.5</td>
</tr>
<tr>
<td>2.5</td>
<td>+7.2</td>
<td>+2.8</td>
</tr>
<tr>
<td>3.0</td>
<td>+8.1</td>
<td>+3.4</td>
</tr>
<tr>
<td>5.0</td>
<td>+10.2</td>
<td>+5.0</td>
</tr>
</tbody>
</table>
Figure 27. Injecting different concentrations of CaCl$_2$ and MgCl$_2$ to gold surface. Solid line shows frequency change and dashed line shows resistance change. The arrow shows the injection time and the data in the parentheses represent the concentration ratio of Ca$^{2+}$ or Mg$^{2+}$ to Na$^+$ in the solution.
Figure 28. Injecting different concentrations of CaCl₂ and MgCl₂ to neutravidin surface. Solid line shows frequency change and dashed line shows resistance change. The arrow shows the injection time and the data in the parentheses represent the concentration ratio of Ca²⁺ or Mg²⁺ to Na⁺ in the solution.
Control experiment using BSA. It is known that biotinylated calmodulin solution contains 1mg/ml BSA when it was bought from VWR. Therefore, it is necessary to confirm that signals were not due to the presence of BSA. From Figure 29, when BSA was injected onto the neutravidin surface, the frequency signal decreased slightly. When buffer was used to wash the surface after BSA attachment, the frequency returned to the original value before BSA injection. A similar trend for the resistance change has been observed and is shown in Figure 29. These results indicate that BSA can not strongly attach onto neutravidin-coated surfaces. This partially eliminates the interaction between BSA and ions that are subsequently injected. Furthermore, when 1 mM Ca\textsuperscript{2+} solution was injected onto the surface, the frequency was slightly changed; whereas, when 1 mM Ca\textsuperscript{2+} solution flowed over calmodulin surface, the frequency was increased. As a result, this experiment fully supports that the frequency changes shown in Figure 25 and Figure 26 are induced by the conformational change of CaM.
Figure 29. Control experiment of BSA with calcium ions. Solid line shows frequency change and dashed line shows resistance change. The arrow shows the injection time.
3.3.3 Response of the shear acoustic wave device to binding of calcium with biotinylated calmodulin

Figure 25 shows that there is a definite response of the device to the addition of calcium ions to surface-bound calmodulin. With addition of CaCl$_2$, an increase in $f_s$ and a decrease in $R_m$ are observed. A portion of this response is due to ionic effects in the liquid, as demonstrated by the addition of the same ionic salt to neutravidin resulting in a smaller shift (see Figure 22). Accounting for these effects by subtracting the control response in Figure 22 from the response in Figure 25 (see Table 6), a device response of $\Delta f_s = +4.5$ Hz and $\Delta R_m = -0.6$ $\Omega$ has been calculated. This is due to changes in the acoustic character of the calmodulin at the device surface that results from calcium binding to CaM. When the sensor is viewed as a microbalance, an increase in frequency would indicate a loss of mass from the device surface, which is clearly absurd in this situation since ions are being added to the solution. It is instructive instead to view both $f_s$ and $R_m$ together.

Generally speaking, there are five major changes induced by calcium in calmodulin that could have influenced the acoustic response of the TSM sensor after the effect of the ionic salt has been considered. These are summarized in Table 7, including their likely effect on $f_s$ and $R_m$. 

92
Table 6. Acoustic response on addition of the indicated sample to the sensor surface. In the first two rows, calcium ions are introduced to the surfaces indicated. The third row shows the response of calcium binding to calmodulin after the effect of the electrolyte has been removed by subtraction.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Surface</th>
<th>$\Delta f_s$ (Hz)</th>
<th>$\Delta R_m$ (Ω)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl$_2$</td>
<td>Neutavidin</td>
<td>6.1 (± 0.4)</td>
<td>−1.2 (± 0.1)</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>Biotin-CaM</td>
<td>10.6 (± 0.2)</td>
<td>−1.80 (± 0.04)</td>
</tr>
<tr>
<td>Calculated:</td>
<td>Ca$^{2+}$-CaM</td>
<td>4.5 (± 0.5)</td>
<td>−0.6 (± 0.1)</td>
</tr>
</tbody>
</table>
Table 7. Summary of known conformational shifts of calmodulin on binding calcium, and their likely acoustic effect and device response

<table>
<thead>
<tr>
<th>Response of CaM to Ca(^{2+})</th>
<th>Acoustic effect</th>
<th>Likely response of TSM device</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decrease in conformational entropy</td>
<td>Increased order of adsorbate</td>
<td>Small decrease in (R_m)</td>
</tr>
<tr>
<td>Increase in rigidity of central helix</td>
<td>Increase in elastic storage at interface</td>
<td>Small increase in (f_s)</td>
</tr>
<tr>
<td>Increase in length by 4 Å</td>
<td>Increase in effective wavelength of device</td>
<td>Decrease in (f_s)</td>
</tr>
<tr>
<td>Increase in (R_g) by 0.9 Å</td>
<td>Increase in roughness</td>
<td>Increase in roughness couples to increase in hydrophobicity to lead to slip</td>
</tr>
<tr>
<td>Increase in hydrophobic contact</td>
<td>Increase in slip (coupled to roughness)</td>
<td>Increase in (f_s), decrease in (R_m)</td>
</tr>
</tbody>
</table>
The decrease in conformational entropy corresponds to an increased order in the adsorbate, which reduces the amount of scatter at the adsorbate boundary. This tapers the resonant peak, which corresponds to a decrease in $R_m$. The corresponding increase in rigidity leads to a change in the energy storage properties, and an increase in $f_s$, which can be described using Hooke’s law for the fundamental frequency of solid material $\omega = (K/m)^{1/2}$, where $K$ is the stiffness of the material and $m$ is the mass. As the stiffness increases, the resonant frequency will increase as well.\(^5\)

As mentioned above, calcium-binding leads to an exposure of the hydrophobic domains in calmodulin. This would lead to a decrease in coupling at the adsorbate-liquid interface, or a manifestation of partial slip,\(^184\) which corresponds to a decoupling of the adsorbate-viscous liquid interaction, resulting in an increase in $f_s$ and a proportional decrease in $R_m$. The change in $R_g$ and the elongation of the molecule, leads to an increase in roughness. A rough, hydrophobic surface generally corresponds to greater partial slip than one that is smooth and hydrophilic. Normally, on a smooth and hydrophilic surface, slippage effects could be neglected as in the previous DNA experiments. In the calmodulin experiments, the increases in $R_g$ and hydrophobicity of the calmodulin surface have potentially led to a manifestation of partial slip. Therefore, the slippage effect has to be taken into account in this work.

As discussed above, for changes involving the interaction of the acoustic wave with liquid under the no slip condition, a change in frequency will be accompanied by an equal and opposite change in the resonance width, $\Delta \Gamma$. The width is proportional to the change of motional resistance as

$$\Delta \Gamma = \frac{8K^2C_0f_0^2\Delta R_m}{\pi}$$

where $C_0$ is the parallel capacitance characteristic of the TSM device (see, for instance, ref \(^{185}\)), $f_0$ is the fundamental resonant frequency, and $K^2 = 7.74 \times 10^{-3}$ is an electromechanical coupling
coefficient of quartz. In this experiment, $C_0$ is $12.43 \pm 0.02$ pF.

However, when there is slippage at the interface of adsorbate and electrolyte, $\Delta \Gamma$ will not be equal to the frequency change $\Delta f_s$ induced by the liquid interaction effect such as interfacial coupling and viscosity change. As mentioned in the introduction section, the following two equations below should be used to calculate $\Delta f_s$ and $\Delta \Gamma$ separately:

$$
\Delta f_s = \Delta f_a + \Delta f_{i+sl} = -\frac{2f_0^2 \Delta m_g}{(\rho_q \mu_q)^{1/2}} A - \frac{f_0^{3/2}(\rho \eta)^{1/2}}{(\pi \rho_q \mu_q)^{1/2}} \left[ \frac{1}{(1 + a)^2 + a^2} \right], \quad (17)
$$

and

$$
\Gamma = \frac{f_0^{3/2}(\rho \eta)^{1/2}}{(\pi \rho_q \mu_q)^{1/2}} \left[ \frac{1 + 2a}{(1 + a)^2 + a^2} \right], \quad (18)
$$

where $\Delta f_a$ is the frequency induced by the weight of adsorbate according to the Sauerbrey equation, $\Delta f_{i+sl}$ is the frequency induced by viscosity of the liquid environment and the interfacial slippage, and $a$ is the ratio of the slip length ($\lambda$) and the velocity decay length ($\delta$) in the liquid. Based on the equations above,

$$
\Delta f_{i+sl} = \frac{-\Gamma}{1 + 2a}, \quad (24)
$$

Accordingly, $a$ can be written as:

$$
a = \frac{\eta}{\chi \delta} = \frac{\lambda}{\delta}, \quad (19)
$$

where the velocity decay length of the shear wave $\delta = (2 \eta \rho_0 \omega_0) / \sqrt{(\rho \eta)} \approx (177 \text{nm} - 250 \text{nm})$ for dilute aqueous solutions at room temperature. The slip length $\lambda$ can be written as:

$$
\lambda = f(\varepsilon_{u//}/\varepsilon_{u\perp}) \Gamma_a/\Gamma_m + (1 - \Gamma_a/\Gamma_m) \approx f(\varepsilon_{u//}/\varepsilon_{u\perp}) \Gamma_a/\Gamma_m \quad (20)
$$

where $\varepsilon_{u//}$ is the characteristic energy between the adsorbate and liquid interaction, $\varepsilon_{u\perp}$ is the characteristic energy between liquid and liquid interaction, $\Gamma_a$ is the surface excess of
adsorbate and \( \Gamma_m \) is the maximum surface excess of adsorbate. When the surface is hydrophobic, \( \varepsilon_{\text{wa}} / \varepsilon_{\text{il}} < 1 \), \( a \approx 10^{-2} \). Therefore, \( \Delta f_{\text{s+sl}} \approx -\Gamma/1.02 \).

As shown in Table 6, after the effect of the electrolyte was eliminated from the device response, an acoustic signal of \( \Delta f_s = +4.5 \) Hz and \( \Delta R_m = -0.6 \) \( \Omega \) is obtained, due to \( \text{Ca}^{2+}-\text{calmodulin} \) interaction. Referring to Eq. (23), one can find that the corresponding change in bandwidth is \( \Delta \Gamma = -11.7 \) Hz. Assuming that the change in bandwidth is due entirely to a change in coupling of the wave into viscous liquid at the surface-liquid interface, the decrease in bandwidth appears to be the evidence of a decrease in coupling and an increase in partial slip at the interface. To a first approximation, an increase in slip decouples the liquid from the surface, which would appear as a viscous response, proportional to \( (\rho \eta)^{1/2} \). A viscous response is characterized by opposite shifts of the resonant frequency. To match the viscous response, the decrease in \( \Gamma \) must be offset by an increase in \( f_s \) of 11.5 Hz. Since the total frequency change induced by the binding of calcium and CaM is 4.5 Hz, there are obviously some other effects that are counteracting the increase in frequency, which corresponds to a compensating decrease in frequency of \( \Delta f_s = -7.0 \) Hz. This change has previously been hidden by the viscous effect.

An assumption above was made that the change in dissipation that occurs when calcium is introduced to the calmodulin monolayer is due entirely to a change in interfacial coupling. As a result, the remaining shift in frequency, \( \Delta f_s = -7.0 \) Hz, should then be due to either a mass or extension of wavelength response in the monolayer, which is the \( \Delta f_s \) shown in Equation (17). The Sauerbrey equation\(^{19} \) gives the frequency drop for a mass added to the surface, that is \( \Delta f_s = -2.26 \times 10^{-6} f_0^2 (\Delta m/A) \), for an 9 MHz AT-cut quartz crystal. The molar mass of calcium is 40 g\( \cdot \)mol\(^{-1} \) and the electrode area is 0.28 cm\(^2 \). If assuming as above that the neutravidin surface concentration is of 1 pmol\( \cdot \)cm\(^2 \), and there is a 2:1 ratio of calmodulin to neutravidin, as well as a 4:1 ratio of \( \text{Ca}^{2+} \) to calmodulin, the mass response would be \( \Delta f_s = -0.2 \) Hz. Since the response
actual response is $\Delta f_s = -7.0$ Hz, it is evident that the added mass of the calcium is almost a negligible factor.

Here one can use a transformed Sauerbrey Equation to consider the thickness change of the layer responds to the quartz oscillation:

$$\Delta f_s = -\frac{2f_0^2 \Delta m_a}{\left(\rho_q \mu_q\right)^{1/2} A} = -\frac{2f_0^2}{\left(\rho_q \mu_q\right)^{1/2}} \rho_a h_a$$  \hspace{1cm} (14)

The frequency decrease must therefore be due to an increase in the effective wavelength of the substrate, which is associated with the thickness increase of the surface-attached biolayer. It is reported that the average density of protein with less than 20 kDa molecular weight is 1.35g/cm³. The molecular weight of calmodulin is 18.5 kDa. Therefore, in this calculation, 1.35g/cm³ will be used as the $\rho_a$. If the initial frequency is $f_0 = 9$ MHz and $2/(\rho_q \mu_q)^{1/2}$ equals to $2.26 \times 10^{-6}$ cm²·g⁻¹·Hz⁻¹, the change in thickness $h_a$ is calculated to be about 3 Å. This result underestimates the literature value for the change in length of calmodulin of 4 Å, but it is in the correct direction and within the same order of magnitude. As we know, a number of assumptions and approximations were made in the analysis, which could attribute to the error. A quantitative validation of the viscoelastic and coupling phenomena that would result from the calcium-CaM conformational shift would require a detailed molecular analysis of the interfacial physics, which is out of the scope of this work.

3.3.4 Binding of biotinylated calmodulin with peptide

As a direct consequence of the conformational change of CaM instigated by the binding of Ca²⁺, the protein is able to interact irreversibly with the peptide, smMLCK. Figure 30 shows introduction of the peptide to calcium-bound calmodulin. An increase in $f_s$ of 9.0 ± 0.5 Hz can be found indicating attachment of smMLCK to calmodulin. Return to buffer flow resulted in an apparent slow removal of the peptide from the device surface, an observation in
keeping with known solution chemistry.\textsuperscript{188, 189} The corresponding control experiments depicting simple addition of the peptide to the surface, without the presence of calmodulin, are shown in Figure 31 and Figure 32. In Figure 31, the signals showed that the peptide interacted with the gold surface and therefore added mass onto the crystal, which increased the wavelength of acoustic wave and thus decreased the frequency. In Figure 32, a small transient increase in $f_s$ could be observed together with a significant decrease in $R_m$, after washing with buffer. This was related to the temporary attachment of peptide onto the surface of neutravidin.
Figure 30. Biotinylated CaM interacting with Ca$^{2+}$ followed by binding with peptide. Solid line shows frequency change and dashed line shows resistance change. The arrow shows the injection time.

Figure 31. Injecting smMLCK peptide to gold surface. Solid line shows frequency change and dashed line shows resistance change. The arrow shows the injection time.
Figure 32. Injecting smMLCK peptide to neutravidin surface. Solid line shows frequency change and dashed line shows resistance change. The arrow shows the injection time.
3.3.5 Binding of SAM linked calmodulin with ions and peptide

As mentioned above, it is reported that biotinylated biomolecules will reserve most of their bioactivities when they are attached onto surfaces\textsuperscript{179}. For comparison, a self-assembled monolayer was also used in this experiment. Here 11-MUA linked CaM was used to interact with CaCl\textsubscript{2} (1 mM), MgCl\textsubscript{2} (1 mM) and smMLCK peptide. As shown in Figure 33, the binding of CaCl\textsubscript{2} and MgCl\textsubscript{2} to CaM induced a frequency increase and a motional resistance decrease, and the signals were reversible after buffer washing. These are similar to the signals given by biotinylated CaM. It is known that biotinylation does not affect the general activity of calmodulin in solution and on surfaces\textsuperscript{179}. And based on the TSM experimental results, it is reasonable to assume that biotinylated CaM can reserve its native state when attached onto surface, whereas SAM linked CaM may also retain its native conformation and orientation. Therefore, the binding between ions and CaM could induce similar signals compared to the interaction between ions and biotinylated CaM.

XPS has been used to analyze the different surfaces in my experiments (as shown in Figure 34 to Figure 36 below) to investigate the homogeneity of SAM layer and attachment of calmodulin onto the SAM surface. As mentioned in the Experimental section, three types of surfaces were prepared including 11-MUA, 11-MUA with NHS/EDC linker; and calmodulin attached onto the SAM modified surface. From Table 8, one can see that the nitrogen signal from the protein surface is the highest among the three surfaces. The 11-MUA surface does not contain any indication of a nitrogen signal, as expected. After NHS/EDC interaction, each 11-MUA molecule binds to one nitrogen. Therefore, the nitrogen signal should be increased. In addition, after protein was attached, the nitrogen signal would be significantly increased because of relatively high nitrogen content in protein molecule. These results have confirmed that 11-MUA SAM layer could be used for attaching calmodulin onto gold surfaces.
Figure 33. SAM linked CaM interacting with ions and peptide. Solid line shows frequency change and dashed line shows resistance change. The arrow shows the injection time.
In summary, the calmodulin conformational change has been detected by a TSM sensor. The slippage effect at the interface has controlled the observed signal, which is largely different from the previous DNA experiments. When analyzing the signals from a TSM experiment in liquid, it is very important to understand the relevant interfacial properties. In the following section, TSM will be used to detect cell behavior. Since the complexity of living cell is quite different from DNA and protein, it is not surprising that use of TSM to detect cells has historically made very slow progress. Furthermore, individual cells even from the same species under the same experimental condition will behave quite differently. However, it is hoped that with the development of instrumentation and advanced theoretical knowledge of interfacial processes, TSM could be widely used in the field of cell research. In the following section, TSM is used to detect the attachment and properties of smooth muscle cells.
Figure 34. XPS spectra for 11-MUA modified surface.
Figure 35. XPS spectra for EDC/NHS linked 11-MUA modified surface.
Figure 36. XPS spectra for calmodulin-coated surface.
Table 8. XPS data for three surfaces in terms of major elemental ratios.

<table>
<thead>
<tr>
<th></th>
<th>11-MUA (%)</th>
<th>11-MUA + EDC/NHS (%)</th>
<th>Calmodulin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Au</td>
<td>6.4</td>
<td>6.8</td>
<td>6.9</td>
</tr>
<tr>
<td>C</td>
<td>59.6</td>
<td>58.4</td>
<td>57.4</td>
</tr>
<tr>
<td>Cr</td>
<td>6.3</td>
<td>7.0</td>
<td>5.4</td>
</tr>
<tr>
<td>N</td>
<td>0.0</td>
<td>1.8</td>
<td>4.3</td>
</tr>
<tr>
<td>O</td>
<td>24.1</td>
<td>23.5</td>
<td>23.4</td>
</tr>
<tr>
<td>S</td>
<td>3.7</td>
<td>2.5</td>
<td>2.7</td>
</tr>
</tbody>
</table>
3.4 Cells

3.4.1 Surface characterisation and cell detection

Three different surface modifications were used to facilitate aortic smooth muscle cell attachment in this study\textsuperscript{69}. They were laminin, fibronectin, and bare gold. Figure 37 below is a sample set of results for deposition of the surface modifications, adhesion of cells to the surface, and subsequent washing with buffer. These experiments were repeated in triplicate and similar frequency shifts were obtained in all cases. However, the resistance decrease upon buffer washing from the fibronectin surface with cells attached was not always reproducible, and the reason for this remains unclear. For the laminin and fibronectin modified surfaces, there was a decrease in $f_s$ and an increase in $R_m$ when laminin or fibronectin was applied. The changes in $R_m$ indicate some interfacial and viscoelastic effects in the monolayers. The shifts for laminin were $\Delta f_s = -401.0$ Hz and $\Delta R_m = 23.6$ $\Omega$, whereas those for fibronectin were $\Delta f_s = -306.3$ Hz and $\Delta R_m = 2.6$ $\Omega$.

Figure 37(a) shows the device response for cell addition onto the laminin modified surface, which induced a small decrease in $f_s$ and a large increase in $R_m$. However, upon washing with buffer, the signals returned to their original values, indicating that the cells attached on a temporary basis only, and did not adhere strongly to the laminin surface. These results suggest that laminin provides an unsuitable modification for the attachment of smooth muscle cells on the device surface. It is unclear why cells do not adhere to the laminin surface.

Using the same method, another extracellular protein, fibronectin, has been studied. Figure 37(b) shows the signal when the cells attached onto fibronectin surface. Addition of the cells resulted in a frequency decrease of 140 Hz and a resistance increase of 14 $\Omega$. Subsequent washing with buffer resulted in net shifts of $\Delta f_s = -110$ Hz and $\Delta R_m = 4.3$ $\Omega$. This indicates that fibronectin is likely a suitable surface for aortic smooth muscle cell
Figure 37. TSM graphs for cells immobilization at different surfaces. Black line represents frequency change. Grey line represents resistance change. L, F, C, D represent laminin, fibronectin, cells, and DPBS respectively.
attachment and it seems that most of the cells remain attached even after being washed by the buffer.

The bare gold surface has also been investigated as shown in Figure 37(c). It is observed that the cells have been attached onto the bare gold surface directly, which is consistent with previous reports\textsuperscript{154,155} even though different cell types are utilized in these studies. In addition, after cell deposition and subsequent washing with buffer, the net frequency and resistance shifts were \(-172\) Hz and \(4.0\ \Omega\), respectively, indicating that good attachment of cells to the surface is reported in this thesis.

3.4.2 The kinetics of cell attachment and detachment

Using the same experimental data as shown in Figure 37, the attachment kinetics for these cells were compared. This information reveals the dynamics of cell attachment, as well as provides information to study cell detachment using trypsin. The kinetics are determined from changes in the differential frequency and resistance shifts over time. Since the frequency shift is proportional to the surface adsorption of the cells, the magnitude of the differential value is linked to the attachment/detachment kinetics\textsuperscript{190}. As seen in Figure 38, the attachment of cells onto a gold surface has the fastest kinetics, while attachment to fibronectin has the slowest. The laminin surface has the least cell capacity, even though it has higher attachment kinetics (Figure 38(a)). In contrast, the fibronectin surface showed much better cell capacity with the slowest kinetics (Figure 38(b)). Surprisingly, the gold surface has both the best cell capacity and the fastest kinetics (Figure 38(c)). The comparison is also shown in Table 9. Since fibronectin and gold have better capacity for cell attachment than laminin, in the latter experiments, I chose to only use these two surfaces. As seen from Figure 39, the cell detachment instigated by trypsin from the gold surface showed lower kinetics than for fibronectin surface. Trypsin is a serine protease that cleaves cell adhesion proteins at arginine (Arg) and lysine (Lys) residues, to break
cell-cell adhesion molecules which attach them together and with the surface. Fibronectin has both Arg and Lys residues, therefore it could be firstly digested by trypsin and make the cells detach from the surface. That might be one of the major reasons for the higher cell removal kinetics from the fibronectin surface. But, this result does confirm the availability of gold surface for the cell attachment.
Figure 38. Kinetics associated with attachment of cells. Black line represents differential frequency change. Grey line represents differential resistance change. L, F, C, D represent laminin, fibronectin, cells, and DPBS respectively.
Table 9. Comparison of cell attachment to different surfaces.

<table>
<thead>
<tr>
<th>Surface</th>
<th>$\Delta f_0$(Hz)</th>
<th>$\Delta R_m$(Ω)</th>
<th>Kinetics(Hz/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laminin</td>
<td>~0</td>
<td>~0</td>
<td>-44</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>-110</td>
<td>4.3</td>
<td>-25</td>
</tr>
<tr>
<td>Gold</td>
<td>-172</td>
<td>4.0</td>
<td>-72</td>
</tr>
</tbody>
</table>
Figure 39. Detachment kinetics of the surface-attached cells when washed by trypsin. Black line represents differential frequency change. Grey line represents differential resistance change. F, C, D represent fibronectin, cells, and DPBS respectively.
3.4.3 Acoustic response of cell adsorption

The analysis began with investigation of deposition of the surface modification reagents, laminin and fibronectin. Based on a previous publication, it is possible to obtain a very rough estimate of the extent of surface binding by using the Sauerbrey equation and then dividing the result by a factor of two as used in the previous DNA project. As shown in equation (25), the Sauerbrey formula is given by

$$\Delta f_s = -\frac{2f_0^2}{Z_q} \left( \frac{\Delta m}{A} \right)$$  \hspace{1cm} (25)

where \(2/Z_q \approx 2.26 \times 10^{-6} \text{ cm}^2 \cdot \text{g}^{-1} \cdot \text{Hz}^{-1}\), the fundamental frequency \(f_0\) is 9.0 MHz and the area is 0.28 cm\(^2\). Therefore, the equation (25) can be written as:

$$\Delta f_s = -2.26 \times 10^{-6} \left(9.0 \times 10^6\right) \left(\frac{\Delta m}{0.28 \times 10^{-4}}\right)$$  \hspace{1cm} (26)

Since the molecular weights of laminin and fibronectin are 600 and 500 kDa, respectively, the shifts of 400 Hz for laminin (Figure 37(a)) and 300 Hz for fibronectin (Figure 37(b)) correspond to surface coverages of 1.8 and 1.6 pmol/cm\(^2\). This is similar to values reported for other protein surface interactions.

Furthermore, it is possible to produce a rough idea of the change of mass at the device surface from changes in frequency. Accordingly, it is potentially feasible to arrive at an estimate of the number of cells chemisorbed at the gold surface of the electrode, fibronectin surface and laminin surface. According to Sauerbrey, the frequency shifts for cell attachment onto laminin (-4.0 Hz), fibronectin (-110 Hz), and bare gold (-172 Hz) correspond to mass deposition of 6 ng (laminin), 167 ng (fibronectin) and 261 ng (gold) respectively. According to previously published data, the dry weight of a single cell is about 100 pg and the ratio of the fresh weight to the dry weight of plant cells is at least 10. Therefore, the fresh weight of a single cell is about 1 ng. It has made the number of cells at the surface nearly ten times smaller. As a
result, the number of cells at the surface as calculated from Sauerbrey equation is respectively 6, 167 and 261 for the three surfaces. However, it is notable that there are some difference between plant cells and animal cells. The weights of single cells and dry cells are just an estimation to derive the discussion below. With the estimation, even the error is 100%, the calculations from the Sauerbrey equation alone are still not going to be correct.

Using a electronic cell counter, the number of cultured cells on a TSM surface with full, 100% coverage is approximately 10,000. Since a flow injection method is used in TSM experiments for attachment of cells onto different surfaces, the number of cells that adhere should be less than that observed using the direct cell culture method. Under an optical microscope, it reveals that only 50% cell coverage of the surface by cells was achieved on both bare gold and fibronectin surfaces (corresponds to 5,000 cells), and less than 20% coverage was seen for laminin coated surfaces. For the bare gold and fibronectin surfaces, as mentioned above, the calculated number of cells on the surfaces are 261 and 167, respectively. They are lower than 5,000 by a factor of twenty, indicating that the device is not measuring cell mass. In the following discussion, the laminin surface is not considered further, since the frequency shift caused by attached cells on this surface were negligible.

As mentioned in the introduction, TSM is sensitive to more factors than adsorption, and is affected by a wide range of surface characteristics, including acoustic properties of materials, interfacial effects, and the presence of a contacting liquid. With cells present on the device surface, all of these factors come into play. The most notable fact is that the acoustic wave only penetrates a few hundred nanometres into the contacting liquid. This is as shown schematically in Figure 40. This penetration distance, known as the decay length of thickness shear mode acoustic wave, is on the order of 230 nm from the crystal surface. Since aortic smooth muscle cells are 0.5–1 μm thick on the surface and are comprised
mostly of liquid cytoplasm, the acoustic device is probing the ECM, the cell membrane, and some distance into the cytoplasm. Consequently, the device does not “feel” the entire cell, but instead detects changes in acoustic properties directly above the sensor surface. In fact, the increases in $R_m$ values indicate that changes in dissipation into the contacting liquid are occurring. Since the increases are similar for both fibronectin and bare gold (as shown in Figure 37), this likely corresponds to changes in the viscosity and density of the liquid near the surface, in this case a change from buffer to cytoplasm. As well, upon cell adhesion, the acoustic wave must now travel through the thickness of the cell membrane.

Therefore, the frequency shift due to cell adhesion should be caused by a combination of the increase in thickness on the surface due to adhesion of the cell membrane, and the change in $\left( \rho \eta \right)^{1/2}$ as the liquid in contact with the surface changes from buffer to cytoplasm. This situation is displayed in Figure 40.
Figure 40. Schematic representation of the shear wave penetration depth into a muscle cell on a TSM surface. Of interest is that the shear wave does not penetrate through the entire cell, but instead dissipates within the cytoplasm. Cell sizes and shear wave dissipation into the cytoplasm are not to scale. $\delta$ is the viscous decay length into the fluid. This representation shows that when traveling through cells, the acoustic wave could not penetrate the whole cell and therefore does not reach the bulk buffer, and instead is dissipated with the cytoplasm. In reality, the measured signals are an average of acoustic wave propagation over a heterogeneous surface.
As a result, the observed frequency shift can be modelled as in two separate portions: a surface adsorption effect and a liquid interaction effect, $\Delta f_s \approx \Delta f_a + \Delta f_{liq}$. The surface adsorption $\Delta f_a$ could be estimated from Sauerbrey equation shown above; however, since this thesis is interested in the thickness of the layer, an alternate form can be utilized, where the $\Delta m/A$ term is replaced by $\rho_s h_a$, the density-thickness product of the surface-adsorbed cell membrane:

$$\Delta f_a = -\frac{2f_0^2}{Z_q} \rho_s h_a \tag{27}$$

The shift due to the liquid can be modelled with the transformed Kanazawa and Gordon equation:

$$\Delta f_{liq} = -\frac{f_0^{3/2}}{Z_q} \frac{\rho_{liq} \eta_{liq}}{\pi}, \tag{28}$$

where $\rho_{liq}$ and $\eta_{liq}$ are the density and viscosity of the contacting liquid. Similarly, the change in resistance is given by

$$\Delta R_m = \frac{1}{8K^2C_0Z_q} \sqrt{\frac{\pi \rho_{liq} \eta_{liq}}{f_0}}, \tag{29}$$

where $K^2 = 0.00774$ is a dimensionless electromechanical coupling coefficient and $C_0 \approx 10$ pF is the parallel capacitance of a 9 MHz quartz device. Note that the resistance $R_m$ shift in the above equation (29) is determined solely from dissipative effects in the contacting liquid, and also is proportional to the liquid-induced frequency shift according to Kanazawa and Gordon equation (shown as equation (28) above). Therefore, any change in $R_m$, assumed to be due to changes in the viscosity and density of the liquid will be accompanied by a proportional change in $\Delta f_{liq}$. Any remaining shift in frequency is then due to adsorption onto
the surface, such as the presence of the cell membrane or the deposition of ECM. This change from the transformed Sauerbrey equation (27) can be estimated as follows.

Using the above strategy, a semi-quantitative analysis of cell attachment on a surface can be considered. If it is assumed that all the change in $R_m$ is due to dissipation into the contacting liquid, any remaining shift in $f_s$ is due to a change in the location of the effective acoustic reflecting boundary. Referring to Table 9 and Eqs. (28) and (29), the changes in $f_s$ and $R_m$ from cell adsorption to fibronectin are -110 Hz and 4.3 $\Omega$. This shift in $R_m$ corresponds to a $\Delta f_{\text{liq}}$ of approximately -69 Hz, which is proportional to changes in the density-viscosity product of the contacting liquid. Assuming that the density of the plasma membrane is comparable to that of water, the remaining frequency shift $\Delta f_a = -41$ Hz corresponds, from Eq. (27), to a thickness increase of 2.2 nm. This is likely due to the presence of the cell membrane on the device surface. While this is an estimate, and does not account for viscoelastic properties of the membrane or interfacial effects between the membrane and the cytoplasm, it is close to the expected value for cell membrane thickness (the lipid bilayer is likely between 2 and 8 nm thick). This shows that, by measuring storage and dissipation parameters, the TSM sensor is a suitable semi-quantitative technique for measuring cell deposition onto a surface. In addition, since 2.2 nm is a typical value for the thickness of cell membrane, it follows that the cell likely does not excrete a significant amount of extracellular matrix above the surface of fibronectin.

On the bare gold surface, the change in $R_m$ due to cell adhesion was 4.0 $\Omega$, a similar value to the fibronectin study. This indicates that the sensor probably detects similar cytoplasm-induced frequency and resistance shifts as in the fibronectin case. The 4 $\Omega$ shift corresponds to a frequency decrease of -64 Hz, so the remaining thickness-based shift is 108 Hz, or 5.9 nm. This value is larger than that found for fibronectin, but still within the
range of expected values for the cell membrane thickness, providing further evidence that this sensor is detecting the presence of the cytoplasm and cell membrane on surface adhesion.

As mentioned above, the difference between the calculated fibronectin and bare gold thickness changes could be due to the ECM deposited by the cells upon adhesion to the surface, or viscoelastic effects near the surface. Hydrophobic properties of bare gold surfaces may prevent the relaxation and flat attachment of the cell membrane. Compared to the hydrophilic surface of fibronectin, the thickness of the cell membrane may be higher when attached onto bare gold. Therefore, this may be one of the major reasons for the difference of cell membrane thickness attached onto bare gold and fibronectin detected by TSM. However, further study is required to fully characterise the liquid properties of the cytoplasm and the thickness and density of the cell membrane on the device surface.

3.4.4 Ions and peptides affecting cell morphology

To further test the TSM device response, the effect of divalent ions on cell attachment was studied. Integrins are intramembrane proteins that are believed to play an important role in the attachment and spreading of animal cells. They require divalent cations in the extracellular medium to bind to specific recognition sites on ECM proteins. In this study, Ca$^{2+}$ (1 mM) and Mg$^{2+}$ (1 mM) were used to affect the attachment of smooth muscle cells. Figure 41 shows the effect of divalent ions. Binding of 1 mM CaCl$_2$ and MgCl$_2$ both resulted in decreases in $f_i$ of $\pm 12.1$ Hz (CaCl$_2$) and $\pm 18.7$ Hz (MgCl$_2$). The $R_m$ shifts were more difficult to determine due to the drifting baseline. Accordingly, the changes in $R_m$ are below the limit of detection so a decaying exponential of the form \[ y = C e^{(\beta x + \alpha)} + D \] was used to fit the baseline. This yielded increased shifts in $R_m$ of 0.7 $\Omega$ and 0.1 $\Omega$ for CaCl$_2$ and MgCl$_2$, respectively.
Figure 41. Ion effect on the attachment of cells and on cell depolarization. Black line represents frequency change. Grey line represents resistance change.
Decreases in \( f_s \) and slight increases in \( R_m \) could be due to a variety of factors. Most likely, the shifts are due to an increase in the number of focal adhesions from the increased integrin activity on the cell membrane. The change in \( f_s \) could be due either to an increase in the surface thickness from the added cell membrane attachment, or to a change in viscosity of the contacting liquid, coupled to the \( R_m \) shift, that results from more cytoplasmic contact as more focal adhesions are formed. It is, however, not immediately apparent which of these factors is more prevalent.

In addition to divalent ions, KCl (60 mM) was used to depolarize the cell membrane. Both frequency and resistance were decreased, as seen in Figure 41, which is different from the signals caused by the lower concentration of \( \text{Ca}^{2+} \) (1 mM) and \( \text{Mg}^{2+} \) (1 mM). There was a frequency decrease of –10.0 Hz, and a baseline–corrected resistance decrease of –1.7 \( \Omega \). This result, whereby the shifts of both \( f_s \) and \( R_m \) are in the same direction, is uncommon in TSM operation, although it has been observed previously and attributed to a number of different processes\(^{141,193,197,198}\). This will be discussed in detail in the following section.

RGDS (Arg-Gly-Asp-Ser) tetrapeptides, which are believed to be a common recognition sequence, act as an anchor for cell attachment to the ECM surface.\(^{196}\) Adding these tetrapeptides to the surface could result in competition between the binding of cells-peptides and the binding of cells-ECM at the surface, which should be detectable using TSM. However, the addition of peptides to cell-coated surface resulted in decreased frequency of -8.5 Hz and nearly no changes for the resistance, as shown in Figure 42. Further injection of cells onto the surface both induced frequency decrease and resistance increase. The change of frequency and resistance were -13.3 Hz and 2.1 \( \Omega \) for the first cell injection cycle and -26.1 Hz and 0.3 \( \Omega \) for the second subsequent cell injection cycle after
Figure 42. The effect of RGDS peptide to the cells attached onto fibronectin surface. Black line represents frequency change. Grey line represents resistance change. The arrow shows the injection time.
peptide addition. However, when DPBS buffer was flowing over the surface at the end of the experiment, the frequency returned almost to a value observed before the second cell injection cycle. There was a negligible change for the resistance. The peptides did not detach the cells from the surface as seen from the TSM signals. Cells added after peptide injection seemed to be able to attach onto the surface, even though the cell attachment was not as effective as it was before peptide injection.

Two possible reasons are given here: Firstly, there may be a multilayer structure on the sensor surface. Before the injection of peptides, cells were covering the full sensor surface. After the injection of peptides, the peptides bound to the top surface of the cell and did not detach the cells by competing for sites underneath the cells. The further injection of cells resulted in binding to the peptides, and the structure on the surface became multilayer of cells. However, this scenario is unlikely. The decay length of TSM sensor in liquid is between 177nm and 250nm, and this instrument could not detect any objects beyond this length. Therefore, even though there may be a multilayer structure on the surface, the second layer of cells is far beyond the detection range of the penetrating wave and there should not be a change in the TSM signals. Then what caused the observed signals? The second explanation below may be reasonable. Initially before peptide injection, the surface was not fully covered with cells. It is known that the attachment during the experiment was realized in a very short time compared to the cell culture process; as well as the capability of ECM for cell attachment was affected because of the attachment of ECM to the gold surface. Therefore, on the TSM sensor surface, there should be some area without cell attachment. When the peptides were injected, the peptides did not detach the cells from the surface but bound to the bare gold surface without cell coverage. Further cell injection
made more cells attach onto the peptide surface. This model is consistent with the progression of the TSM signals observed in Figure 42.

3.4.5 Hydrogen peroxide affecting cell morphology

Exposure of the cells to \( \text{H}_2\text{O}_2 \) was studied by TSM. The comparison of the acoustic signals from the TSM device to visual images (obtained through optical microscopy, electron microscopy and atomic force microscopy) is now discussed. Figure 43(a) shows the images of the crystal surface obtained from optical microscope. For the TSM study, cells were first cultured directly onto the bare gold surface and the signal was allowed to stabilise by injecting IMDM onto the surface (approx. 25 minutes). Hydrogen peroxide (200 \( \mu \text{M} \)) was flowed over the cells, resulting in decreases in both \( f_s \) and \( R_m \) of \(-74.6 \) Hz and \(-9.9 \) \( \Omega \), respectively, as shown in Figure 43(c). The device surface was then washed with culture medium (IMDM), leading to a return to near–baseline values for \( f_s \) and \( R_m \).

The acoustic signals induced by 60 mM KCl (Figure 41) and those caused by hydrogen peroxide (Figure 43) were surprisingly similar with respect to the direction of the changes, while the signals caused by \( \text{H}_2\text{O}_2 \) were much greater than those from KCl. These two reactions are likely due to related factors. It is known that membrane depolarisation can initiate oxidant formation in the endothelial cell.\(^{199}\) It is then possible that KCl-induced membrane depolarisation also generates small amounts of \( \text{H}_2\text{O}_2 \) or other oxidants, leading to similar chemical or morphology changes at the surface of smooth muscle cells, as detected by the TSM. The oxidation caused by KCl would not generate as much hydrogen peroxide as was used in the \( \text{H}_2\text{O}_2 \) studies, so the decreases from KCl are not as large. An alternative explanation is that the disruption of the cell membrane by \( \text{H}_2\text{O}_2 \) could appear as a massive depolarisation, yielding a much larger event than that caused by KCl. Both
descriptions are consistent with the observations and future study is needed to clarify the precise mechanism.
Figure 43. (a), (b) are the microscope images of aortic smooth muscle cells on TSM crystal surface. Circles in (b) show the shrunken cells. (c) is the TSM graph. H, M represent H₂O₂ and cell culture medium respectively. Black line represents frequency change. Grey line represents resistance change.
To observe the changes of cell morphology on the surface upon interaction, an optical microscope was used in this study and hydrogen peroxide was added directly onto the crystal surface by pipette. The concentration of hydrogen peroxide (200 μM) used in optical microscopy experiments was the same as used in the previous TSM experiment. Initially, there was no significant change to the surface-attached cells under the microscope. After 10 min, since there was still no change, the concentration of hydrogen peroxide was increased to more than 500 μM and an aliquot was added onto the crystal surface again. Figure 43(b) is the image taken at 15 min after the concentration increased. It is clearly noticed that the membrane of the cells was damaged and some of the cells collapsed.

The signals from TSM were noted immediately after hydrogen peroxide (200 μM) had been injected. It would appear that TSM was quite sensitive for detection of slight changes of the cells. In addition, the TSM results also indicate that long after hydrogen peroxide was added, the structure of the cells was changing. Yet optical microscopy was not able to recognize these changes. To prove this, the surface scanning electron microscope (SEM) has been utilized in this study. Cells were compared under SEM before and after interacting with hydrogen peroxide. Figure 44(a) shows healthy flat aortic smooth muscle cells before hydrogen peroxide interaction. However, the structure of the cells was changed after interaction with 200 μM hydrogen peroxide, which is shown in Figure 44(b) and Figure 44(c). It is noticed that the membranes of these cells appear damaged and the cells have swollen.
Figure 44. The images of surface-attached cells under the SEM. (a) shows the structure of the cells before interacting with H$_2$O$_2$. (b) and (c) are the structures seen after interaction.
The atomic force microscopy (AFM) has also been used to confirm the results from TSM and SEM. The procedure of running AFM experiments was similar to that for SEM experiments. The results from AFM showed us that these cells were flat when cultured on the surface, which confirmed the results obtained by SEM. After interacting with hydrogen peroxide, the cells collapsed and the average thickness of the cells was increased. However, after these damaged cells were washed by the buffer, the cells became flat again. These can be seen from Figure 45 to Figure 47.
Figure 45. AFM images of the healthy cells before H₂O₂ interaction. Right: height image, Left: phase image.
Figure 46. AFM images of cells after H$_2$O$_2$ interaction. Right: height image, Left: phase image.
Figure 47. AFM images for cells after both H₂O₂ interaction and medium solution washing. Right: height image, Left: phase image.
Based on the observations of SEM and AFM, the structure of the cells was changed immediately after injecting hydrogen peroxide. The signals obtained from the TSM experiment were indeed related to the interaction between hydrogen peroxide and the cell surface. The actual acoustic mechanism leading to concurrent frequency and resistance decreases is quite complex. It is likely caused by a combination of factors, including a decrease in the number of focal adhesions to the device surface and changes in the density and viscosity of the cytoplasm due to the depolarisation. Structural changes in the cell membrane, morphology of the cell membrane in contact with the device surface, and the density and viscosity of the contacting liquids could also be responsible. Inner slip has also been proposed as a mechanism that can also lead to this type of signal\textsuperscript{197, 200}.

While it is currently impossible to determine the precise mechanism for these signals, it is possible to arrive at a phenomenological description by referring to the SEM and AFM images of the cells, before and after addition of hydrogen peroxide. Before the addition of H\textsubscript{2}O\textsubscript{2}, the cells appear flat and healthy on the gold surface (Figure 44 (a) and Figure 45). After addition of H\textsubscript{2}O\textsubscript{2} (Figure 44 (b), (c) and Figure 46), the cell surface has been disrupted, and the cells have swollen and become slightly detached from the surface. This could result in numerous effects, of which at least two are likely important. First, when the cells become swollen, some of the focal adhesions between cell and the substrate surface are lost. Therefore, the outside cell medium can penetrate closer to the device surface and, since the medium likely has a lower viscosity than the cytoplasm, $f_i$ would increase and $R_m$ would decrease, both proportionally to $(\rho \eta)^{1/2}$ (Eqs.(28) and (29)). This is a main contributor to the decrease in $R_m$. Second, it appears from Figure 44 to Figure 47 that the cells have swollen and become thicker and rougher after interaction with hydrogen peroxide. This essentially results in a longer average effective wavelength for the acoustic wave, corresponding to a decrease in frequency.
These two effects appear to occur concurrently, and the frequency increase due to the change in viscosity is offset by the increase in the effective thickness of the surface film. Another possible contribution to the frequency decrease may arise from the thickening of the cell membrane due to the swelling of the cells.

When the surface was washed with IMDM medium (Figure 43(c)), $R_m$ almost returned to baseline, while $f_s$ increased to a value above baseline. It is likely that most cells return to their original configuration once the cell–growth medium was restored as shown in Figure 47. However, some cells likely died and detached on exposure to $\text{H}_2\text{O}_2$, resulting in the increase in frequency due to the loss in cell mass at the surface. In addition, those cells lost from the surface made the density, and thus the viscosity of the medium became larger than the original without any cells. Since viscosity is related to resistance of the signals, this might be a reason why the resistance also slightly increased. However, another reason raised here might be more plausible. After injecting the medium, the normal morphology of the cells recovered. This made the cells attach to the surface closely and the physical connection between each other reappear. Therefore, the signals returned to the original values. Further acoustic, SEM and AFM studies are required to validate this result.
4. Conclusions

This thesis demonstrates the broad usage of the thickness shear mode acoustic wave technology in the bioanalytical field. TSM devices have been successfully employed to detect lesions in DNA, a conformational change of calmodulin, as well as the attachment and properties of rat aortic smooth muscle cells in a liquid environment. In the following paragraphs, the conclusions from these main research areas are presented in sequence.

Firstly, the cleansing solution of SDS has proved to be an effective method to wash away most of the biomaterials from the TSM crystal surface. As proven from XPS data, this cleansing solution can be used to make old sensors reusable for up to five times. Therefore, using this method, the cost of running TSM experiments will be reduced. Thus, it makes the TSM device more suitable for industrial applications.

In DNA experiments, DNA probes with 1, 2, 3 AP sites have been used to discover the influence of AP sites on local base stacking energy and geometry. By using TSM biosensors, it was found that these AP sites can cause a dramatic destabilization of the DNA duplex structure. Among these damaged double strand DNAs, the one with 3 AP sites had the least stability as determined by changes of series resonance frequency $f_s$ and motional resistance $R_m$ using TSM device. Simple calculation and analysis of DNA data are given by using the Sauerbrey equation. However, the quantitative results could only be used as an approximation. This is because when using TSM device in the liquid environment, surface and interfacial properties play big roles in the observed TSM data. Therefore, a more comprehensive mathematical model should be used in future attempts to explain the contributing sources of TSM signal.

The changes detected by the acoustic wave method correlated well with the thermostability of DNA duplexes in solution. With respect to the latter, UV-monitored melting curves indicated that both the number of AP sites and their localization in the double-stranded
structure affected the degree of destabilization of a 19 b.p. duplex. It was found that the presence of 3 abasic sites completely destabilized the DNA duplex.

The high sensitivity of the acoustic shear wave technique can be used to detect the hybridization between DNA strands and damaged complementary DNA probes containing AP sites. This technique appears to be a useful tool for the detection of DNA damage generated by organophosphate pesticides or other agents causing DNA depurination.

In calmodulin experiments, this thesis has reported the detection of a conformational shift in this calcium-binding protein using a thickness shear-mode acoustic wave biosensor in an on-line format. The sensor was able to detect an overall increase in length of the protein, as well as exposure of a hydrophobic region, as it went from the closed, unbound state to the open, calcium-bound state. The increase in length was determined from an increase in the effective wavelength of the acoustic wave, and was also measured as a decrease in the series resonant frequency, $f_s$, of the compound resonator. From the acoustic data, a change in length of about 3 Å was deduced using the model introduced by Gileadi et al. $^{36,37}$ This change is considered to be consistent with the literature value of 4 Å$^{135}$. To account for the exposure of hydrophobic areas when calmodulin binds calcium, I also included the possible change in coupling at the surface/liquid interface, which I believe is attributable to the occurrence of partial slip. Furthermore, the comparison between biotinylated calmodulin and SAM-linked calmodulin using the TSM device was provided. The experimental data showed that biotinylated calmodulin is likely to reserve its native conformation and orientation as well as SAM-linked calmodulin when the biomolecules are attached on surfaces.

Calmodulin is an ideal candidate for study, since it is relatively small and its conformational shifts are well characterised, both experimentally and through simulation. Even though, this work might be the first semi-quantitative detection of a protein conformational shift using an acoustic wave device, there are several assumptions made through data analysis.
As a result, further study is required to fully validate and characterise the conformational shift of the surface-bound protein.

Information on protein binding events, including peptide binding kinetics and enzyme kinetics, is of great interest for a systematic biological approach to determine of protein chemical binding. In the near future, acoustic wave biosensing can become an analytical technique for the detection of protein conformational shifts on a modified surface in solution. As such, it can measure protein behaviour in a simulated natural environment, as well as observe changes over time. In addition, due to its small size, acoustic sensors are well-suited to miniaturisation and, with further development, would be ideal for lab-on-a-chip applications.

The TSM biosensor has also been used to measure the adsorption of rat aortic smooth muscles cells, and their subsequent response to various chemical stimuli. This work has demonstrated that, by studying smooth muscle cells, this acoustic biosensor has the potential to detect cell deposition, morphological changes due to ion effects, and membrane depolarisation and denaturation.

Along with the bare gold surface, two common extracellular matrix proteins, laminin and fibronectin, were tested as possible surface modifications. Cells were easily washed from the laminin surface, indicating poor binding, but both fibronectin and the bare gold surfaces performed well in cell adhesion studies. However, further study is required to confirm the results and elucidate the underlying mechanisms. The Sauerbrey equation alone was unable to describe cell binding as mentioned in the above DNA and calmodulin experiments, as well as is not a suitable model for studying complex biological surfaces. Instead, measurements of separate storage and dissipation parameters (in my case $f_s$ and $R_m$) are required to fully characterise the acoustic system. By employing both measurements, a semi-quantitative model of cell adsorption behaviour has been constructed.

Ions, RGDS peptide and hydrogen peroxide were used to affect the attachment and
morphology of immobilized cells. Based on corroboration with SEM and AFM images, a phenomenal explanation for the observed shifts of TSM signals in terms of cell properties has been proposed. As opposed to using the TSM device as a pure mass detector, this device has the capability to detect and measure a wide variety of cell behaviors in an on-line format.

At present, cell behaviour on surfaces remains a significant problem for study and, as such, this thesis can only present a partial description of the complex mechanisms that contribute to the observed acoustic signals. Full characterisation of the sensor will require more complete models and predictors of cell structure under given perturbations, both mechanical and chemical, to be able to link the observed signals to cellular properties. However, with improved models and a better understanding of the complex behaviour of cells on surfaces, and in the presence of high frequency acoustic fields, this device can be used as an on-line, label-free measurement technique to test cell deposition and metabolism throughout its lifecycle, including morphological changes, cell death, and interactions with drugs and other small molecules. Real-time information on cell behaviour, including adsorption, kinetics, and morphological changes, is of great interest to molecular biologists and in drug development. Acoustic biosensing, in conjunction with imaging techniques such as SEM and AFM, promises to become a useful bio-analytical technique for the detection of morphological and metabolic changes in cells in response to a wide range of stimuli.
5. Outlook

This thesis sheds new light on the use of the acoustic technique in clinical research. The advantages of this technique over many existing methods are that it can be conducted in real time and it is non-destructive. These characteristics ensure the biological responses are monitored as they happen. However, more work needs to be done before commercialization of this technique as a diagnostic tool.

By running the DNA experiments, it is found that TSM instrument could be used as a promising method to investigate the influence of pollution on organisms. DNA duplexes can be immobilized on the surface of the TSM crystal by affinity binding. Water samples with different concentrations of the environmental pollutants, such as nitrates or organophosphate pesticides, could be injected onto the surface of TSM. Based on signals in the form of resonant frequency change and motional resistance change, the degree of damaged DNA could be measured. As a result, the concentrations of environmental pollutants in the water samples could be deduced. More work should be done in the lab to determine detection limits of this sensor to various pollutants, since different species may induce quite different TSM signals. This factor could be a main point for consideration before using this sensor in the future.

From the calmodulin experiments, it is concluded that this sensor could be used to detect the conformational change of proteins. The advantage of using TSM to study protein is that this technique is a label-free method. Unlike the traditional techniques for protein research (such as fluorescence and radioactivity), this method does not alter the protein of interest. Even though traditional labeling methods have advantages, the efficiency of labeling varies from protein to protein, making comparisons a challenge. In addition, attaching fluorophores may influence the way in which proteins bind to other molecules and cause background signals. As a result, label-free detection methods, such as TSM, have been employed to overcome some of
these flaws.

Calmodulin belongs to the EF hand proteins, which have a helix-turn-helix structural domain found in a large family of calcium-binding proteins. As a result, other types of EF hand proteins could also be immobilized onto the surface of TSM and any conformational changes would be detected. For example, troponin-C and S100B could be used for similar experiments as done for calmodulin in this thesis. Adding calcium ions should induce the resonant frequency change and motional resistance change. Exploring these signals given by the TSM sensor could allow us to better understand the underlying detection mechanism of this device. Furthermore, considering calmodulin is a relatively small protein in size, other larger proteins could be tested for their conformational change using TSM detection, such as haemoglobin. It is found that cationic haemoglobin (Hb) could interact with the organic phase electrolyte anions at immiscible electrolyte (water-organic) interfaces using electrochemical method. This interaction could be due to association of the anions with hydrophobic regions of the Hb molecule as a result of its conformational change. Therefore, we could use TSM to detect this interaction and further explain the interaction mechanisms given by this large biomolecule. In addition, other techniques could be used to observe the change of protein conformation (such as SIMS) on surface and thus to further aid the TSM experiments.

The preliminary results given by this thesis about the smooth muscle cells has shown us that TSM has the potential to be used for the study of cell behavior on surface. This area could be further extended to investigate cell-cell communication and interaction. Future work could include: (1) continuing study of the morphology change of smooth muscle cells, (2) using different cell types, such as cardiomyocytes and neuron cells, and (3) employing other imaging techniques to observe cells on surfaces. It is known that pH change could induce morphological changes of cells. Possible experimental design could include utilizing buffers with different pH values to flow over the surfaces of cells. The morphological changes of the cells could then be
detected by TSM. In addition, other drugs such as norepinephrine, which can also induce changes in cell morphology, could be used. Another possibility would be to use different cell types in order to explore the application of TSM in the area of cell research. Cardiomyocytes could be detected by TSM of their own spontaneous contraction. Since this contraction will be affected by an electrical field or chemical reagents (drugs), corresponding drugs could be used to induce the change of the beating rate of cardiomyocytes. This change could be detected by the TSM sensor. The results would be compared with the signals given by healthy cells. Drugs that could be used for this type of TSM experiment include negative inotropic drugs (used to treat patients with hypertension, such as ethanol) and positive inotropic drugs (used to treat patients with congestive heart failure, such as levosimenden, isomazole, milrinone, zinterol). In addition, the communication of neuron cells could also be explored by the use of the TSM device. Also, by running contact mode AFM experiments, the morphological changes of live cells could be observed directly on the surface in a liquid environment. Choosing suitable immobilization methods for AFM experiments would be required before parallel comparisons between the results of TSM experiment and the images from AFM experiments could be made.

With the development of the design of TSM instrument and the mathematical model used in analyzing TSM data, it is expected that TSM could be used widely in medical research in the near future.
References


2000, 45, 3615-3621.


79. C. Werner, H. J. Jacobasch and G. Reichelt, *Journal of Biomaterials Science-Polymer*


125. J. Haiech, M. C. Kilhoffer, T. A. Craig, T. J. Lukas, E. Wilson, L. Guerra-Santos and D.


York, 1981.


