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Characterization & Application of Immobilized Biomacromolecules using Microcantilever and QCM Sensors

by

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ABSTRACT

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The structure and function of immobilized biomacromolecules are likely to be altered because of the solid surface. The long-term objective of this thesis is to develop surface-based biosensors for the characterization and application of biomacromolecules at the liquid-solid interface. In this study, two analytical surface-sensitive sensors are utilized: microcantilevers and quartz crystal microbalance with dissipation (QCM-D). Each offers unique information regarding the molecules of interest. In particular, the systems that are covered in this thesis include the detection of target analytes using specific recognition elements and the characterization of supported lipid membranes. This research has led to a better understanding of the effect of solid surfaces on protein structure and function, as well as the ability to engineer biomolecular surfaces with great control.

There are two detection systems that were studied: a phage-derived peptide system for the detection of pathogenic bacteria Salmonella and an antibody displacement assay for the detection of an explosive, 2,4,6-trinitrotoluene (TNT). The microcantilever responds to changes in the surface free energy on the sensor surface by monitoring changes in its deflection. The physisorption or chemisorption of molecules to the cantilever surface induces a mismatch in the surface stress, causing the cantilever to
bend. The multiplexed measurement is able to quickly determine the binding affinities of various phage-derived peptides, improving the screening efficiency of the peptides derived from phage display libraries for Salmonella detection. The microcantilever-based technique provides a novel biosensor to rapidly and accurately detect pathogens and holds potential to be further developed as a screening method to identify pathogen-specific recognition elements. QCM measures mass changes on the sensor surface by monitoring the frequency change of the crystal. The combination of a competition assay with QCM using an anti-TNT antibody is able to distinguish a TNT molecule among molecules of similar structure at low concentrations, leading a sensitive and selective assay. The reliability of this method was further investigated in more real environments simulated by fertilizer solution and seawater. Furthermore, this method could be also applied in gas phase detection of TNT, as well as the detection of other chemicals, such as environmental pollutants and illegal drugs. In both of these detection assays, a mathematical model was developed to quantify the binding of the target molecules with the molecules of interest.

In the second half of the thesis, the microcantilever sensor is applied to characterize supported lipid bilayers (SLBs), an interesting biomacromolecular assembly that holds great importance as a model system for membranes. Through monitoring the cantilever deflection, the formation of the SLB, its temperature induced phase transitions, and its interactions with membrane-active molecules are investigated. With increasing temperature, the lipid acyl chains transition from an ordered state to a disordered state, accompanied by a changes in the surface stress that can be readily detected using microcantilever. The phase transition temperature of SLBs is different from that of a lipid
monolayer, indicating that the existence of the solid support affects the monolayer structure. Two amphipathic membrane-active molecules, peptide (PEP1) and a triblock copolymer (Pluoronic), are studied for their associations with SLBs. PEP1’s association with SLBs highly depends on the ratio of peptide over lipid, while the Pluoronic interacts with SLBs as a function of temperature and the length of lipophilic block in the copolymer. Therefore, the microcantilever sensor is capable of measuring the conformational change of surface-bound molecules, as well as characterizing the kinetics of membrane-peptide interactions with great sensitivity.
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Chapter 1

Introduction and Objectives

1.1. Motivation

The development of sensors for molecular interactions that present high sensitivity and selectivity has been receiving increasing attention in applications, including detection of environmental or food contaminants and characterization of structure-function relationships for proteomics and pharmaceutics. Sensors can be classified based on the nature of signals monitored\(^1\). Particularly, biosensors utilize biological components, such as DNA, enzyme, or antibodies, in the detection process. Sensitivity, or the limit of detection (LOD), refers to the minimum amount of target molecule or molecular interaction that can be detected with a certain sensor. Sensitivity is defined as the ratio of true positives over the sum of true positives (TP) and false negatives (FN) (“Sensitivity = TP/(TP+FN)”, where TP is the number of true positives...
and FN is the number of false negatives). In addition to sensitivity, specificity is also used to evaluate the sensor performance. Specificity is defined as the ratio of true negatives over the sum of true negatives (TN) and false positives (FP) (“Specificity = TN/(TN+FP)”, where TN is the number of true negatives and FP is the number of false positives). In summary, sensitivity describes the ability of a sensor to measure and confirm a true positive signal, while specificity describes its ability to distinguish a true negative signal.

Another parameter, sensor stability, indicates the performance of a sensor over time under various conditions. Surface-sensitive sensors are used in this work: the targets of interest are bound close to solid surfaces and their native structures and properties might be altered. Thus the challenge and motivation of this project is to understand how solid surfaces affect the molecular structure and function of various targets, as well as to study the intermolecular interactions at the liquid-solid interfaces. This dissertation focuses on two types of surface-sensitive sensor platforms: microcantilevers and quartz crystal microbalance (QCM). These sensors will be adapted for two main applications: i) accurate and rapid detection of target analytes; and ii) characterization of lipid membrane and its interaction with small molecules. This introductory chapter will provide the background on current biosensors and specific description of the sensors used in this work.

1.2. Introduction to Sensors

1.2.1. Background Review of Current Sensors
Sensors penetrate almost every corner of our life, such as the food industry, medicine, safety, and transportation. Due to the rapid progress of science and technology, an increasing number of sensors have been developed to ameliorate our life. So what is a sensor? From the American National Standards Institute (ANSI) definition, a sensor is a device that can translate a measured property into an output signal that in turn can be monitored by an equipment or an observer.

A sensor contains three basic parts: input signal, output signal, and signal transducer and processor (Figure 1.1). A number of properties can be detected as input signals, such as temperature, pH, light, and specific molecules. Normally, the input signal is physical property. Even if the input is as complex as a reaction between two chemicals or the interaction between antigen and antibody molecules, the chemical reaction or biological binding is transformed into an easily measurable physical property, such as mass or heat. The output signal can be current, voltage or digital signals. Digital output signals are preferred, because they are easily quantified. Furthermore, the output signal of one sensor can be used as input signal for another sensor. So both input and output signals can be either analog or digital. The transducer coupled to a processor is the key component of a sensor that determines the type of output signal, and the sensitivity of the device. Besides these three parts, other features can be added to the sensor, such as signal amplification and noise processing, which may further improve the performance of sensor.
Figure 1.1. A typical sensor is composed of three parts: input signal, output signal, and signal transducer and processor.

One desired property of an ideal sensor is to have a high limit of detection, or in other words, a large range between lower and higher limit of detection. An ideal sensor should also not be sensitive to other factors, which may lead to detection of false positives. A sensor should be designed to minimize detection of false positive signals, and respond only to the specific input signal for which is was specifically designed. In short, a sensor should have high sensitivity and selectivity. Lastly, the performance of a sensor should be stable under various conditions, such as temperature, humidity and altitude. Such a sensor is economically advantageous and can be used widely without modifications.

Besides the basic techniques for scientific analysis, such as thermometers and pH meters, sensors can be ameliorated for very specific applications, such as environmental\textsuperscript{4}, clinical\textsuperscript{5} and food\textsuperscript{6} research. Electrochemical sensors are widely used to typically measure the changes in electrochemical properties, such as current, voltage and conductivity\textsuperscript{1}. If one or more biological elements are included in the detection process, the sensor is typically referred to as electrochemical biosensor. Because biosensors combine the high sensitivity of sensors with high selectivity of biological reactions, they have been widely used in a number of applications, particularly to study enzymatic reactions and antibody
mediated protein-protein interactions.\textsuperscript{7} Besides electrochemical sensors, other types of sensors can also be coupled with biological components, generally referred as biosensor or immunosensor\textsuperscript{8}. Three widely applied sensors are discussed in the following session: microcantilever, quartz crystal microbalance (QCM) and surface plasmon resonance (SPR). Based on the signal transducer, they are respectively classified as optical, acoustic, and micromechanical sensors.

1.2.2. Microcantilevers

Cantilevers are familiar to all of us as they are commonly found in our daily life. For instance, a diving board, balcony, or dock utilizes the principle of a cantilever. Because only one end of cantilever is fixed, there are variations in the moment and surface stress of the beam. At the micro scale, changes in the mass or surface stress of cantilever beams can be measured quickly and accurately. Therefore, microcantilevers are applied frequently in various research areas\textsuperscript{9}. As shown in Figure 1.2, spatial scaling of cantilever is associated with respective scaling of masses, frequencies, and energies,\textsuperscript{10} indicating the potential of cantilever based sensors in various applications.

![Figure 1.2. Spatial scaling of cantilevers.\textsuperscript{10}](image)


Microcantilevers can operate in two different modes: dynamic and static mode\textsuperscript{11}. The dynamic mode measures a mass change on the cantilever surface through a change in the resonance frequency, while the static mode measures changes in surface stress by monitoring cantilever bending. Both modes present advantages and disadvantages. The dynamic mode indicates a mass change on the surface, leading to a relatively easy data analysis. However, liquids present a low quality factor which dampens the oscillations, making it difficult to conduct sensitive mass based detection in liquids. Some groups have overcome this limitation by shrinking the cantilever from micron length scales to nanometer length scales\textsuperscript{12}. Other groups have created a cantilever in which liquids flow inside the cantilever so that the cantilever is surrounded by air instead of liquid\textsuperscript{13}.

In the dynamic mode, the resonance frequency of a vibrating microcantilever is measured. The resonance frequency can be expressed using following equation\textsuperscript{14}.

\[ f = \frac{1}{2\pi} \cdot \sqrt{\frac{K}{m}} \]  
(1.1)

where \( K \) is the spring constant and \( m \) is the effective mass of the microcantilever. This equation can be simplified as follow:

\[ f_0 = \sqrt{\frac{k}{m}} \]  
(1.2)

where \( k \) and \( m \) are effective stiffness and mass of the cantilever, respectively\textsuperscript{11}. Equation 1.2 can be rewritten as\textsuperscript{11}

\[ \Delta m = -\frac{2m}{f_0} \cdot \Delta f \]  
(1.3)
Equation 1.3, in which $\Delta m$ is the mass change corresponding to frequency change $\Delta f$, can be used to analyze data acquired in dynamic mode.

In static mode, the change of surface stress is caused by a free energy change, making this mode more sensitive for the detection of small molecules. In addition, free energy is normally related to the structural conformation of molecules, thus the static mode is ideal for probing protein conformational changes.

Four kinds of forces affect deflection of the cantilever: conformational entropy, electrostatic repulsion, osmotic pressure, and a mechanical energy penalty. In static mode, the bending of cantilever is caused by compressive force or tensile stress among molecules on the surface\textsuperscript{15}. During the bending process, the force on cantilever $F$ acts at a distance $x$ and the bending moment is calculated as $M = Fx$. The radius of curvature is expressed as

$$1/R = \frac{d^2 z}{dx^2} = \frac{M}{E I} \tag{1.4}$$

where $E$ is the apparent Young’s modulus and $I$ is the moment of inertia for a rectangular cantilever. The static bending is caused by the change of surface stress, so the bending momentum can be written as

$$M = \Delta \sigma wt/2 \tag{1.5}$$

where $\Delta \sigma$ is the differential surface stress at the upper and lower side of cantilever. Stoney’s formula is obtained by combining Equation 1.4 and 1.5:

$$1/R = \frac{6(1 - \vartheta)}{Et^2} \cdot \Delta \sigma \tag{1.6}$$
Considering the cantilever boundary conditions, the equation above can be solved as follows:

\[
\Delta \sigma = \frac{Et^2 \Delta z}{3(1-\nu)L^2}
\]  

(1.7)

where \( \Delta \sigma \) is change of surface stress, \( \nu \) is Poisson’s ratio for the thick layer, \( E \) is Young’s modulus for the thick layer, \( t \) is the thickness of the thick layer, \( L \) is the length of cantilever and \( \Delta z \) is the deflection of cantilever. Equation 1.7 shows the linear relationship between change of surface stress and cantilever deflection.\(^{16}\)

The microcantilever device used in this study (Concentris GmbH, Figure 1.3) consists of eight parallel cantilevers for each sensing array. The cantilever array (CLA-500-010-08, Concentris GmbH) is 1 \( \mu \)m thick, 100 \( \mu \)m wide, and 500 \( \mu \)m long. The cantilevers are normally silicon or silicon nitride, with one side is coated with a 3 nm layer of titanium followed by a 20 nm gold layer. Hence, the values of \( t \) and \( L \) are 1 \( \mu \)m and 500 \( \mu \)m, respectively. Poisson’s ratio \( \nu \) and Young’s modulus \( E \) are 0.25 and \( 1.32 \times 10^{11} \) N/m\(^2\). So a 1 nm deflection of the cantilever corresponds to a surface stress change of 0.234 mN/m, as calculated using Equation 1.7. Target chemicals can be immobilized onto either gold surface or silicon dioxide surface.

![Image](image1.png)

**Figure 1.3.** Concentris GmbH (Basel, Sweden) and Cantilever arrays.
Microcantilevers operating in static mode have several advantages for research applications, including high sensitivity and selectivity, high-throughput measurement, label-free and real-time detection. Microcantilevers can be used for thermal, physical, and chemical sensing (Figure 1.4). Since cantilevers have two different layers, they are very sensitive to temperature changes due to bimetallic effect. Therefore, cantilever measurements need to be conducted in a temperature-controlled environment to avoid background noise caused by temperature fluctuations. Static mode cantilevers can detect forces in the order of magnitude of the picoNewton and displacements of angstrom. Therefore, AFM-based cantilevers can be useful to detect parameters such as force, pressure, acceleration, acoustics and flow rate\textsuperscript{14}. In addition, microcantilevers are widely used for the detection of chemicals, in both gas and liquid phases\textsuperscript{10}. Fritz \textit{et al.}\textsuperscript{17} first reported the detection of DNA hybridization on microcantilevers (Figure 1.5). Biswal \textit{et al.}\textsuperscript{18} later utilized microcantilever to detect DNA melting temperatures. Microcantilevers can also be used to detect the conformational change of proteins or peptides\textsuperscript{19}, providing a tool to study the relationship between protein folding and activity, which is particularly relevant for biomedical application, as several human diseases, such as Alzheimer’s and Parkinson’s disease, are caused by protein misfolding.
Figure 1.4. Schematic drawings of cantilever transduction principles.\textsuperscript{15} (a) temperature and heat sensor due to bimetallic effect, (b) surface stress sensor due to chemical adsorption, (c) mass sensor due to additional mass loading.

Figure 1.5. Schematic representation of the hybridization experiment\textsuperscript{17}. Each cantilever is functionalized on one side with a different oligonucleotide base sequence (red or blue). Two complementary oligonucleotides (green and yellow) match the red and blue sequences, respectively.
1.2.3. Quartz Crystal Microbalance

Quartz Crystal Microbalance (QCM) is a mass sensitive device, which measures the mass change per unit area on a sensor surface, through monitoring the frequency change of quartz oscillation. The QCM system used in this study is shown in Figure 1.6. The piezoelectric quartz crystal is the key component of this device. Early in 1880, the Curie brothers discovered that a quartz crystal of Rochelle salt produced electricity when subjected to pressure\(^2^0\), and if electricity was applied to the crystal first, a strain would be produced. In short, there is an electromechanical interaction between mechanical strain and the electricity (piezoelectricity). Taking advantage of this effect, an alternating current to the quartz crystal induces oscillation at a preferred frequency. Furthermore, this frequency can be interrupted by the addition or removal of chemicals at the crystal surface. As a result, a device with quartz crystal is able to monitor the adsorption or desorption of chemicals on the crystal surface. Based on this idea, the first quartz crystal controlled oscillator was constructed in 1921\(^2^1\). The first generation of these oscillators was so sensitive to temperature that prevents generating steady signals, because the crystal was X-cut (major plane surface is perpendicular to X axis, while surface normal is parallel to Z axis, also known as zero-angle cut). To overcome this drawback, an AT-cut (the angle between surface normal and Z axis is 54°45’, which is complementary to AT angle) crystal was generated in 1934, which can be operated for a wider temperature range\(^2^2\). In 1959, Sauerbrey\(^2^3\) found the linear relationship between frequency change of quartz crystals and mass added on the surface. With this finding, quartz crystal based sensors can be used to quantify the signal change in terms of material properties.
As mentioned above, the quartz crystals’ piezoelectric properties result in their oscillating at a specific frequency due to alternating currents. The harmonic motion of quartz crystals are common and similar to the vibration of a spring, a pendulum, or a string. What characterizes quartz crystals is that it consumes minimal energy during oscillation, which makes QCM appealing to conduct accurate frequency measurement with low energy requirements. The oscillation frequency can be disturbed by surface mass changes. An increase of mass on surface causes a decrease in frequency. The relation between these two variables is given by Sauerbrey’s equation:

\[
\Delta f = \frac{-2\Delta m f^2}{A(\mu_\rho)^{0.5}}
\]

(1.8)

where \(\Delta f\) is the measured resonant frequency change in Hz, \(f\) is the intrinsic crystal frequency, and \(\Delta m\) is the elastic mass change. This equation can be rewritten as:
In Equation 1.9, $C$ is the combination of parameters $\mu$, $\rho_q$, and $f$, which are known for certain crystals, so $C$ can be considered as a constant. If the value of $C$ is known, the frequency change can be easily converted into the mass change. Another mode of operation is quartz crystal microbalance with dissipation (QCM-D), where the dissipation of the crystal signal can be related to the stiffness of the material. Changes in the dissipation, $\Delta D$, can be used to study viscoelastic properties of attached materials by monitoring the decay of oscillation after a quick excitation, or by impedance analysis\textsuperscript{25}. QCM-D can be used to quantify viscoelasticity through the decay in the amplitude over time, expressed using following exponentially damped sinusoidal equation:

$$A(t) = A_0 \exp\left(-t/\tau\right) \sin(2\pi ft + \phi)$$

(1.10)

where $A$ is the amplitude, $\tau$ is the decay time, and $\phi$ is the phase. The change of dissipation ($\Delta D$) is defined as\textsuperscript{25}:

$$\Delta D = \frac{1}{Q} = \frac{E_{\text{dissipated}}}{2 \pi E_{\text{stored}}}$$

(1.11)

$$\Delta D = \frac{1}{\pi f \tau}$$

(1.12)

where $Q$ is quality factor, $E_{\text{dissipated}}$ is the energy dissipated during one oscillatory cycle and $E_{\text{stored}}$ is the energy stored in the oscillating system. Equation 1.12 represents an expression for the dissipation change, which is an indication of the rigidity of the layer on gold surface.
The QCM-D equipment used in this work is Q-SENSE E4 system (Västra Frölunda, Sweden), featured with four parallel sensors. The crystal used is quartz crystal coated with 100 nm of gold (QSX 301), shown in Figure 1.7. On one side of the crystal there is the gold surface, which is used as reaction substrate. On the other side, there are two electrodes: the contact and the counter electrode. For this kind of crystal, the intrinsic crystal frequency is $5MHz$. The electrode area $A$ is about $196mm^2$, while $\rho_q$ and $\mu$ are $2.65g/cm^3$ and $2.95 \times 10^{11} dyn/cm^2$, respectively. With these values, the constant $C$ is $17.7ng \cdot cm^{-2} \cdot Hz^{-1}$. The frequency change can be converted to mass change through Sauerbrey’s equation. For unit area ($1cm^2$), when there is $1Hz$ frequency change, the mass change on the gold surface is about $17.7ng$.

![Figure 1.7. Forward and backward view of crystal.](image)

Early detection using QCM were mostly conducted in gas phase. It was not until 1980s that solution based QCM was developed, making it possible to measure frequency changes and dissipation. QCM is also currently used to measure mass change at liquid-solid interface. The advantages of QCM are the following: high sensitivity, label-free detection, feasible application in complex (such as optically opaque solution),
interface detection, and ease of operation with low cost\textsuperscript{22}. Because of these advantages, QCM is commonly used in many different areas, such as thin film formation, surfactant adsorption, analytical electrochemistry, biosensing, and drug research. Ullevlg \textit{et al.}\textsuperscript{27} demonstrated that QCM can be used for electrodeposited films containing significant stress. QCM has also been successfully applied in studies of the sputtering behavior of linear polymers, metals, metal oxides, and plasma-deposited polymers. In addition, studies about viscoelastic and dielectric properties of complex samples using QCM have been reported\textsuperscript{28}. Both frequency and dissipation changes are taken into account. Regarding biological applications, QCM-D can also be used to detect conformational changes. Particularly, Zhang and Wu\textsuperscript{29} reported a study of conformational changes of polymer chains via QCM-D, which is especially suited to this research because it can measure grafting kinetics of polymer chains on liquid-solid interface in real time. Furthermore, QCM-D has also been used to study DNA, protein, lipids, and cells\textsuperscript{25}.

\textbf{1.2.4. Surface Plasmon Resonance}

The principle of surface plasmon resonance (SPR) is summarized in these three words: surface, suggesting that this sensor is normally used for the study of phenomena or reactions occurring at the surface; plasmon, indicating the movement of free electrons\textsuperscript{30}; and resonance, which refers to the energy transfer from incident light to surface plasmons. SPR can be used to study surface interactions via surface plasmons caused by resonance with incident light. The incident light is normally p-polarized visible light or infrared light. P-polarized light is used because its polarization is in the direction parallel to the incidence plane. Therefore, electronic surface plasmons can be excited. Wood \textit{et al}.\textsuperscript{31-32} first reported the anomalous diffraction of surface plasmon excitation.
Later, Kretschmann\textsuperscript{33} and Otto\textsuperscript{34} presented the optimal excitation of surface plasmon through attenuated total reflection. In SPR, when resonance occurs, the energy is transferred from incident light to surface plasmons, and the intensity of reflective light is reduced. So there is an angle at which the intensity of reflection is minimal. This angle changes as a function of surface substrate. Molecules in a sample adsorb and desorb on the surface. Since molecules and buffer typically present different refractive indices, the angle with minimal reflection will be changed (Figure 1.8), making it possible to monitor the interactions on the surface.

\textbf{Figure 1.8.} Tracking surface absorption by SPR.\textsuperscript{35} (a) Prism-coupled configuration and (b) Resonance shift in the reflected light spectrum.

SPR is already widely used in different areas (\textit{e.g.} immunoassays), because it presents a series of advantages, including high sensitivity, high selectivity after coupled with biological elements, label-free, real-time, recycled measurement, and feasible applications for miniaturization and multi spot detecting\textsuperscript{36}. Among numerous applications of SPR, different formats of detection are chosen based on the characteristics of the
analyte and detection system. Here, four main detection formats are described: direct detection, sandwich detection, competitive detection, and inhibition detection (Figure 1.9), which can also be transferred to the other sensors, such as microcantilever and QCM. SPR is used for the detection of different types of analytes, such as explosives, pollutants, and some biomolecules of different sizes. In addition, the development of SPR allows for the study of dynamic processes. For example, SPR has been reported to study the elongation of Aβ fibrils, which are associated with the development of Alzheimer's disease.

**Figure 1.9.** Main detection formats used in SPR biosensors. (A) Direct detection; (B) sandwich detection format; (C) competitive detection format; (D) inhibition detection format.

In summary, the sensors discussed above share some useful advantages: high sensitivity and specificity, label-free and real-time measurement. Although some molecular interactions (e.g. antigen-antibody binding and membrane-active molecules)
have been previously studied, the influence of the solid surface on these interactions still remains elusive to some extent. By utilizing these sensors, the work in this thesis not only studies the interfacial interactions of molecules, but also understands the kinetics, equilibrium configuration, and even structure properties of molecules of interest.

1.3. Research Objectives

Molecular interactions regulate a variety of chemical and biological processes. In this work, I focus on molecular interactions occurring at the liquid-solid interface that can specifically modify the solid surface with functional chemicals in order to achieve the desired properties of sensors. However, the structure of a target molecule might be altered upon interaction with solid surfaces, leading to changes in the chemical and/or biological properties of the target molecule. It is critical to maintain or predict the properties of target molecules at the liquid-solid interface. Therefore, the objective of this work is to develop surface-sensitive sensors-based techniques to investigate the behavior of surface-bound molecules, as well as to understand the important mechanisms that regulate the molecular interactions at interfaces. I envision that the related findings could facilitate applications of various sensors in food, environmental and medical areas. This work utilizes two surface-sensitive sensors, microcantilever and quartz crystal microbalance with dissipation (QCM-D) to detect target analytes and characterize lipid membrane. Cell membranes are complex macromolecular structures that is difficult to be purified and studied in isolation. Supported lipid bilayers (SLBs) provide a simplified artificial membrane model to study cell membrane. The SLB is the planar lipid bilayer supported on a solid support, so one leaflet in SLB faces an ambient aqueous solution and
the other leaflet faces a rigid solid. On one hand, the water layer between SLB and solid support enables SLB to behave as a fluid, similar to the free-standing lipid bilayer. On the other hand, because of membrane asymmetry, the SLB exhibits different properties from free-standing lipid bilayer. The integration of molecular interactions with surface-sensitive sensors to probe molecular interfacial characteristics underscores the novelty of this work.

1.4. Dissertation Outline

The research described here is based on the use of surface-sensitive sensors, such as microcantilevers and QCM, and is organized under the following specific objectives.

Pathogenic bacteria were first detected using an array of microcantilevers. The specific binding of Salmonella to peptides derived from phage display libraries is reported in Chapter 2. As molecules adsorb onto the surface of the microcantilever, the microcantilever bends due to induced compressive or tensile stresses, which is a result of the surface free energy change. Selectivity of these phage-derived peptides for Salmonella spp. was compared with a commercially available anti-Salmonella antibody and the antimicrobial peptide alamethicin. A multiplexed screening system to quickly determine the binding affinities of various peptides highly improved the efficiency of the peptide screening process. Combined with phage-derived peptides, this microcantilever-based technique provides a novel biosensor to rapidly and accurately detect pathogens and holds potential to be further developed as a screening method to identify pathogen-specific recognition elements. The development of methods for pathogen detection is a
collaborative work with Dr. Nitsara Karoonuthaisiri (Queen’s University Belfast, United Kingdom; National Science and Technology Development Agency, Thailand).

A QCM-based method is also developed in Chapter 3 for the sensitive detection of commonly used explosive, 2,4,6-trinitrotoluene (TNT), which might contaminate soil or ground water and severely affect human health. I designed a QCM-based biosensor that measures the displacement of a TNT-specific antibody and made it possible to distinguish TNT from molecules with similar chemical properties. The reliability of this method was investigated by using fertilizer solution and artificial seawater as dirty environment. A Langmuir kinetic model was developed to describe the molecular interactions on the sensor surface and to establish a standard curve to estimate on-site TNT detection. QCM detection of anti-TNT antibody displacement provides a method for rapid detection of TNT with high sensitivity and specificity.

After developing the detection methods of target analytes for microcantilever and QCM techniques, I worked on utilizing another macromolecular assembly, supported lipid bilayers (SLBs), as model membranes in the following chapters. The solid-liquid phase transition of supported membranes using microcantilevers is discussed in Chapter 4. With increasing temperature, the lipid acyl chains transition from an ordered state to a disordered state, accompanied by a free energy change, which is coupled to changes in the surface stress in the underlying solid support layer. These surface stress changes can be readily detected using microcantilevers. For an MPPC monolayer, this phase transition temperature was shifted, indicating that the existence of the solid support affects the monolayer structure. Furthermore, the microcantilever also detected the cholesterol induced decrease of the phase transition temperature. Thus the microcantilever is proven
to be sensitive enough to detect the conformational change of surface-bound molecules, and can be used to study interfacial interactions.

The SLB-coated microcantilever is also used to investigate the lipid interaction with the membrane-active peptide PEP1 in Chapter 5. PEP1 is a synthetic amphipathic peptide resembling a segment of the nonstructural protein (NS5A) of hepatitis C virus. A critical concentration of PEP1 for its association with the SLBs was observed. At low concentrations, PEP1 peptides adsorb onto the lipid membranes in parallel. At concentrations greater than the critical concentration, PEP1 peptides begin to aggregate and form pores. Finally PEP1 peptides at even higher concentrations destabilize and induce the solubilization of the supported membranes. The membrane-coated microcantilever sensor is capable of characterizing the kinetics and dynamics of membrane-peptide interactions with great sensitivity. The work reported in Chapter 4 and 5 was initiated by a previous lab colleague, Dr. Kai-Wei Liu.

In addition to membrane-active peptides, the association of an amphiphilic triblock copolymer Pluronic with lipid membranes is reported in Chapter 6. Pluronics are known to interact with cellular membranes in interesting ways, such as permeabilization versus sealing of membranes. The solubility of these triblock copolymers with free lipid membranes can be altered with temperature, leading to the ability to create a tunable insertion within the membrane. With the use of microcantilevers and fluorescence recovery after photobleaching (FRAP) measurements, the solubility and interactions of triblock copolymers (F68 and F98) with the SLBs was investigated as a function of temperature and the length of lipophilic block in the copolymer. Compared with free membranes, the results suggest that the solid support
affects the polymer-lipid interactions. A Langmuir isotherm model and a free mean area theory were applied to describe the polymer-lipid interactions at the microcantilever surface, determine association constants, and analyze the effect of triblock copolymers on lipid lateral diffusion.

Finally a brief summary of the work discussed in this dissertation is presented in Chapter 7, as well as the suggestions to further utilize surface-sensitive sensors for the interfacial characterization.

1.5. References

3. Sobhan, S., Introduction to Sensors. *RAISE Teachers Workshop @ Polytechnic University* **2005**.


Chapter 2

Rapid Detection of Pathogenic Bacteria and Screening of Phage-derived Peptides Using Microcantilevers

2.1. Introduction

The use of an array of microcantilevers to measure the specific binding of *Salmonella* to peptides derived from phage display libraries is reported in this chapter. Microcantilevers can be powerful biosensors with various advantages and applications, as discussed in Chapter 1. Selectivity of these phage-derived peptides for *Salmonella* spp.
and other pathogens (*Listeria monocytogenes* and *Escherichia coli*) are compared to a commercially available anti-*Salmonella* antibody. This microcantilever-based method provides a novel technique to study pathogen detection, as well as the molecular interactions on the solid surface.

The rapid and sensitive detection of pathogenic bacteria remains critical for the prevention of foodborne illnesses. Among these pathogens, *Salmonella*, is one of the most common causes of food contamination\(^1,2\). Due to the number of different serovars and the low infectious dose\(^3\), preventing *Salmonella* contamination requires quick and accurate pathogen testing. Conventional methods, including culture and colony counting methods\(^4\), immunology based methods\(^5\), and polymerase chain reaction (PCR)\(^6\), have been widely used for reliable, sensitive, and inexpensive detection\(^1\). However, these techniques are either tedious, or time-consuming, or require pretreatment or labeling of samples.

Label-free biosensing technologies that promise improved performance and shorter analysis time have been recently developed\(^2\). These biosensors are composed of a recognition element, such as an antibody specific for the pathogen, and a transducer that translates the binding of the pathogen to the recognition element into a easily detectable output signal\(^7\). Highly specific recognition elements ensure selectivity, while sensitivity is achieved through transducer-mediated signal amplification. Antibodies are the most commonly utilized recognition elements due to their high binding affinity and specificity. However, antibodies lack stability under harsh environmental conditions and can be expensive to generate\(^8,9\). DNA and aptamers have been used for pathogen detection\(^10,11\); however, the processing can be labor intensive. A high throughput screening technology
is typically needed to first isolate recognition elements with high affinity and low cross reactivities\textsuperscript{12}. Phage display has been widely used to generate combinatorial libraries of antibodies\textsuperscript{13} or peptides\textsuperscript{8}, which are subsequently screened by ELISA\textsuperscript{14} or flow cytometry\textsuperscript{15} to isolate recognition elements with desired binding properties, but simplified screening processes remain a priority\textsuperscript{16, 17}.

Piezoelectric biosensors have been widely explored for pathogen detection. Particularly quartz crystal microbalance (QCM)-based biosensors were reported\textsuperscript{18}: despite considerable effort has focused on enhancing the limit of the detection through the use of displacement assays\textsuperscript{19}, QCM biosensors still lack the sensitivity needed for detection of ultra-low pathogen concentrations that can cause foodborne diseases. Surface plasmon resonance (SPR) has also been successfully used for the detection of microbial species with high sensitivity, but it requires an extra pretreatment step to first separate the bacteria using antibody coated magnetic nanoparticles\textsuperscript{20,10}. Electrochemical biosensors promise fast read-out, but suffer from limited sensitivity and selectivity\textsuperscript{2, 21}. Nanomechanical cantilevers provide an alternative transducer for the development of high sensitivity biosensors for bacteria detection. The attachment of target molecules onto one of cantilever surfaces causes a surface stress mismatch between the upper and lower surfaces of the cantilever, leading to bending of the cantilever\textsuperscript{22-23}. This method offers high sensitivity and holds promise for multiplexed analysis based on simultaneous testing of microcantilever arrays.

In this Chapter, I report the development of a multiplexed microcantilever biosensor that utilizes phage-derived peptides\textsuperscript{24} for Salmonella detection. An array of cantilevers, individually functionalized with different peptides, enables the detections of
multiple pathogens in parallel\textsuperscript{25}. The selectivity of the phage-derived peptide was determined by quantifying its cross-reactivity to other relevant bacteria species, such as \textit{L. monocytogenes} and \textit{E. coli}, and compared to a commercial anti-\textit{Salmonella} monoclonal antibody. The antimicrobial peptide alamethicin was used as a control due to its ability to bind to bacteria nonselectively\textsuperscript{26}. The selectivity of the peptide toward various \textit{Salmonella} serovars is also reported. A Langmuir adsorption based model\textsuperscript{27} was used to determine the binding affinity constants and characterize the peptide-pathogen interaction. This multiplexed detection enables detection with high selectivity. Moreover, simultaneous testing of multiple peptides has the potential to be further developed into a screening technology for pathogen-specific recognition elements.

\textbf{2.2. Materials and Methods}

\textbf{2.2.1. Materials}

Anti-\textit{Salmonella} monoclonal antibody was purchased from \textit{Abcam} (UK cat# ab8273). The sequences of the peptides MSal 020401, MSal 020404, MSal 020415, and MSal 020417 are listed in Table 2.1. These peptides were derived from phage clones screened against a cocktail of eight commonly found \textit{Salmonella} serovars (Enteritidis, Typhimurium, Dublin, Infantis, Senftenberg, Hadar, Mbandaka, Virchow)\textsuperscript{24}. They were chemically synthesized by \textit{GL Biochem} (China) at >85\% purity with a glycine-glycine-glycine-serine-cysteine space at the C-terminus as suggested by the manufacturer (PhD-12 phage display library; \textit{New England Biolabs}, UK). DSP (dithiobis[succinimidyl propionate]) was purchased from \textit{Pierce}, and PEG-SH (dithiol-alkane-aromatic PEG3-OH) was from \textit{SensoPath Technologies}. Alamethicin was purchased from \textit{VWR}.
Bacteria used in this study include a cocktail of eight commonly found *Salmonella* serovars (Enteritidis, Typhimurium, Dublin, Infantis, Senftenberg, Hadar, Mbandaka, Virchow), *Listeria monocytogenes*, and *Escherichia coli* K12. Each culture was inoculated from a single colony in 10 mL of nutrient broth (CM-0001, Oxoid Ltd., UK) overnight at 37 °C. The overnight cultures were washed with phosphate buffered saline (PBS), pH 7.4, by centrifugation (14,400 rpm, 15 min, 4 °C) and resuspended in PBS to a concentration of $2 \times 10^9$ cfu/mL, as determined using previously collected optical density measurements (the number of cells per milliliter at an OD of 1 measured at 600 nm for each bacterium). The bacterial suspension was subjected to a 10-kGy dose of gamma radiation (Gamma 650 cobalt irradiator) to kill the bacteria while preserving the outer structure of the whole cell.

Table 2.1. *Salmonella*-specific phage-derived peptides.

<table>
<thead>
<tr>
<th>Name</th>
<th>Amino acid sequence</th>
<th>Theoretical pI (a)</th>
<th>Theoretical MW (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSal 020401</td>
<td>SEAYKHRQMHMSGGGSC</td>
<td>7.97</td>
<td>1866.07</td>
</tr>
<tr>
<td>MSal 020404</td>
<td>VPWVTTYEPWGMGGSC</td>
<td>4.00</td>
<td>1827.06</td>
</tr>
<tr>
<td>MSal 020415</td>
<td>GPADNTSKHVIRGGGSC</td>
<td>8.23</td>
<td>1655.81</td>
</tr>
<tr>
<td>MSal 020417</td>
<td>NRPDSAQFWLHHGGGSC</td>
<td>6.91</td>
<td>1869.00</td>
</tr>
</tbody>
</table>

\(a\) Isoelectric point; \(b\) Molecular weight.

2.2.2. Preparation of Microcantilever Surfaces
Microcantilever chips were purchased from Concentris GmbH. Each chip contains eight rectangular silicon cantilevers coated with 3 nm of titanium followed by a 20 nm gold layer, resulting in a bimetallic structure. The cantilevers are 500 µm in length, 100 µm in width, and 1 µm thick. Before functionalization, the microcantilever chip was washed with a mixture of hydrogen peroxide and ammonia hydroxide at 75 ºC and cleaned using a UV-ozone cleaner (Novascan) under 5 psi oxygen. Dithiobis [succinimidyl propionate] (DSP) was used as cross-linker to immobilize the recognition element to the gold surface of microcantilevers. The chip was first immersed in DSP (1 mg/mL in DMSO) for 30 min, and then in the recognition element solution (2 µg/mL for antibody, 5 µg/mL for peptide, or 5 µg/mL for alamethicin) for 2 h to form a “sandwich” structure: “Au↔DSP↔Recognition element”, on the surface. Finally, the chip was incubated in a 1M Tris solution overnight to block uncoated sites. The cartoon schematic in Figure 2.1 depicts the chemical functionalization. A reference cantilever was functionalized with PEG-SH (1 mM in ethanol), which has no affinity to the bacterial solutions. For multiplexed detection, each cantilever was individually functionalized with a different peptide using glass microcapillaries. After use, the microcantilever chips were treated with 0.1M glycine-HCl solution at pH 2.2 for at least 1 h to release the cells from the immobilized peptides on the cantilever surface, and then regenerated by equilibrating with PBS buffer.

2.2.3. Microcantilever Assay for Pathogen Detection

Microcantilever responds to the change in surface free energy on sensor surface by monitoring its deflection change. The physical- or chemisorption of molecules to cantilever surface induces a mismatch in the surface stress, causing the cantilever to bend.
The relationship between cantilever deflection and the change in surface stress is described by Stoney’s equation\textsuperscript{32}. Real-time deflection of microcantilevers was monitored using a Cantisens system from Concentris GmbH, which utilizes a scanning laser diode aligned to the tip of the microcantilevers. The position of the reflected laser beam was captured using a position-sensitive detector (PSD), with a sampling frequency of 1 Hz\textsuperscript{28}. The various bacterial solutions (Salmonella spp., Listeria monocytogenes, or Escherichia coli) were flowed past the functionalized microcantilevers via a syringe pump at 0.42 µL/s at 25 °C. Small variations in the material properties of the cantilevers, such as stiffness or thickness of the gold layer, result in different signals. Thus the deflections of microcantilevers were normalized by each cantilever’s thermo-mechanical sensitivity, which uses the change in deflection due to a 1 °C change in temperature\textsuperscript{22}. Each experiment was repeated at least three times on either the same or different chips, with a minimum of five cantilevers on one chip.

2.2.4. Mathematical Modeling of Bacteria Binding on Microcantilevers

The binding of the bacteria to the recognition element on the cantilever surface was modeled by assuming a Langmuir isotherm model\textsuperscript{27}. The kinetic of the binding is shown as:

\[
\text{Binder} + \text{Bacteria} \xrightleftharpoons{K_A} \text{Binder} - \text{Bacteria}
\]  

(2.1)

In Equation 2.1, “Binder” represents the recognition element: anti-Salmonella antibody or a phage-derived peptide. \(K_A\) is the binding affinity constant. Affinity constant plays an important role in adsorption process, and can be used to study interaction between two molecules. The deduction steps of the model have been previously
reported. There are three more assumptions in this model: the deflection is proportional to the coverage of the recognition element on the microcantilever surface; the recognition elements bind only one molecule of targets (monovalency); and the adsorption of bacteria to the recognition element reaches equilibrium after a certain time. Afterwards, the following equation is obtained:

\[ k_A[Bacter]a(1 - \theta) = k_{-A}\theta \]  

(2.2)

In the equation above, \( \theta \) is the fraction of the surface with attached Salmonella, and \((1 - \theta)\) represents the sites available for further Salmonella binding. Define \( K_A \) as the binding affinity constant, and the expression for \( \theta \) is obtained. Finally the equation with relation between Salmonella concentration and deflection signal from microcantilevers is obtained, as shown in Equation 2.3.

\[
\frac{[Bacter]}{\Delta Def} = \frac{[Bacter]}{\Delta Def_{max}} + \frac{1}{\Delta Def_{max}K_A} 
\]  

(2.3)

In Equation 2.3, \([Bacter]\) is the molar concentration of bacteria, while \(\Delta Def\) is the change of normalized deflection caused by a given concentration of Salmonella and \(\Delta Def_{max}\) is the maximal change of normalized deflection when all the recognition elements are occupied indicating full coverage of bacteria on sensor surface (\(\theta = 1\)). Since \([Bacter]/\Delta Def\) is linear to \([Bacter]\), \(K_A\) is obtained from the slope and intercept of the plot of \([Bacter]/\Delta Def\) with respect to \([Bacter]\).

2.3. Results and Discussion

Phage-derived peptides have increasingly been employed as recognition elements to detect specific analytes. Several rounds of biopanning are typically employed to
screen a phage display library and isolate high affinity binders. Previously, a PhD-12 phage display library containing $10^{13}$ phage forming unit (pfu)/mL expressing 12-amino acid peptides was biopanned against *Salmonella* spp. and several phage clones were selected that present high affinity for *Salmonella* spp. The corresponding peptides from some of the phage clones were chemically synthesized and proven to be specific receptors to *Salmonella* spp. for magnetic separation.

In this study, four peptides of the previously identified phage-derived peptides specific to *Salmonella* spp. (MSal 020401, MSal 020404, MSal 020415, and MSal 020417) were investigated as bacterial recognition elements on microcantilevers. Previous ELISA results using phage-displayed peptides, showed that MSal 020401, MSal 020415 and MSal 020417 exhibited higher binding affinity to the *Salmonella* serovars than to other non-target bacteria tested (*L. monocytogenes* and *E. coli* K12). Although MSal 020404 did not display high affinity binding toward *Salmonella* spp. in phage ELISA analyses, it was found to have the lowest cross-reactivity to the non-target bacteria in studies conducted using chemically synthesized peptides.

### 2.3.1. Microcantilevers for Pathogen Detection

Detection of *Salmonella* was first investigated using a microcantilever functionalized with the anti-*Salmonella* antibody to determine selectivity of the antibody. Three different bacterial solutions at concentrations of $1 \times 10^7$ cfu/mL were flowed past the functionalized cantilever, causing a deflection, as shown by Figure 2.1. A decrease in cantilever deflection of 24 nm is observed upon the binding of *Salmonella* spp. to the anti-Salmonella functionalized cantilever, compared to 10 nm for *L. monocytogenes*, and
3 nm for *E. coli*. The extent of binding, which is directly proportional to the magnitude of deflection change, reflects the relative affinity of each bacterium for the anti-Salmonella antibody. The results obtained using microcantilevers are in agreement with the relative affinity measured by ELISA: *Salmonella* spp. > *L. monocytogenes* > *E. coli*.

**Figure 2.1.** Selective detection of *Salmonella* using microcantilevers. (A) Schematic representation of the microcantilever-based sensor. (B) Anti-Salmonella antibody (2 μg/mL) was immobilized on the cantilever surface and three bacterial solutions (1x10⁷ cfu/mL, *Salmonella* spp.: red lines; *Listeria monocytogenes*: blue lines; *Escherichia coli*: green lines). Each experiment was conducted in quadruplicate. The arrow represents the time point at which the bacterial solution was added.

2.3.2. *Comparison of Antibody and Peptides: A Potential Screening Method of Pathogen Specific Recognition Elements*
Four phage-derived peptides (MSal 020401, MSal 020404, MSal 020415, and MSal 020417) were individually immobilized onto microcantilevers. The binding behavior of these peptides was compared to the anti-*Salmonella* antibody, alamethicin, and a PEG-coated microcantilever surface. The anti-*Salmonella* antibody remains the standard for comparing the selectivity of the peptides. Alamethicin serves as a control since it interacts nonspecifically with bacterial cell membranes mainly through electrostatic attraction\(^3\). The PEG-coated microcantilever serves as the negative control as it prevents any nonspecific adsorption to the surface. The normalized deflections (\(\Delta \text{Def}/\Delta \text{Def}_{\text{PEG-SH}}\)) of the various recognition elements to *Salmonella* spp. at concentrations of 1x10^6, 1x10^7, and 1x10^8 cfu/mL are given in Figure 2.2(A). The extent of deflection increases with increasing concentration of *Salmonella* spp. Deflections at concentrations below 1x10^6 cfu/mL were too small to be accurately detected, suggesting that 1x10^6 cfu/mL *Salmonella* is the limit of detection of this biosensor under the conditions tested in this study. The relative deflection compared to the PEG-coated reference cantilever, \(\Delta \text{Def}/\Delta \text{Def}_{\text{PEG-SH}}\) is shown in Figure 2.2(B). The sensitivity of detection at different concentrations of *Salmonella* can be determined by normalizing the normalized signals to that of the reference cantilever. I found that the sensitivity decreased when the concentration of *Salmonella* was increased from 1x10^7 cfu/mL to 1x10^8 cfu/ (Figure 2.2(B)), most likely due to multilayer adsorption at concentrations above the absorption capacity of the microcantilever.

Because 1x10^7 cfu/mL of *Salmonella* proved to be the optimal concentration for detection, the binding behavior of various recognition elements to the three different bacterial species was tested under these conditions (Figure 2.2(C)). As expected, minimal
deflection was detected on the PEG-SH coated cantilever, indicating poor binding of any of the bacterial solutions. Alamethicin-coated cantilevers displayed similar binding affinity for *Salmonella* spp., *L. monocytogenes*, and *E. coli*. The anti-*Salmonella* antibody presented high binding affinity for *Salmonella* spp., but low affinity for *L. monocytogenes* and *E. coli*, confirming previously reported results\(^2\). Three of the four peptides, MSal 020401, MSal 020415, and MSal 020417, exhibited similar relative affinity for the different bacterial species tested. The MSal 020417-coated cantilever resulted in the largest deflection upon *Salmonella* spp. binding, indicating that MSal 020417 presents the highest affinity to *Salmonella* spp. compared to the other phage-derived peptides tested, and compared to the anti-*Salmonella* antibody. These results confirm previously reported studies using peptide-displayed magnetic beads\(^2\). As a comparison with the deflections for anti-*Salmonella* antibody in Figure 2.1(B), the direct cantilever deflections for MSal 020417 caused by three bacterial solutions are given in Figure 2.2(D), indicating its better sensitivity and selectivity. The MSal 020401-coated cantilever, on the other hand, displayed the smallest deflection upon binding of *L. monocytogenes*, and *E. coli*, suggesting that MSal 020401 presents the highest selectivity toward *Salmonella* spp. and the lowest cross-reactivity with the other bacterial species tested. The only inconsistency between the results obtained with microcantilevers and ELISA is the relative affinity of MSal 020404 towards *L. monocytogenes*. This difference is likely due to differences in affinities of peptides when tested individually or displayed on a phage capsid.
Normalized $\Delta\text{Def} = \frac{\Delta\text{Def}}{\Delta\text{Def due to } 1^\circ C}$

Relative $\Delta\text{Def} = \frac{\Delta\text{Def of recognition element}}{\Delta\text{Def of PEG - SH}}$
**Figure 2.2.** Selective detection of bacterial species using peptide-coated microcantilevers. (A) Normalized deflection of microcantilevers coated with recognition elements and upon binding of *Salmonella* spp. at a concentration of $1 \times 10^6$, $1 \times 10^7$, or $1 \times 10^8$ cfu/mL. (B) Relative deflection of microcantilevers under conditions described in (A). Relative deflection is defined as the deflection of the recognition element-coated cantilever normalized to the deflection of the PEG-SH-coated cantilever. (C) Relative affinities of various recognition elements towards different bacteria *Salmonella* spp. (*Sal*), *L. monocytogenes* (*LM*), and *Escherichia coli* (*E.coli*) at $1 \times 10^7$ cfu/mL. The equations used to calculate the normalized and relative deflection are also reported. (D) Selective detection of *Salmonella* using peptide MSal 020417. Three bacterial solutions ($1 \times 10^7$ cfu/mL, *Salmonella* spp.: red lines; *Listeria monocytogenes*: blue lines; *Escherichia coli*: green lines) were tested. Each experiment was conducted in quadruplicate. The arrow represents the time point at which the bacterial solution was added.

### 2.3.3. Detection of Various Salmonella Serovars

The experiments described above were conducted using solutions of *Salmonella* spp., which is a mixture of various *Salmonella* serovars. To further evaluate the selectivity of the cantilever-displayed peptide technology for biosensing and biopanning applications, eight different *Salmonella* serovars was tested individually (Figure 2.3). Microcantilevers were coated with the peptide with highest affinity towards *Salmonella* spp. (MSal 020417) to quantify the relative affinity of MSal 020417 to various *Salmonella* serovars tested at optimal concentration ($1 \times 10^7$ cfu/mL). The eight serovars induced different signals (normalized deflections), indicating different binding affinities of MSal 020417 to *Salmonella* serovars. The relative normalized deflections detected using microcantilevers are in agreement with the relative binding affinities observed in the previously reported phage-peptide ELISA analyses. Moreover, the deflection observed using each one of the eight *Salmonella* serovars tested was much larger than that observed using *L. monocytogenes* and *E. coli*, demonstrating that the MSal 020417 peptide can be used for detection not only of *Salmonella* spp., but also of individual
Salmonella serovars. Interestingly, I found that the average normalized deflection induced by the Salmonella serovars equals the normalized deflection observed using Salmonella spp., underscoring the potential of microcantilever-displayed peptide biosensors for reliable detection of bacteria.

Figure 2.3. Detection of Salmonella serovars using microcantilevers. The peptide MSal 020417 is used as recognition element, and the concentration of bacterial solution is \(1 \times 10^7\) cfu/mL. Salmonella serovars: shades of red; Salmonella spp.: red; Listeria monocytogenes: blue; Escherichia coli: green.
2.3.4. Mathematical Modeling and Calculation of the Binding Affinity Constants

The binding affinity of the peptide for the different bacterial species can be determined by modeling the process using a Langmuir isotherm model, which has previously been used to describe antigen-antibody interactions on biosensor surfaces\textsuperscript{37-38}. This binding occurs due to specific antigen-antibody interactions. We assume a monolayer of bacteria binds to the recognition elements. Moreover in the plot of binding signal versus bacterial concentration (Figure 2.4), the binding signal initially increases significantly with bacterial concentration and then reaches a plateau at higher concentrations. This type of behavior is characteristic of Langmuir adsorption process\textsuperscript{39} and a Langmuir adsorption model can be utilized to describe the binding of the bacteria to the recognition elements immobilized on the sensor surface. The binding affinity constant $K_A$ refers to the relative affinity of the peptide to a specific bacterium, as described in Equation 2.3. The selected plots of $[\text{Bacteria}]/\Delta\text{Def}$ as a function of $[\text{Bacteria}]$ for the four peptides are shown in Figure 2.5. In each plot at least six data points were used to optimize the data fitting. The binding affinity constant $K_A$ was calculated from the slope and intercept of each plot (Table 2.2): the values of $K_A$ represent the relative affinity of a recognition element for each bacterium, with the largest value corresponding to the highest affinity. Thus, the relative affinity values obtained using the Langmuir isotherm model recapitulate the experimental results described above for cantilevers coated with the anti-Salmonella antibody and MSal 020417 ($\text{Sal > LM > E. coli}$), MSal 020401 and MSal 020415 ($\text{Sal > LM \approx E. coli}$), MSal 020404 ($\text{LM > Sal > E. coli}$), and almethecin and PEG ($\text{Sal \approx LM \approx E. coli}$). The model was therefore considered reliable and applied to evaluate the affinity constants of various recognition
elements. MSal 020417 was found to have the highest affinity constant for *Salmonella* spp., confirming the superior binding properties of this peptide even compared to the commercially available antibody. These results, taken together, suggest that the mathematical model developed in this study can be used to reliably determine the relative affinities of various recognition elements for different bacterial species and to determine the kinetics properties of phage-derived peptide screened using microcantilevers.

**Figure 2.4.** Selected plots of normalized deflection of microcantilevers coated with four phage-derived peptides. The *Salmonella* or *L. monocytogenes* solution was tested at concentrations ranging from $1 \times 10^6$ cfu/mL to $1 \times 10^8$ cfu/mL.
Figure 2.5. Data fitting plots to determine the binding affinity constant $K_a$. $\frac{[\text{Bacteria}]}{\Delta \text{Def}}$ values are plotted with respect to $[\text{Bacteria}]$ and the equations for trend lines and R-squared values are reported in each graph. From this linear relationship, the slope and intercept of this plot were obtained, and binding affinity constant $K_a$ was calculated using Equation 2.3.

2.3.5. Multiplexed Screening of Peptides on Microcantilevers

To determine whether the microcantilever-based biosensor developed in this study could be used to screen combinatorial libraries of peptides, I investigated binding of *Salmonella* spp. to a random array of cantilevers coated with different recognition elements. An array of seven cantilevers was prepared on a single chip in which each
cantilever was functionalized with a different recognition element, as illustrated in Figure 2.6(A). *Salmonella* spp. at a concentration of $1 \times 10^7$ cfu/mL was flowed past the chip. As shown in Figure 2.6(B), the deflection of each cantilever was observed to be proportional to the binding affinity of the specific recognition element coated onto the cantilever as determined above, suggesting that the binding properties of the different recognition elements are not altered upon immobilization onto the array of microcantilevers. This simultaneous testing of multiple peptides on a single chip improves the efficiency of detection. These results illustrate the high potential of this microcantilever-based sensor for the screening of phage-derived peptides as well as other recognition elements.

**Table 2.2.** Binding affinity constants $K_A \times 10^9$ (mL/cfu) to *Salmonella* spp. (Sal), *Listeria monocytogenes* (LM), and *E.coli* K12.

<table>
<thead>
<tr>
<th>Constants $K_A$</th>
<th><em>Sal</em></th>
<th><em>LM</em></th>
<th><em>E. coli</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>MSal 020401</td>
<td>20.7</td>
<td>0.358</td>
<td>0.251</td>
</tr>
<tr>
<td>MSal 020404</td>
<td>7.33</td>
<td>42.9</td>
<td>0.335</td>
</tr>
<tr>
<td>MSal 020415</td>
<td>21.6</td>
<td>0.518</td>
<td>0.293</td>
</tr>
<tr>
<td>MSal 020417</td>
<td>55.4</td>
<td>4.41</td>
<td>0.180</td>
</tr>
<tr>
<td>Antibody</td>
<td>33.6</td>
<td>5.28</td>
<td>0.288</td>
</tr>
<tr>
<td>Alamethicin</td>
<td>25.3</td>
<td>27.1</td>
<td>26.5</td>
</tr>
<tr>
<td>PEG-SH</td>
<td>0.0689</td>
<td>0.0277</td>
<td>0.0411</td>
</tr>
</tbody>
</table>
Figure 2.6. Multiplexed screening of recognition elements on microcantilevers. (A) Schematic representation of individual functionalization of cantilevers with various recognition elements on a single chip. (B) Multiplexed detection of *Salmonella* spp. using microcantilevers. The concentration of *Salmonella* spp. is $1 \times 10^7$ cfu/mL. Various recognition elements were immobilized on a single cantilever chip, and tested simultaneously. The lines represent the deflection of cantilevers functionalized with different recognition elements as reported in the figure legend, and the hollow pink circles represent data obtained from experiments conducted using individual recognition elements.
2.3.6. Performance Discussion of Microcantileve-based Technique

The experiments and models used to determine the binding affinity constant show that peptide MSal 020417 has better sensitivity and selectivity than anti-Salmonella antibody. In addition to the normalized deflection in Figure 2.2(C), the direct cantilever deflections for antibody (Figure 2.1(B)) and MSal 020417 (Figure 2.2(D)) are also given for a more direct comparison. The difference between specific Salmonella binding and non-specific binding (E. coli or L. monocytogenes) is obviously larger for MSal 020417 than for anti-Salmonella antibody. As a result, the microcantilever-based technique using the peptide MSal 020417 demonstrates high selectivity for Salmonella detection. Another important factor in biosensor detection is the surface grafting density of immobilized chemicals. The differences in molecular size, charge and concentration might lead to different grafting density on sensor surface, which could interfere with cantilever detection. From Figure 2.1(B) and Figure 2.2(D), compared to MSal 020417, although anti-Salmonella antibody apparently has smaller surface density due to its larger size, it results in a larger non-specific binding towards E. coli and L. monocytogenes. The above comparison might suggest that the affinity of recognition elements plays a more important role in cantilever deflection than the surface density. To enhance the performance of this microcantilever-based technique, one possibility is to improve the detection sensitivity by controlling the orientation of recognition elements immobilized on sensor surface\textsuperscript{40-41}. The random immobilization of the recognition element used in this study might lead to a steric hindrance effect, potentially blocking available active sites for specific binding to the bacteria. Therefore, an oriented immobilization method can be explored to minimize the steric hindrance which may further improve the detection.
sensitivity\textsuperscript{20}. Moreover, the results reported above suggest that the microcantilever sensor can distinguish \textit{Salmonella} from other bacteria, such as \textit{L. monocytogenes} and \textit{E. coli}, when they are present in solution at similar concentrations. However, the deflection associated with a solution containing low \textit{Salmonella} concentration is expected to be similar to that of a solution containing high \textit{E. coli} concentration. To potentially address this issue, the binding of one bacterium to seven recognition elements using multiplexed detection is considered. Although \textit{Salmonella} at a low concentration and \textit{E. coli} at a higher concentration might induce similar binding signals to a certain recognition element, the relative magnitudes of bindings of two bacteria to different recognition elements are likely to be different because they have different affinities towards various recognition elements. Therefore, the deflection caused by one bacterium when binding to seven recognition elements is unique and can be used as “fingerprint” to distinguish \textit{Salmonella} from other bacteria.

2.4. Conclusion

A rapid and specific microcantilever-based technique has been developed for the detection of the pathogenic bacterium \textit{Salmonella} by exploiting the binding affinities of \textit{Salmonella}-specific recognition elements. In this study, four phage-derived peptides selected from the screening of a commercial phage display library were tested. The peptide MSal 020417 was found to have better binding affinity and specificity than a commercially available anti-\textit{Salmonella} antibody. In addition to distinguishing \textit{Salmonella} spp. from \textit{Listeria monocytogenes} and \textit{Escherichia coli}, this microcantilever-based technique also enables to distinguish between eight \textit{Salmonella} serovars. The
binding properties of the various recognition elements to the different bacterial species were subsequently investigated using a Langmuir isotherm model, and the binding affinity constants $K_A$ were calculated. The relative affinities of recognition elements evaluated with the Langmuir isotherm model were consistent with the experimental results. Furthermore, I show that immobilizing different peptides on an array of microcantilevers in a single chip allows for rapid screening of multiple peptides for the isolation of high affinity binders. I envision that by immobilizing a collection of peptides that present high selectivity for different bacterial pathogens, a multiplexed detection system could be generated to detect multiple pathogens simultaneously, largely improving the detection efficiency of the biosensor. Therefore, the microcantilever-based technique has been proved to be a useful sensor to characterize molecules on liquid-solid interface. In summary, multiplexed measurement on microcantilevers can be used to rapidly and accurately investigate molecular properties on the solid surface, as well as to efficiently evaluate various molecular interactions.

2.5. References

4. Fratamico, P. M., Comparison of culture, polymerase chain reaction (PCR), TaqMan Salmonella, and Transia Card Salmonella assays for detection of Salmonella spp. in naturally-contaminated ground chicken, ground turkey, and ground beef. Molecular and Cellular Probes 2003, 17 (5), 215-221.


Chapter 3

Sensitive Detection of TNT using Competition Assay on Quartz Crystal Microbalance

3.1. Introduction

The rapid and reliable detection of explosives has gained increasing attention, due to health and public safety reasons\(^1\). Most types of currently used explosives are toxic to living systems, even when present in trace amounts. This study focuses on the detection of 2, 4, 6-trinitrotoluene (TNT). Different from the detection of foodborne bacteria studied in last chapter, TNT is a very small molecule compared to the bacterial cell, making its detection more difficult, particularly at low concentration. Therefore a
competition assay is introduced to detect TNT. Instead of measuring the actual binding of TNT, this method is based on the displacement of an anti-TNT antibody, which allows quantifying the concentration of TNT in solution with higher sensitivity. Currently available TNT sensors are characterized by high sensitivity, but low specificity, which limits the detection of TNT in dirty environments. Here quartz crystal microbalance (QCM) is designed to measure the displacement of a TNT-specific antibody.

A variety of technologies have been developed for the detection of TNT. They can be broadly classified as physical, chemical and biological methods, based on the detection mechanism and output signal. Physical methods, such as laser, mass spectroscopy and NMR\(^2\), allow achieving high sensitivity of detection, but involve time-consuming and costly procedures. Particularly, TNT concentrations as low as 1 pg/mL can be detected in vapor phase with low false positive signals using laser photoacoustic spectroscopy\(^2\). The specificity of chemical methods, including electrochemical sensors\(^5\)\(^6\) and fluorescence spectrophotometry\(^7\)\(^9\), is relatively low. Fluorescence spectrophotometry is based on the electron deficiency of TNT, which as many nitroaromatic compounds, functions as electron acceptor and causes quenching of a number of photoluminescent\(^10\), fluorescent\(^11\), and phosphorescent\(^12\) materials by electron transfer. Among these materials, fluorescent polymers were first reported to allow detecting saturated TNT vapor (0.1 ng/mL) within seconds\(^13\). The performance of fluorescence spectrophotometry was later improved using mesostructured silica films\(^14\), nanocrystals\(^15\), and quantum dots\(^16\). Particularly, the limit of detection for TNT was reduced to \(~0.023\) ng/mL using a hybrid material composed of gold nanorod and quantum dots. This method, however, exhibits relatively low specificity, which prevents distinguishing TNT from other nitroaromatic
compounds with similar chemical properties\textsuperscript{17}. Biological methods typically present enhanced specificity due to the use of TNT specific molecules, such as antibodies\textsuperscript{18-19}, and molecularly imprinted polymers (MIPs)\textsuperscript{20-21}. Most of these reported sensors lack data comparing their performance in a dirty environment. The purpose of this study is to develop a rapid and accurate sensor that combines the high sensitivity of chemical methods with the high specificity of biological methods for the detection of TNT in aqueous solutions containing similar molecules.

One of the critical issues in the development of a TNT sensor is the small size of the TNT molecule that often precludes high sensitivity of detection at low concentrations. To overcome this limitation I designed a sensor based on the detection of a TNT-specific antibody. Particularly, the concentration of TNT in solution was measured by quantifying the antibody displacement from the surface by TNT, a strategy that allows amplifying the output signal compared to the signal associated with direct detection of TNT\textsuperscript{22-25}. The detection of TNT using displacement assay was previously reported using Enzyme-Linked Immunosorbent Assay (ELISA)\textsuperscript{26-28}, which is used as comparison in this study. In addition, the method of antibody displacement was also previously used for TNT detection in a flow system (microcapillary)\textsuperscript{29}.

A number of techniques have been used for the detection of TNT including surface plasmon resonance (SPR)\textsuperscript{30} and fluorescence resonance energy transfer (FRET)\textsuperscript{31}. In this study I used quartz crystal microbalance (QCM) couple with the antibody displacement method described above. QCM has been widely used as biosensor, in studies of affinity estimation\textsuperscript{32} and polymer conformational changes\textsuperscript{33}, due to its high sensitivity, label-free, real-time measurements, portability, and ease of operation\textsuperscript{34}. In this
study, I describe the development of a QCM based sensor for TNT detection that allows measuring TNT with high sensitivity in pure solution as well as in dirty environments, such as fertilizers and seawater\(^35\). The kinetics of antibody displacement during the detection process was studied through a Langmuir adsorption based model.

### 3.2. Materials and Methods

#### 3.2.1. Materials

2, 4, 6-Trinitrotoluene (TNT), 1, 3, 5-Trinitrobenzene (TNB), 2, 6-Dinitrotoluene (DNT), 2-Amino-4, 6-Dinitrotoluene (2-a-DNT), and ovalbumin were purchased from Sigma-Aldrich. Anti-TNT monoclonal antibody A1.1.1 was from Strategic Diagnostics. HRP conjugated anti-mouse antibody was purchased from Assay Designs. 2, 4, 6-trinitrobenzene sulfonic acid (TNBS) and Dithiobis[succinimidyl propionate] (DSP) were from Pierce. TMB substrate was purchased from BioLegend.

#### 3.2.2. Synthesis of TNB-ovalbumin Complex

The TNB-ovalbumin complex was prepared by conjugating the sulfonic group of 2, 4, 6-trinitrobenzene sulfonic acid (TNBS) to the primary amines of ovalbumin molecules as previously described\(^36\). Briefly, a solution of 10.2 mM TNBS and 0.67 mM ovalbumin in PBS (\(pH\) 8.0) was stirred at 30rpm for one hour at room temperature. The reaction product was dialyzed overnight against PBS to eliminate free TNBS and stored at -80°C until use.

#### 3.2.3. Preparation of TNT and TNT Analogs
Acetonitrile was evaporated from the stock solution of TNT (1000 µg/mL) and TNT analogs. Then PBS buffer, fertilizer solution, or seawater, was used to dissolve them before use. Commercially available fertilizer powder was dissolved in PBS buffer at a concentration of 1 mg/mL (0.1% w/v), and the pH adjusted to 7.4. Artificial seawater was prepared by dissolving 100% natural sea salt in deionized water (26.7 g/L) to obtain a solution containing the same concentration of sodium as natural seawater (0.469 mol/kg).

3.2.4. ELISA Assay

100µL of TNB-ovalbumin complex at 10 µg/mL in 0.1M sodium bicarbonate were added to each well of a 96-well plate and plates incubated overnight at 4 °C. After washing with 0.1% PBST, PBS 4% milk (200 µL/well) was added to block uncoated sites. TNT or TNT analogs at concentration specified in each experiment and mouse anti-TNT antibody (0.5 µg/mL) were added to each well and plates incubated for ~2 hours with gentle shaking. After washing with 0.1% PBST, HRP-conjugated goat anti-mouse antibody (100 ng/mL) was added to each well, plates incubated for 1 hour, and washed again. 100 µL/well of TMB substrate were added, and, after 10 minutes, 50 µL/well of 1 M phosphoric acid were added to stop the reaction. The absorbance at 450 nm was measured with a GeneMate UniRead 800 plate reader. The cross reactivity \((CR)^{37}\) of the anti-TNT antibody with each compound was evaluated as follows:

\[
CR = \frac{C_0}{C} \times 100\% 
\]

where \(C_0\) is the concentration of TNT upon 50% of antibody displacement, and \(C\) is the concentration of compound used to achieve 50% displacement.
3.2.5. Functionalization of the Crystal Surface

Crystals were washed with a mixture of hydrogen peroxide and ammonia hydroxide at 75 °C, and UV-ozone cleaner (novascan) under 5 psi oxygen. Dithiobis-[succinimidyl propionate] (DSP) was used as cross-linker to immobilize TNB-ovalbumin complex on the gold surface of crystals. Crystals were first immersed in DSP (1 mg/mL in DMSO) for 30 min, and then in TNB-ovalbumin complex (100 µg/mL) for 2 hour to form a “sandwich” structure: “Au↔DSP↔TNB-ovalbumin”, on the surface. Crystals were incubated in 1 M Tris overnight to block uncoated sites.

3.2.6. QCM Assay

The QCM system used in this study is a Q-sense E4 system (Q-Sense, Västra Frölunda, Sweden), which measures changes in mass and related viscoelastic properties. The AT-cut QCM crystal used has a resonance frequency of 5 MHz. Using Sauerbrey equation\(^{38}\), 1 Hz frequency change can be converted to a mass change of 17.7 ng/cm\(^2\) on the crystal surface. The viscoelastic properties can be obtained from energy dissipation measured by the decay of oscillation\(^{39}\). After immobilization of TNB-ovalbumin complex, anti-TNT antibody and solutions of TNT or TNT analogs were flowed sequentially at 50 µl/min at 25 °C.

The frequency change measured after addition of TNT was divided by the frequency change caused by the addition of antibody (\(\Delta f_{\text{dis}}\)). \(\Delta f_{\text{dis}}\) obtained from each compound tested was divided by the \(\Delta f_{\text{dis}}\) of the control sample (crystal without TNT or TNT analog), and the new parameter obtained, the normalized frequency change (\(\Delta f_N\)), was used for the data analysis.
The ratio of frequency change over a chosen time interval, defined “quick” slope $k$, was calculated as follows:

$$k = \frac{\Delta f_N}{\Delta t}$$

where $\Delta f_N$ is the normalized frequency change during the time interval $\Delta t$.

### 3.2.7. Mathematical Modeling of Antibody Displacement on QCM

There are totally two steps in QCM displacement assay: attachment step and displacement step. “Displacement” takes place between TNT and TNB-ovalbumin towards anti-TNT antibody. The process includes two immunoreactions:

1. **Attachment**: $Ab + TNB \rightleftharpoons Ab - TNB$ (Antibody is attached.)

2. **Displacement**: $Ab - TNB + TNT \rightleftharpoons Ab - TNT + TNB$ (Antibody is displaced.)

“$Ab$” above represents anti-TNT antibody. “$K_a$” is binding affinity constant. Affinity constant plays an important role in adsorption process, and can be used to study interaction between two molecules. So $K_a$ is expected to announce critical factor and improve detection performance.

In the process above, the first step (attachment) is ignored, because in QCM assay crystal surface is saturated by antibody. So only the second step (displacement) is considered. The displacement of antibody on QCM is simulated by assuming a Langmuir isotherm model. The deduction steps of the model have been previously reported$^{40-41}$. The following assumptions are used in the model: the crystal surface is saturated by anti-TNT
antibody before the addition of TNT; the antibody binds one molecule of TNT or TNB so
that if one of them is bound to antibody, the others will be free; the displacement of
antibody caused by TNT reaches equilibrium after a certain time; and the concentration
of TNB is constant on surface. When the displacement step reaches equilibrium, the
following equation is satisfied.

\[ k_A[TNT](1 - \theta) = k_{-A}[TNB]\theta \Rightarrow k_A[TNT](1 - \theta) = k_{-A}\theta \]  

(3.6)

In the equations above, \( \theta \) is the fraction of the surface without coverage of
antibody. On the contrary, \((1 - \theta)\) represents the sites that are available for TNT. The
effect of TNB concentration was ignored, because it was immobilized on surface.
Defining \( K_A \) as the binding affinity constant, with the expression for \( \theta \), the equation
describing relationship between TNT concentration and the frequency change was finally
obtained.

\[ K_A = \frac{k_A}{k_{-A}} = \frac{\theta}{(1 - \theta)[TNT]} \Rightarrow \theta = \frac{k_A[TNT]}{1 + k_A[TNT]} = \frac{\Delta f}{\Delta f_{max}} \]  

(3.7)

\[ \Rightarrow \frac{1}{\Delta f} = \frac{1}{\Delta f_{max}} + \frac{1}{\Delta f_{max}K_A[TNT]} \]

\[ \Rightarrow \frac{[TNT]}{\Delta f} = \frac{[TNT]}{\Delta f_{max}} + \frac{1}{\Delta f_{max}K_A} \]  

(3.8)

where \( \Delta f \) is the frequency change at a given concentration of TNT, \( \Delta f_{max} \) is the maximal
frequency change when the antibody is completely displaced, and \( K_A \) is the binding
affinity constant (Equation 3.7). \( \Delta f_{max} \) and \( K_A \) were calculated from the plot of \([TNT]/\Delta f\)
with respect to \([TNT]\) (Equation 3.8). In order to simulate the kinetics of QCM
displacement process, the rate of adsorption was taken into account. For the Equation 3.5,
the expression of the forward reaction rate (describing the formation of antibody-TNT complex) was given by\(^4\)

\[
\begin{align*}
    r_{Ab-TNT} &= k_A[TNT](1 - \theta) - k_{-A}\theta = k_A[TNT] - (k_A[TNT] + k_{-A})\theta \\
    r_{Ab-TNT} &= -\frac{d[TNT]}{dt} = \frac{d\theta}{dt} = k_A[TNT] - (k_A[TNT] + k_{-A})\theta
\end{align*}
\] (3.9)

In the ODE above, the concentration of TNT was assumed to be constant, because TNT in solution was flowed continuously into the reaction chamber. As a result, Equation 3.9 was integrated with respect to \(\theta\), from 0 to time \(t\). The result was further simplified with constant \(A\) and \(B\) (Equation 3.10). With the values of \(K_A\) and \(\Delta f_{max}\), \(A\) and \(B\) were calculated. By plotting the right part of this equation as a function of time, the forward reaction rate \(k_A\) was calculated,

\[
\Rightarrow t = \frac{1}{k_A[TNT]+k_{-A}} \ln \frac{k_A[TNT]}{k_A[TNT]-(k_A[TNT]+k_{-A})\theta}
\]

\[
\theta = \frac{\Delta f}{\Delta f_{max}} \Rightarrow k_A t = \frac{1}{AB} \ln \frac{1}{1-A\Delta f}
\] (3.10)

where \(A = (1 + (1/K_A[TNT]))/\Delta f_{max}\), \(B = [TNT]\Delta f_{max}\). A new ODE describing the change of \(\Delta f\) as a function of time was obtained from the expression of \(k_A\) (Equation 3.11), and analyzed in Matlab. The model derived was used to analyze the experimental data, and predict the kinetics of displacement under different conditions, such as different pH and temperature.

\[
\frac{d\Delta f}{dt} = k_A[TNT]\Delta f_{max} - \left( k_A[TNT] + \frac{k_A}{K_A} \right) \Delta f
\] (3.11)
3.3. Results and Discussion

3.3.1. Detection of TNT through Antibody Displacement by ELISA

The anti-TNT monoclonal antibody A1.1.1 was chosen because of its high binding specificity to TNT\(^42\) compared to other nitroaromatic compounds with similar structures\(^37\). ELISA analyses were first conducted to evaluate the feasibility of the displacement assay and determine the affinity and limit of detection of this antibody with a currently well-established technique. The principle of the displacement assay is based on the ability of the anti-TNT antibody to cross react with TNT analogs. TNB, which was previously reported to bind to this anti-TNT antibody with low affinity\(^37\) was used as reference. The ability of TNT and other TNT analogs (including TNB) to displace the antibody from TNB was evaluated. Measurements of antibody displacement are reported in Figure 3.1 using TNT, TNB, DNT, and 2-a-DNT (Table 3.1). TNT was observed to cause the maximum antibody displacement and the limit of detection was estimated to be 1 ng/mL.

The anti-TNT antibody used was isolated based on its high specificity to TNT, but, similarly to most antibodies, it displays cross-reactivity to compounds structurally similar to TNT. To quantify the binding affinity, the cross reactivity (CR) of the antibody for each compound was calculated as shown in Equation 3.1 (Table 3.2). The results obtained illustrate the antibody relative affinity: TNT>TNB>2-a-DNT>DNT, and confirm previously reported measurements\(^27, 37, 43-44\).
Figure 3.1. ELISA measurements of anti-TNT antibody displacement from TNB by TNT and TNT analogs. Compounds were tested at concentrations ranging from 0.1 ng/mL to 100 µg/mL and anti-TNT antibody A1.1.1 at 10 µg/mL. Data points were obtained from at least three independent experiments and normalized to the signal measured from control samples containing only antibody. The detection limit for each molecule corresponds to the lowest concentration that causes loss of absorbance.

Table 3.1. Chemical structure of TNT and TNT analogs used in this chapter.

<table>
<thead>
<tr>
<th>Name</th>
<th>Chemical Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4,6-Trinitrotoluene (TNT)</td>
<td><img src="image" alt="Chemical Structure" /></td>
</tr>
</tbody>
</table>
1, 3, 5-Trinitrobenzene (TNB)

\[ \text{O}_2\text{N} \text{NO}_2 \]

2, 6-Dinitrotoluene (DNT)

\[ \text{CH}_3 \text{NO}_2 \]

2-Amino-4, 6-Dinitrotoluene (2-a-DNT)

\[ \text{O}_2\text{N} \text{NH}_2 \text{NH}_2 \text{NO}_2 \]

Table 3.2. Cross reactivities for the compounds using antibody A1.1.1.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$\Delta f_{\text{max}}$</th>
<th>$K_\lambda (\text{mL/μg})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNT</td>
<td>0.73±0.05</td>
<td>5.09±0.61</td>
</tr>
<tr>
<td>TNB</td>
<td>0.66±0.04</td>
<td>1.44±0.62</td>
</tr>
<tr>
<td>DNT</td>
<td>0.24±0.11</td>
<td>0.47±0.14</td>
</tr>
</tbody>
</table>

3.3.2. Development of a QCM based Displacement Assay for TNT Detection

The method developed for QCM detection of TNT is based on the principle of antibody displacement described above. The TNB-ovalbumin complex was first immobilized onto the surface of the crystal. The surface was then saturated with anti-TNT antibody, and subsequently a flux of TNT or TNT analogs was used to displace the
antibody. The change in frequency was recorded until a plateau was reached, indicating maximum displacement of antibody. Because the molecular weight of the antibody is much greater than that of the TNT molecule, detecting the frequency change caused by the antibody displacement, rather than that associated with the binding of TNT, gives rise to significant amplification of the detection signal. This enables the sensor to achieve higher sensitivity and lower limit of detection. Control studies were conducted to measure the frequency change caused by flowing TNT on a crystal coated with TNB-ovalbumin but without anti-TNT antibody, which resulted in signal indistinguishable from the background (e.g. the frequency change associated with flux of PBS buffer on to a TNB-ovalbumin coated crystal), indicating the absence of non-specific binding (data not shown).

The antibody displacements by TNT and two analogous compounds, TNB and DNT, at a concentration of 10 μg/mL were tested by QCM (Figure 3.2). A sharp decrease of frequency (around 45 Hz on average) is observed upon binding of the antibody to the TNB-ovalbumin complex on the crystal surface, which corresponds to a mass change of 506.25ng from the Sauerbrey equation. The number of antibody molecules attached on sensor surface is 2.03×10^{12}. Given the size of crystal (9mm diameter) and ovalbumin molecule (6.1nm diameter), I estimated that 2.18×10^{12} molecules of ovalbumin are immobilized on the sensor surface. Hence, I can assume that virtually all TNB-ovalbumin molecules form a complex with the antibody. After immobilization of the antibody, the solution of compound was flowed on to the crystal and the displacement of antibody was monitored (Figure 3.2). To minimize the variability of surface immobilization, Δf_{dis} (frequency change of TNT / frequency change of antibody) was used in the data analysis.
The displacement caused by TNT was about 3-fold higher than that observed using DNT. The extent of displacement (the magnitude of frequency change) obtained at equilibrium reflects the binding affinity of each compound for the anti-TNT antibody. The antibody displacement measured by QCM is in agreement with the relative affinity of the three compounds calculated by ELISA: TNT>TNB>DNT.

Figure 3.2. QCM measurements for the detection of TNT. Antibody displacement from TNB by TNT and TNT analogs. Anti-TNT antibody (10 μg/mL) was immobilized on the crystal surface and three compounds (10 μg/mL, TNT: red line; TNB: green line; DNT: blue line) were tested. The arrows represent the time points when TNT and its analogs were added. Results are representative of three independent experiments.

The results presented above suggest that this sensor allows distinguishing TNT from molecules with similar chemical structure when they are present in solution at similar concentrations. However, the frequency change associated with a solution
containing low TNT concentration is expected to be similar to that of a solution containing high TNB concentration. To address this issue I considered the energy dissipation. The change of energy dissipation $D$ was related to the change of frequency $F$, thereby removing the time dependency of the data$^{45}$. The slope in $\Delta D$ versus $\Delta f$ plot indicates different states (conformations) of proteins$^{46}$ (anti-TNT antibody in this study). Results from QCM revealed that 1 $\mu$g/mL TNT ($\Delta f = 26.65$ Hz) induces a frequency change similar to 10 $\mu$g/mL TNB ($\Delta f = 28.85$ Hz). However, the $\Delta D/\Delta f$ ratio of 1 $\mu$g/mL TNT ($\Delta D/\Delta f = 0.0051 \pm 0.0011 \times 10^{-6}$/Hz) was significantly lower than that of TNB ($\Delta D/\Delta f = 0.0111 \pm 0.0007 \times 10^{-6}$/Hz), as easily appreciated comparing the slopes of the $\Delta D$ versus $\Delta f$ plot reported in Figure 3.3, which indicates that TNT can be distinguished from TNB even when they induce similar signals on QCM. In sum, I demonstrated that QCM can be used to effectively distinguish TNT from other molecules with similar chemical structure.

### 3.3.3. Limit of Detection of the QCM based TNT Sensor

The QCM based sensor described here allows detecting TNT with high specificity in solution, and distinguishing it from molecules with similar chemical structure. I next investigated the detection limit (sensitivity) of this sensor by testing solutions of TNT ranging from 0.1 ng/mL to 10 $\mu$g/mL. The extent of antibody displacement was proportional to the concentration of TNT in solution. The data analysis was based on the use of the normalized frequency change $\Delta f_{\text{N}}$ (Equation 3.2), which was calculated by dividing the $\Delta f_{\text{dis}}$ of each compound by $\Delta f_{\text{dis}}$ of a control sample without TNT. Thus the resulting values are always equal or greater than one. The normalized frequency changes of TNT at different concentrations are reported in Figure 3.4(A). The lowest detectable concentration of TNT in this assay was 0.1 ng/mL ($p<0.01$), which is one order of
magnitude lower than what previously determined by ELISA (1 ng/mL), demonstrating the superior properties of a QCM based sensor for applications in the field.

Figure 3.3. $\Delta D$ versus $\Delta f$ plot for TNT at 1 μg/mL (A) and TNB at 10 μg/mL (B). Colored points represent different overtones of frequency in QCM measurement. The slopes are obtained from the trend lines of these points. And the difference in slopes distinguishes TNT from its analogs.
Figure 3.4. (A) Limit of detection of the QCM based TNT sensor. A solution of TNT at concentrations ranging from 0.1 ng/mL to 10 µg/mL was flowed on to the QCM crystal and the normalized frequency change was calculated. The data points reported were obtained at equilibrium from at least three independent experiments. (B) “Quick” slope analysis for rapid detection of TNT. The concentration of TNT in the solutions analyzed ranged from 0.1 ng/mL to 10 µg/mL, and the time interval chosen was 10 minutes. The data points reported were obtained from at least three independent experiments.

3.3.4. **Accelerated Detection using the Rate of Antibody Displacement**

The analysis described above is based on the measurements of the frequency change at equilibrium caused by the displacement of anti-TNT antibody after the addition
of TNT, which usually requires several hours. This time scale is typically not considered practical for rapid on-site detection. Hence, I introduced a new parameter, the “quick” slope \( k \) (Equation 3.3), which allows estimating the dependence of the rate of displacement on the concentration of TNT.

The “quick” slope \( k \) was calculated for solutions of TNT ranging from 0.1 ng/mL to 10 \( \mu \)g/mL, and a time scale of 10 minutes (Figure 3.4(B)). The “quick” slope was observed to increase with the concentration of TNT even at low TNT concentrations (0.1 ng/mL, \( p<0.01 \)). Therefore, the “quick” slope \( k \) can be used to reliably quantify the detection of TNT in solutions of different TNT concentrations (0.1 ng/mL – 10 \( \mu \)g/mL) while considerably decreasing the time of detection (~10 minutes).

### 3.3.5. Detection of TNT in Dirty Environments

The experiments described above were conducted using solutions of TNT in PBS buffer, which is an ideal solvent and hardly recapitulates the conditions of on-site analysis. Thus, in an attempt to evaluate the reliability and robustness of this sensor for use in the field, solutions of TNT crowded with molecules with similar chemical structure, which might interfere with the detection, were tested. Particularly, I used a solution of commercially available fertilizer, which contains nitrogenous compounds with chemical reactivity potentially similar to TNT and TNT analogs, and seawater\(^{35} \), which represents a commonly contaminated environment.

The rate of displacement and normalized frequency change at equilibrium for solutions of TNT in PBS buffer, in fertilizer, and in artificial seawater were found to be comparable (data not shown), demonstrating the robustness of this detection method.
Then the limit of detection of TNT in dirty environments was investigated and compared to PBS (0.1 ng/mL) (Figure 3.5). The normalized frequency changes obtained from the fertilizer solution and seawater were 1.50±0.15 and 1.56±0.16, respectively (p<0.05), which are comparable to the normalized frequency change measured using PBS (1.92±0.27, p<0.01), indicating that the QCM based detection of TNT is not limited by the composition of the solution.

**Figure 3.5.** Detection of TNT in dirty environments. The limit of detection of TNT in PBS (green), fertilizer (blue), and seawater (red) was 0.1 ng/mL. The black line represents the value of control sample (solutions without TNT). The data analysis was conducted as described in the text (Equation 3.2), and data points were obtained from at least three independent experiments.

3.3.6. **Mathematical Modeling and Calculation of the Binding Affinity Constants**

The displacement process can be modeled with a Langmuir isotherm model, which was previously used to describe antigen-antibody interactions on a surface. The
binding affinity constant $K_A$ refers to the relative affinity of the anti-TNT antibody to a specific compound and was determined based on Equation 3.8 (Figure 3.6). The maximum frequency change $\Delta f_{\text{max}}$ caused by antibody displacement and TNT binding affinity constant $K_A$ are $0.73\pm0.05$ and $5.09\pm0.61$, respectively. The positive reaction rate $k_A$ (Equation 3.9, Figure 3.7) was $(5.247\pm0.027)\times10^{-5}$. The data obtained with solutions of TNB and DNT were analyzed in the same fashion and results are reported in Table 3.3. The values of both $\Delta f_{\text{max}}$ and $K_A$ represent the relative affinity of each molecule for the anti-TNT antibody, with the largest value corresponding to the highest affinity. It is important to notice that the relative affinity values calculated in this study refer to the ability of each molecule to displace anti-TNT antibody specifically from TNB. Thus, as shown in Table 3.3, the relative affinity values (TNT>TNB>DNT) are consistent with the experimental results obtained.

Figure 3.6. Calculation of competitive binding affinity $K_A$. $[\text{TNT}]/\Delta f$ is plotted with respect to $[\text{TNT}]$, according to Equation 3.8. Only four data points were used to maximize data fitting. R-squared value for this plot is 0.9977. From this linear relationship, the slope and intercept of this plot can be obtained. And the values of parameter maximal frequency change ($\Delta f_{\text{max}}$) and competitive binding affinity ($K_A$) are calculated.
Figure 3.7. Calculation of positive reaction rate $k_A$. In Equation 3.9, the right part is plotted as a function of time. R-squared value for this plot is 0.9983. The slope represents the value of positive reaction rate ($k_A$).

Table 3.3. Parameters obtained from the mathematical model.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$\Delta f_{\text{max}}$</th>
<th>$K_A$ (mL/μg)</th>
</tr>
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<tr>
<td>DNT</td>
<td>0.24±0.11</td>
<td>0.47±0.14</td>
</tr>
</tbody>
</table>

Known the values of $\Delta f_{\text{max}}$, $K_A$ and $k_A$, the ODE describing the change of $\Delta f$ as a function of time (Equation 3.11) can be analyzed in Matlab (Figure 3.8). The detection of TNT in a solution at 10 μg/mL was simulated and observed to fit the experimental data accurately during the initial phase of displacement. Similar results were obtained for simulated and experimental data using lower TNT concentration (1 μg/mL, data not...
shown). The model was therefore considered acceptable and extended to the analysis of TNB and DNT at high concentrations (Figure 3.9 and 3.10). The mathematical model developed allows establishing a standard curve, which can be used to estimate the concentration of unknown TNT solutions.

Figure 3.8. Mathematical model of TNT (10 μg/mL) detection. The solid line represents the simulated data obtained from Equation 3.11, while the open circles are experimental data points obtained from QCM.
**Figure 3.9.** Mathematical model of TNB (10 μg/mL) detection. The solid line represents the simulated data obtained, while the open squares are experimental data points obtained from QCM.

![Graph showing simulated and experimental data for TNB detection](image)

**Figure 3.10.** Mathematical model of DNT (10 μg/mL) detection. The solid line represents the simulated data obtained, while the open triangles are experimental data points obtained from QCM.

**3.4. Conclusion**

A rapid and accurate QCM based displacement assay for the detection of TNT in liquid phase has been developed, by exploiting the cross reactivity of an anti-TNT antibody (A1.1.1) for TNT analogs. In this study, ELISA was first used to evaluate the displacement assay for the detection of TNT, and calculate the relative affinity of the antibody for TNT and selected TNT analogs and the limit of detection of TNT (1 ng/mL). Results obtained were comparable to previously reported data. The displacement principle was subsequently used to develop a QCM based assay for the detection of TNT.
with higher sensitivity and specificity. The limit of detection obtained was an order of magnitude lower than previously reported (0.1 ng/mL). The robustness of this QCM based TNT sensor was confirmed by evaluating TNT detection in dirty environments, such as fertilizer and seawater, and the limit of detection achieved was comparable to that measured in pure TNT solution. As described in the Introduction, fluorescence spectrophotometry has been widely used for TNT detection. This method, however, exhibits low specificity compared to the displacement assay-based QCM sensor reported herein. It was previously demonstrated that fluorescence spectrophotometry can be used to distinguish TNT from nitrobenzene (NB) and DNT because of higher electron deficiency of TNT \(^{17}\). However, the specificity of this method is likely to be significantly lower when used to detect TNT in a mixture containing molecules with similar electron transfer properties, such as TNB. In summary, the QCM based displacement assay developed in this study allows detecting TNT at a concentration as low as 0.1 ng/mL within 10 minutes, even in dirty environments containing structurally similar molecules. The kinetics of detection can be predicted using the model described, based on which a standard curve can be established and facilitate on-site detection.

The assay described is based on the competition between two immunochemical interactions: the binding TNB-antibody and the binding TNT-antibody. Therefore, the extent of antibody displacement depends on the ratio between the relative binding affinities of TNT and TNB for the antibody. The limit of detection could be further increased by using a TNT analog with lower affinity than TNB as surface competitor. On the other hand, using a competitor with high affinity is likely to increase the specificity of the assay for detection of TNT in dirty environments. The use of protein engineering
technologies for the selection of an anti-TNT antibody with enhanced affinity for TNT or lowered cross-reactivity with TNT analogs would also allow further enhancing the sensitivity and specificity of this detection method.

Similarly to the microcantilever, QCM also proves to be a sensor with excellent performance to characterize the molecular properties on the liquid – solid interface. Finally, I anticipate that the optimized displacement method (coupled with QCM) can be modified for the detection of other molecules of interest (e.g. imazethapyr	extsuperscript{49} or cocaine	extsuperscript{50}), provided that an antibody specific for the molecule of interest, and displaying cross-reactivity for analogous molecules that typically limit its detection, has been isolated or engineered.

3.5. References

Chapter 4

Lipid Bilayer Phase Transformations Detected using Microcantilevers

4.1. Introduction

Artificial models of cell membrane attract increasing attention, because they can be used to study the lipid bilayer, the protective envelope of cell, as well as the interaction between lipid bilayer and other molecules. Among various models, supported lipid bilayers (SLBs) have served as a useful model for complex biological membranes and have led to novel membrane-on-chip devices for biosensor design\textsuperscript{1-3}. Lipid bilayers and monolayers are known to exhibit phase transitions as a function of temperature. The thermally induced solid-liquid transition is particularly important because of its role in membrane permeability and electrical conductivity\textsuperscript{4}. During the solid-liquid phase transition, a supported lipid bilayer or monolayer undergoes a conformational change in
which the lipid acyl chains transition from an ordered state to a disordered state. This process is accompanied by a free energy change, which is coupled to changes in the surface stress in the underlying solid support layer. These surface stress changes can be readily detected using microcantilevers. In this chapter, the phase transformation of 1-myristoyl-2-palmitoyl-sn-glycero-3-phosphocholine (MPPC) is measured on the microcantilever surface.

Attempts to understand the differences between supported and free-standing lipid membranes, and how a solid support interacts with and influences the stability of an SLB, are ongoing\(^5\)\(^{-10}\). One important difference between supported and free-standing membranes is the phase transition temperature \(T_m\). It is believed that the solid-liquid transition of a lipid membrane is a first-order process at \(T_m\)\(^{11\text{-}12}\), where the acyl chains in the lipid molecules are organized into a crystal-like lattice in the solid state and disordered in the liquid phase. However, in an SLB, the two leaflets composing the bilayer face different environments: one leaflet faces an ambient aqueous solution, and the other leaflet faces a rigid solid. This results in membrane asymmetry, which is reported to affect the surface tension, the lipid lateral diffusion coefficient, and the phase transition temperature. Leonenko \textit{et al}\(^\text{13}\) reported that mica-supported SLBs undergo a broader solid-liquid phase transition than do free-standing SLBs and that this transition is accompanied by observable structural changes around the main \(T_m\). Feng \textit{et al}\(^\text{14}\) reported that the solid-liquid phase transition for SLBs is a two-step process, with a primary transition at \(T_m\) and a secondary transition at a temperature 5 °C above \(T_m\). They proposed that the proximal leaflet is stabilized by the ordered thin water film beneath it, resulting in a secondary transition that occurs at a temperature higher than the primary \(T_m\). Oncins \textit{et al}\(^\text{15}\)
confirmed the existence of the secondary \( T_m \) in the proximal leaflet by examining the phase transition of a Langmuir-Blodgett lipid monolayer. Seeger et al\textsuperscript{16, 17} further investigated the factors (ionic strength, incubation temperature and solid support) that influence the SLB solid-liquid phase transition, which switches between a one-step and a two-step process. All of these studies involved applying external forces on the membrane using methods such as force spectroscopy or atomic force microscopy, with their probe tips exerting a force on the soft films during scanning\textsuperscript{16}. Other, less intrusive methods to probe the order/disorder transition of lipids in monolayer, bilayer or vesicle films include vibrational sum frequency spectroscopy (VSFS)\textsuperscript{18-19} and quartz crystal microbalance with dissipation (QCM-D)\textsuperscript{20}.

More recently, microcantilevers have been able to provide a non-invasive method for measuring free energy changes of adsorbates\textsuperscript{21}. Conformational changes in the adsorbed molecular films and temperature-induced melting of dsDNA are readily observed using microcantilevers\textsuperscript{22-24}. The detection principle is based on measuring the mechanical bending of the cantilever as a result of changes in the surfaces stress on the cantilever surface. The source of the surface stress change is a change in surface free energy. Changes in the Gibbs free energy of surface coated films, either physisorbed or chemisorbed on the cantilever surface, are coupled to changes in the surface free energy of the solid surface. These lead to changes in the surface stress, thus resulting in cantilever bending\textsuperscript{25}. Microcantilevers also respond to temperature variations, which enable them to function as temperature or heat flow sensors\textsuperscript{26}. In this study, I use microcantilevers to probe the solid-liquid phase transition temperature \( T_m \) of phospholipid bilayers and monolayers supported on a silicon dioxide surface. I also report how the
microcantilevers can be used to examine the effect of cholesterol, which is known to regulate the physical properties of the lipid membrane. A model considering the bimetallic bending of microcantilevers was developed to characterize the lipid properties in the solid and liquid phases and to interpret the phase transition of lipid bilayers in terms of thermal expansion coefficients. The recent success of microcantilevers over other biosensing techniques is due to their ability to sensitively measure surface stress changes associated with liquid-solid interfacial behavior. Furthermore, microcantilevers have the advantage of being a sensitive, real-time, and label-free technique.

4.2. Experimental Techniques

1-myristoyl-2-palmitoyl-sn-glycero-3-phosphocholine (MPPC) (Avanti Polar Lipids, Alabaster, AL, USA) and cholesterol (Sigma Aldrich, USA) were used as received. MPPC was chosen because its transition temperature ($T_m$~35 °C) is within the working temperature range of the equipment. The inner (surface-facing) and outer (solution-facing) leaflets in MPPC bilayers have different structures, leading to an asymmetrical lipid bilayer structure\textsuperscript{27-28}. Lipid vesicles were prepared by the standard extrusion method and were kept in a hot water bath at least 15 °C above their phase transition temperature at all times\textsuperscript{29}. Briefly, the lipid was dissolved in 0.25 ml of chloroform at a concentration of 5 mg/ml in a glass vial. For lipid-cholesterol mixtures, a specific amount of cholesterol was added to the chloroform solution. The chloroform was evaporated and dried under a gentle ultra-pure nitrogen stream. The resulting lipid film was desiccated in a vacuum chamber for at least two hours and then hydrated in 0.25 ml of a pH 7.4 phosphate-buffered saline (PBS) buffer solution (Sigma-Aldrich, USA) at 50 °C, followed by
vortexing the solution. The solution was then extruded 40 times through a polycarbonate membrane with 100 nm pore-size using a mini-extruder (Avanti Polar Lipids), resulting in a translucent solution of large unilamellar vesicles (LUVs) approximately 100 nm in diameter in PBS. The vesicle solution was further diluted with nine parts of PBS to 1 part of the freshly extruded vesicle solution and stored in a warm water bath at 50 °C. The estimated lipid concentration was 0.5 mg/ml.

The gold surface of the microcantilever was coated with a dithiolaromatic-PEG (C_{25}H_{44}O_{6}S_{2}, MW=504.74 g/mol, Sensopath Technologies, Bozeman, MT, USA) monolayer to prevent vesicle binding. The surface functionalization typically proceeded for two hours. To generate a reference cantilever, a PEG-silane, 2 - [methoxy-(polyethyleneoxy)propyl] - trimethoxysilane (C_{13}H_{30}O_{7}Si, MW=326.46 g/mol, Gelest Inc. Morrisville, PA, USA), was used to prevent vesicles from adsorbing onto the silicon dioxide surface of the cantilever. The cantilever deflection is a result of the changes in the surface free energy associated with physical or chemical adsorption of molecules to cantilever surface. Preferential molecular adsorption on either side of cantilever surfaces induces a mismatch in the surface stress between the two surfaces of the cantilever, causing the cantilever to bend.30

SLBs were formed on the silicon dioxide surfaces of microcantilevers through vesicle fusion at 10 °C above the primary T_m. A solution of MPPC vesicles was injected at 0.42 µl/sec into the measurement chamber to form the SLB on the silicon dioxide surface of the microcantilever. The supported lipid monolayer was prepared outside the measurement chamber. A lipid monolayer was deposited on the silicon dioxide surface using the Langmuir-Blodgett transfer method (KSV 2000 series, KSV instruments Ltd.,
The cantilever was first pre-functionalized with dithiolaromatic-PEG to prevent lipid adsorption to the gold surface. The cantilever was then held on a Teflon clip and immersed in a DI water-filled Langmuir trough. Lipid molecules were dispersed onto the air-water interface, with the total surface area and surface pressure controlled by two barriers. The lipid monolayer was transferred onto the cantilever at room temperature and a surface pressure of 30 mN/m (surface area of 50 Å² per MPPC molecule). The monolayer was dried in a desiccation chamber, and cantilever measurements were performed within 20 minutes in PBS. Phase transition curves were acquired by slowly ramping the temperature from 45 °C to 31 °C for the bilayer or from 31 °C to 45 °C for the monolayer at a rate of ± 0.8 °C/min in PBS buffer.

4.3. Thermal Expansion Coefficient Analysis

The solid-liquid phase transition of lipid bilayers is studied by altering the temperature. The mechanism of microcantilever bending with changing temperature is based on the bimetallic effect, i.e., the difference in the thermal expansion between the gold film and a silicon substrate. The deflection-versus-temperature relation is derived as follows:

\[
\Delta z = 3(\alpha_1 - \alpha_2)L^2 \frac{t_1 + t_2}{t_2^2} \kappa \Delta T
\]  

\[
K = 4 + 6 \left( \frac{t_1}{t_2} \right) + 4 \left( \frac{t_1}{t_2} \right)^2 + \frac{E_1}{E_2} \left( \frac{t_1}{t_2} \right)^3 + \frac{E_2}{E_1} \left( \frac{t_2}{t_1} \right)
\]

where \(\Delta z\) is the change in vertical deflection of cantilever, \(t\) is the thickness of the layer, \(\alpha\) is the thermal expansion coefficient, \(E\) is the Young’s modulus, \(L\) is the cantilever
length, and \( \Delta T \) is the temperature change. The two layers made of different materials are referred to by the subscripts 1 and 2. The values of the parameters for the gold and silicon layers are listed in Table 4.1. The thermosensitivity is defined as the deflection per unit change in temperature. From Equation 4.1, the thermosensitivity of the cantilever used is 79.95 nm/K, which agrees very well with the experimental result (80.6 ± 6.3 nm/K), demonstrating the accuracy of Equation 4.1 in describing the bimetallic effect.

**Table 4.1.** Physical properties of microcantilever layers: gold, silicon, and lipid bilayer.

<table>
<thead>
<tr>
<th>Layer in cantilever</th>
<th>Thickness ( t ), nm</th>
<th>Young’s modulus ( E ), MPa</th>
<th>Thermal expansion coefficient ( \alpha ), K(^{-1} )</th>
<th>Relative deflection per T unit ( \Delta z_{\text{Si-SLB}} / \Delta T ), nm/K</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gold</td>
<td>20</td>
<td>7.9( \times 10^4 )</td>
<td>1.42( \times 10^{-5} )</td>
<td>N/A</td>
</tr>
<tr>
<td>Silicon</td>
<td>1( \times 10^3 )</td>
<td>1.69( \times 10^5 )</td>
<td>2.59( \times 10^{-6} )</td>
<td>N/A</td>
</tr>
<tr>
<td>Lipid bilayer, Solid phase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 mol% chol</td>
<td>4.7( \text{b}) 4.7( \text{b})</td>
<td>28.1( \text{b}) 28.1( \text{b})</td>
<td>0.0068\pm0.0008( \text{a}) 0.0068\pm0.0008( \text{a})</td>
<td>4.0\pm0.5( \text{a}) 4.0\pm0.5( \text{a})</td>
</tr>
<tr>
<td>2 mol% chol</td>
<td>4.72( \text{b}) 35-36</td>
<td>28.19( \text{b}) 28.19( \text{b})</td>
<td>0.0031\pm0.0009( \text{a}) 0.0031\pm0.0009( \text{a})</td>
<td>1.8\pm0.6( \text{a}) 1.8\pm0.6( \text{a})</td>
</tr>
<tr>
<td>5 mol% chol</td>
<td>4.76( \text{b}) 35-36</td>
<td>28.32( \text{b}) 28.32( \text{b})</td>
<td>0.0009\pm0.0006( \text{a}) 0.0009\pm0.0006( \text{a})</td>
<td>0.6\pm0.4( \text{a}) 0.6\pm0.4( \text{a})</td>
</tr>
<tr>
<td>Lipid bilayer, Liquid phase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 mol% chol</td>
<td>3.6( \text{b}) 35-36</td>
<td>19.3( \text{b}) 19.3( \text{b})</td>
<td>0.0236\pm0.0029( \text{a}) 0.0236\pm0.0029( \text{a})</td>
<td>7.7\pm1.2( \text{a}) 7.7\pm1.2( \text{a})</td>
</tr>
<tr>
<td>2 mol% chol</td>
<td>3.64( \text{b}) 35-36</td>
<td>19.36( \text{b}) 19.36( \text{b})</td>
<td>0.0187\pm0.0018( \text{a}) 0.0187\pm0.0018( \text{a})</td>
<td>5.9\pm0.6( \text{a}) 5.9\pm0.6( \text{a})</td>
</tr>
<tr>
<td>5 mol% chol</td>
<td>3.70( \text{b}) 35-36</td>
<td>19.54( \text{b}) 19.54( \text{b})</td>
<td>0.0135\pm0.0025( \text{a}) 0.0135\pm0.0025( \text{a})</td>
<td>4.4\pm0.8( \text{a}) 4.4\pm0.8( \text{a})</td>
</tr>
</tbody>
</table>

\( \text{a}\) Calculated values: average ± standard deviation, obtained from at least three independent experiments.

\( \text{b}\) Estimated values from the literature with references below values, considering both lipid phase and cholesterol content.
To include a lipid bilayer film on the silicon side, the total deflection of the cantilever, $\Delta z_{Au-Si-SLB}$, is divided into two parts: the deflection due to the bimetallic effect between gold and silicon, $\Delta z_{Au-Si}$, and the relative deflection due to the bimetallic effect between silicon and the lipid bilayer, $\Delta z_{Si-SLB}$. This assumption relies on the fact that the thickness of either the gold layer or the lipid bilayer is much smaller than that of the silicon substrate. The relative deflection due to the bimetallic effect between silicon and the lipid bilayer $\Delta z_{Si-SLB}$ is obtained by subtracting the deflection of the reference cantilever $\Delta z_{Au-Si}$ (blank experiment) from the deflection of the lipid bilayer-coated cantilever $\Delta z_{Au-Si-SLB}$. The thermal expansion coefficient $\alpha$ of lipid bilayers can be calculated using Equation 4.2.

$$\alpha_1 = \frac{t_2^2 K}{3(t_1 + t_2)L^2} \frac{\Delta z_{Si-SLB}}{\Delta T} + \alpha_2 = \frac{t_2^2 K}{3(t_1 + t_2)L^2} \frac{(\Delta z_{Au-Si-SLB} - \Delta z_{Au-Si})}{\Delta T} + \alpha_2$$  (4.2)

4.4. Results and Discussion

4.4.1. Solid-Liquid Phase Transition of Supported Lipid Bilayers and Monolayers

The equilibrium deflection of microcantilevers upon the formation of an MPPC SLB with varying cholesterol concentrations at 45 °C is shown in Figure 4.1. The vesicle solution flows through the measurement chamber from 240 sec to 720 sec. As vesicles fuse onto the SiO$_2$ surface and rupture to form a planar lipid bilayer, a compressive surface stress is induced, causing the cantilever to bend toward the gold side. The free energy of adsorption is transferred to the solid as a surface free energy at the solid-water-lipid interface$^{25}$. This surface free energy disturbs the atoms of the solid surface, leading to a surface stress in the solid that deforms the material laterally. Due to a surface stress
mismatch between bulk and surface, the cantilever bends toward the gold surface. After switching back to a vesicle-free buffer, the microcantilever remains deflected, confirming that a stable SLB has formed. As the cholesterol percentage increases, the equilibrium deflection value is reduced, corresponding to a decrease in surface stress that is consistent with previously reported data. 

![Image](image-url)

**Figure 4.1.** Real-time deflection measurement of the cantilever as an MPPC supported lipid bilayer with 0 mol% (solid lines), 2 mol% (dot-dash lines), and 5 mol% cholesterol (dash lines) forms on the SiO$_2$ surface of the cantilever at 45 °C. Arrow (i) indicates the injection of MPPC vesicles into the measurement chamber. SLB forms on SiO$_2$, causing a deflection toward the gold side. Arrow (ii) indicates that the flow is switched from vesicle solution to PBS buffer. Three separate cantilevers on the same array are shown to confirm the formation of SLBs under each condition. For the solid lines, one cantilever (black curve) responds prior to other two (gray curves) because the vesicles reach it earlier in the measurement chamber. 

After the formation of the pure MPPC SLB, the temperature is lowered from 45 °C to 31 °C, and the resulting cantilever responses are shown in Figure 4.2(A). Due to
the mismatch in the thermal expansion coefficients between gold and silicon, the reference cantilever (blank) deflection decreases linearly with increasing temperature. For lipid bilayer coated cantilevers, a discontinuity in the linear bending profile is observed at ~35 °C. This discontinuity, an abrupt step of ~20 nm, can be attributed to the solid-liquid phase transition of the lipid bilayer. By plotting the derivative of the deflection with respect to temperature (d(Deflection)/dT), as in Figure 4.2(B), a distinct peak can be observed that corresponds to the phase transition temperature, Tm. The Tm value for the MPPC SLB measured with microcantilevers (at 34.9 ± 0.1 °C) agrees well with the value reported for MPPC lipids in the literature (35 °C)40. This value corresponds to the temperature where the fluid phospholipid membrane undergoes a liquid-disordered to solid-ordered phase transition. An additional compressive surface stress of ~4.7 mN/m is exerted on the solid support at Tm. It is well known that this phase transition is an exothermic process that results in an abrupt rise in conformational order and reduced mobility of the lipid molecules41. A single phase transition is observed for a SiO2 supported MPPC bilayer compared with a two-step phase transition previously observed for a mica-supported lipid bilayer14,15. The difference in surface roughness between silicon dioxide and mica may change the interactions between the SLB and the solid support17. AFM studies report that the solid support significantly broadens the transition temperature range13, but as shown in Figure 4.2(A), the phase transition is relatively narrow when measured using microcantilevers.
Figure 4.2. Phase transition of a pure MPPC supported lipid bilayer. (A) Deflection of microcantilevers vs. temperature. Inset figure provides finer resolution between 34 °C and 36 °C. (B) The derivative of the deflection with respect to temperature is shown. The $T_m$ value can be determined from the position of the peaks. The derivative plots are offset for clarity. The blank in both A and B represents the reference cantilever without coating of lipid bilayers. Different colored lines represent different cantilevers on a single sensor chip.
I compare the supported MPPC bilayer phase transition with that of a supported MPPC monolayer. The MPPC monolayer prepared on the SiO$_2$ surface of microcantilevers using the Langmuir-Blodgett transfer method is initially kept at 31 °C. As the temperature is slowly increased to 45 °C, there is a change in the slope of the deflection curve at ~41 °C, as shown in Figure 4.3(A). In contrast to the MPPC SLB, the monolayer phase transition results in a slope change rather than an abrupt step. The gradual change of the cantilever deflection suggests a broader phase transition of the lipid monolayer due to its interaction with the solid support$^{13}$. The plot of d(Deflection)/dT (Figure 4.3(B)) shows a clear change at 40.8 ± 0.10 °C for all monolayer coated cantilevers, which is ~6 °C above the bilayer T$_m$. This corresponds to the secondary phase transition temperature for the lower leaflet observed in mica supported bilayers, which is due to the decoupling of the two leaflets$^{15}$. The slope of the deflection with respect to temperature increases by 0.3 ± 0.1 nm/°C when going from the ordered to the disordered phase. The increased T$_m$ for the monolayer is related to the interactions of an MPPC monolayer with the solid support. The slope change for the MPPC monolayer is smaller than that observed for the MPPC bilayer. Because of the interleaflet coupling of the SLB, the monolayer appears to adhere more rigidly to the silicon dioxide surface. This is likely due to the strong electrostatic attraction of the polar lipid headgroups (positively charged choline groups) to the negatively charged silicon dioxide surface$^{18}$ and to the crystalline-like water beneath the lipid layer$^{14}$. Thus, T$_m$ shifts to a higher temperature, from 34.9 ± 0.1 °C to 40.8 ± 0.10 °C.
Figure 4.3. Phase transition of pure MPPC supported lipid (Langmuir-Blodgett) monolayer. (A) Deflection of microcantilevers vs. temperature. Inset figure provides finer resolution between 40 °C to 42 °C. (B) The derivative of the deflection with respect to temperature is shown. The derivative plots are offset for clarity. The blank in both A and B represents the reference cantilever without coating of lipid bilayers. Different colored lines represent different cantilevers on a single sensor chip.
4.4.2. Effects of Cholesterol on the Phase Behavior of an MPPC Supported Lipid Bilayer

Cholesterol alters the physical properties of a lipid bilayer, including the phase transition behavior. The effect of cholesterol on the lipid phase transition is largely related to the symmetry of the two acyl chains. The heat-capacity curves of the symmetric lipids are normally resolved into a sharp and a broad component for analysis\textsuperscript{42}, while the phase transitions of bilayers containing asymmetric (chain mismatched) lipids are simpler and the effects of cholesterol more obvious\textsuperscript{43}. Thus, MPPC is used here to probe the effect of cholesterol on the phase transition measured with microcantilevers. After the formation of MPPC SLBs in the presence of cholesterol, the temperature is decreased, and the abrupt step size, as well as additional compressive surface stress, is obtained from the discontinuity in the linear cantilever responses at $T_m$ (Table 4.2). Both the size of the abrupt step and the surface stress are reduced by the addition of cholesterol. Thus, the phase transition of lipid bilayers depends upon the concentration of cholesterol\textsuperscript{43-45}.

<table>
<thead>
<tr>
<th>Cholesterol content, mol%</th>
<th>Abrupt step, nm\textsuperscript{a}</th>
<th>Surface stress, mN/m\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>22.4±2.1</td>
<td>5.2±0.5</td>
</tr>
<tr>
<td>2</td>
<td>13.3±1.5</td>
<td>3.1±0.4</td>
</tr>
<tr>
<td>5</td>
<td>7.1±1.2</td>
<td>1.7±0.3</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Average value ± standard deviation, obtained from at least three independent experiments.
The phase transition temperatures of SLBs containing 0-5 mol% cholesterol in MPPC are shown in Figure 4.4. Similar to Figure 4.2(B), the phase transition temperature $T_m$ is determined by the position of the peak in the plot of $d(\text{Deflection})/dT$ versus temperature. $T_m$ decreases with increasing cholesterol content at a rate of $\sim 0.38 \, ^\circ\text{C}$ per mol%. The reduced $T_m$ may be explained by the local disordering effects in the lipid solid phase caused by the bulky tetracyclic ring of cholesterol\textsuperscript{43}. Moreover, increasing cholesterol content leads to a smaller change in surface stress, as shown in Table 4.2. As described earlier, changes in surface stress are readily detectable in the coupled lipid leaflets of the SLB. Increasing the cholesterol content stiffens the membrane, which makes it more difficult to detect changes in surface stress. The peak in the $d(\text{Deflection})/dT$ plot disappears at 10 mol% cholesterol, indicating the complete removal of the lipid phase transition. Higher cholesterol content results in less deflection of the microcantilevers, as shown in Figure 4.1. Thus, the surface stress of the microcantilevers is smaller as a result of the smaller free energy changes due to SLB adsorption\textsuperscript{39}, which indicates that the interactions among lipid molecules also become smaller. Therefore, less energy needed for the phase transition, and the transition temperature is lower. In short, the increasing cholesterol content induces a decrease in the microcantilevers’ deflection via lipid physisorption, corresponding to a reduced “melting” energy, so the transition temperature $T_m$ is also decreased.
Figure 4.4. (A) Phase transition temperatures of MPPC supported lipid bilayers in the presence of cholesterol. (B) The phase transition temperature, $T_m$, is determined by the position of the peak in the $d$(Deflection)/$dT$ plot at the corresponding cholesterol content (from left to right: 0%, 2%, 5% cholesterol). The derivative plots are offset for clarity. The data points reported were obtained from at least three independent experiments.

4.4.3. Physical Interpretation of the Temperature-induced Phase Transition in Microcantilevers using Thermal Expansion Coefficients

To understand the mechanism by which cholesterol alters the membrane’s phase transition, I consider the bimetallic effect of the microcantilevers. The difference in
deflection between the coated and reference cantilevers \((\Delta z_{\text{Au-Si-SLB}} - \Delta z_{\text{Au-Si}})\) represents the effect of lipid bilayers on cantilever bending. The relative change in cantilever deflection per unit temperature, \(\Delta z_{\text{Si-SLB}}/\Delta T\), is then used to calculate the thermal expansion coefficient \(\alpha\) (Equation 4.2), which has been reported for different lipid phases\(^{46}\). The change in thermal expansion coefficient with respect to temperature has been reported to be small in the absence of a phase transition \((\sim 5 \times 10^{-6} \, \text{K}^{-1})\)\(^{47}\) and was thus considered to be constant within the temperature range considered in the calculation \((\leq 7 \, \text{K})\). Likewise, for a particular phase, the thickness \(t\)\(^{36}\) and Young’s modulus \(E\)\(^{38}\) are also assumed to be constant with respect to temperature. The values of these parameters are estimated from the literature and are reported in Table 4.1. The SLB can be considered as an elastic thin film and Young’s modulus to be the elastic modulus parallel to the SLB plane that is related to the lateral elasticity of the bilayer. Although I hypothesize that the temperature does not significantly affect the thickness \(t\) and Young’s modulus \(E\) of lipid bilayer outside the phase transition, the phase of lipids and the content of cholesterol do affect these values. To improve the accuracy and reliability of the calculations, both lipid phase and cholesterol content are considered in the estimation of lipid thickness and Young’s modulus (Table 4.1). Using the relative deflection per unit temperature, together with the estimated parameters, the thermal expansion coefficients \(\alpha\) of lipid bilayers under different conditions are calculated and shown in Figure 4.5. The values of \(\alpha\) here compare well with previously reported measurements\(^{36}\). However, they are relatively large compared to other measurements\(^{48-49}\) because \(\alpha\) increases significantly at temperatures near a phase transition\(^{36}\).
Figure 4.5. The cholesterol dependence of the thermal expansion coefficient $\alpha$ of lipid bilayers in both liquid (red circles) and solid (blue squares) phases. The values are all calculated from Equation 4.2. The data points reported were obtained from at least three independent experiments.

Two conclusions are evident from Figure 4.5. First, the thermal expansion coefficient $\alpha$ of lipid bilayers is larger in liquid phase than in solid phase, reminiscent of the change in thermal expansion coefficient $\alpha$ reported previously or after the main phase transition\textsuperscript{46, 50}. Apparently, the lipids in the solid phase are more rigid, so they are less able to expand. Moreover, the small value of $\alpha$ in the solid phase is explained by the combined effects of chain packing and chain tilting\textsuperscript{51}. Briefly, the lipids in the solid phase are ordered and tilted, and the increase in temperature reduces both the ordering and tilting of the lipids. The reduced chain tilting thus offsets the expansion of the chain lattice, and $\alpha$ becomes much smaller. In contrast, there is no tilting effect in the liquid phase, so the thermal expansion coefficient is higher\textsuperscript{48}. Second, the thermal expansion
coefficient $\alpha$ decreases with increasing cholesterol content in both the liquid and solid phases, which is similar to the reported trends$^{36, 50}$. The reason might be that the incorporation of cholesterol fills in the free space among lipids and decreases the flexibility of the lipids, so the thermal expansion coefficient is reduced. Detection of the phase transition on the cantilever is attenuated at higher cholesterol content and disappears at 10% cholesterol content. This is related to the stiffening and reduction in $\alpha$ with increasing cholesterol content. The comparison of values in Table 4.1 further illustrates that the variation of thermal expansion coefficient with cholesterol is more apparent than the variation of other parameters (e.g., thickness and Young’s modulus). In addition, the thermal expansion coefficient of the carbon chain has been reported not to vary significantly with temperature$^{52}$, suggesting that the change in $\alpha$ results from changes in the lipid interactions between phases. Therefore, thermal expansion coefficient can be used to characterize lipid phase behaviors. Some of the parameters used in the calculations are from data involving lipid vesicles, which may differ from SLBs. More precise values of SLB properties (e.g., thickness and Young’s modulus) under various conditions will lead to more accurate results.

4.5. Conclusion

In summary, the microcantilever has been successfully used to probe the solid-liquid phase transitions of supported lipid bilayers and monolayers. The phase transition temperature $T_m$ can be accurately measured for the MPPC SLB, and the $T_m$ for a MPPC supported lipid monolayer can be successfully detected at $\sim$6 °C above the bilayer $T_m$. Cholesterol is found to decrease the phase transition temperature at a rate of $\sim$0.38 °C per
mol%. By considering the bimetallic effect in the microcantilever, the thermal expansion coefficients $\alpha$ of lipid bilayers under various conditions are obtained and used to explain the cantilever deflection. This coefficient is found to be smaller in the solid phase than in the liquid phase and decreases with increasing concentrations of cholesterol. Therefore, the stiffening effect of cholesterol on lipid membranes is reflected in the thermal expansion coefficient $\alpha$, suggesting that $\alpha$ can describe the physical structure of lipid bilayer. Generally speaking, treating the SLB as a thin film is a reasonable way to characterize the bilayer. Besides the detection of molecular interaction, microcantilever has been shown to accurately probe the conformational changes of macromolecular assemblies confined on solid surfaces. I offer this microcantilever-based technique as a promising tool for future studies on the conformational changes of other molecules.

4.6. References


Chapter 5

Investigation of the Interaction between Amphipathic α-Helical Peptides and Supported Lipid Membranes using Microcantilevers

5.1. Introduction

In addition to the temperature induced phase transition, the SLB coated microcantilever sensor is also capable of characterizing the membrane interactions with membrane-active molecules, such as amphipathic peptides. Understanding peptide-membrane interactions is important in elucidating biomolecular mechanisms, such as
membrane fusion, cell signaling, and therapeutic approach of antibiotic and antiviral drugs. Membrane-active peptides are proteins that are known for their association with lipid membranes. They interact predominantly with the hydrocarbon region of the lipid membrane, the polar headgroup region, or both regions of the bilayer, depending on the peptide’s hydropathicity. An amphipathic helix (AH) peptide that is of great interest is the AH segment derived from the N-terminal end of the Hepatitis C Virus’s nonstructural protein NS5A. The Hepatitis C Virus (HCV) infects more than 170 million people worldwide and those who are exposed risk chronic liver disease;¹ ² therefore understanding the infectious mechanism of HCV is of great need.

The association of the NS5A-derived AH peptide with lipid membranes is an essential step in the HCV viral infection. The AH viral attack is able to induce lysis of lipid membranes. Blocking the membrane binding pathway of the AH is a promising therapeutic strategy.³ Cho et al. and Chah et al. investigated the binding mechanism of this AH peptide interacting with model lipid membranes, either lipid vesicles (SUVs or LUVs) or supported lipid membranes (SLBs), with analytical tools, such as quartz crystal microbalance with dissipation (QCM-D), dynamic light scattering (DLS), reflectometry and atomic force microscopy (AFM).⁴ ⁸ The NS5A-derived AH peptide is thought to bind parallel to the lipid membrane surface and penetrate into the lipid membrane, leading to expansion of the outer leaflet. Cho’s AFM images showed that sequential introduction of AH onto the SLBs causes a membrane thinning at AH concentration ranging from 1.63 to 3.25 µM⁶, which indicates that the membrane laterally expands, according to Huang’s geometric model⁹. In addition to insights into the binding mechanisms, the virus-mimetic attack of NS5A-derived AH for rupturing lipid membranes provides a novel SLB
patterning approach onto surfaces that do not easily rupture vesicles, such as gold and TiO$_2$ surfaces, which are known for difficulties in forming SLBs spontaneously without external forces.$^8,^{10}$

Instead of laboriously purifying this AH segment of the NS5A protein from HCVs, I use a synthetic analogue, PEP1 (synthesized by AnaSpec, CA). Similar to the natural NS5A-derived AH, PEP1 exhibits typical amphipathic characteristics by partially penetrating the lipid bilayer and interacting with both polar hydrophilic lipid headgroups and hydrophobic tails. Additionally, the PEP1 peptide displays the potent antiviral activity against certain viruses.$^{11}$ PEP1 is found to destabilize viral membranes and lyse virions.$^2$ Several binding and disruption processes have been proposed empirically, however a physical understanding is still lacking.$^6$ Compared to PEP1, other well characterized amphipathic helical peptides belong to the family of antibacterial peptides, such as melittin and magainin.$^9,^{12-15}$ They are known for adsorbing onto lipid membranes and reorienting to form transmembrane pores, leading to higher membrane permeability or even disruption. I refer to theories and models developed for these amphiphilic peptides to explain the membrane binding and possible pore formation of PEP1. More specifically, I am interested in extracting the membrane lateral expansion caused by the PEP1, because it is an indicator of foreign molecules insertion into the membrane.

Here I describe how membrane lateral expansion caused by peptide adsorption and pore formation can be detected using the microcantilevers. Previously it has been shown that microcantilevers are useful in sensing peptide binding and reconstruction on surfaces; for example, the amyloid fibrils of insulin grow in the in-plane direction and generate a tensile surface stress of 20 mN/m within two hours on microcantilever
surfaces. Ghatkesar et al. observed melittin molecules binding to lipid vesicle membranes deposited on gold surfaces of the microcantilevers. These findings open up possible techniques to monitor protein aggregations using microcantilevers; however, there have not been a quantitative explanation of how the surface stress can be related to changes in the surface-bound proteins. This study establishes a method for quantifying the peptide-induced mechanical response of the microcantilevers.

A systematic characterization of the SLBs adsorption on the microcantilever surface and analysis of the electrostatic and hydrophobic contributions that take parts in the changes in adsorption free energy have been described previously. In addition, the insertion and solubilization effects of amphiphilic surfactants on SLBs were detected using the lipid membrane-coated microcantilevers. In particular, herein is a practical application of the previously developed lipid membrane-coated cantilever sensors in biomolecular recognition at model membrane interfaces. This study focuses on the nonspecific α-helix induced peptide interaction with a supported lipid bilayer. The lipid membrane-coated microcantilever has the potential to probe membrane motions in the lateral directions, thus adding more information other than thickness and bound mass that are studied with ellipsometry or quartz crystal microbalance. I will unravel the concentration dependent interactions between amphipathic PEP1s and phospholipid membranes. The response of the lipid membrane-coated cantilevers is analyzed based on the free energy change of this interaction between model membranes and peptides.
5.2. Materials and methods

5.2.1. Lipid Vesicle Preparation

A zwitterionic lipid, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), was used to form a neutral lipid bilayer. The lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Vesicles were prepared by the extrusion method. Briefly, lipids were dissolved at 5 mg/mL in chloroform. The chloroform was evaporated under a nitrogen stream. The resulting lipid film was then dried in a vacuum chamber for 2 h and then hydrated in a solution composed of 0.5:99.5 (v%) of DMSO and 0.25 mL pH 7.4 phosphate buffered saline (PBS, Sigma-Aldrich) solutions. The solution was then extruded 40 times through a 100 nm polycarbonate membrane using a minieextruder, resulting in a translucent solution of large unilamellar vesicles (LUVs) approximately 100 nm in size. The vesicle solution was further diluted with 9 parts of PBS to 1 part of the freshly extruded vesicle solution and stored at 4 °C until use. Note that the final vesicle concentration may be lower than initially desired due to lipid loss on filter membranes after extrusion; however, the concentration was well above the threshold needed to achieve full surface coverage of the SLB.

5.2.2 Amphipathic Helix Peptide Preparation

The peptide, PEP1, was synthesized by AnaSpec (San Jose, CA). PEP1 has 31 amino acids (MW= 3804.3 g/mol), with the sequence: H–Ser–Gly–Ser–Trp–Leu–Arg–Asp–Val–Trp–Asp–Trp–Ile–Cys–Thr–Val–Leu–Thr–Asp–Phe–Lys–Thr–Trp–Leu–Gln–Ser–Lys–Leu–Asp–Tyr– Lys–Asp–NH₂. It was used as delivered without further purification. The peptide was dissolved in a small amount of dimethyl sulfoxide (DMSO,
(CH₃)₂SO, MW=78.14 g/mol, *EMD Chemicals*, USA) and then slowly diluted in phosphate buffered saline (PBS, *Sigma-Aldrich*, USA). The final peptide solution was prepared in an eluent mixture of DMSO and PBS (0.5:99.5 v%). The peptide solution was kept refrigerated at 4 °C no more than three days prior the experiments. PEP1 is an amphipathic helix peptide that forms a helix structure when adsorbed onto lipid membranes. The helical wheel diagram²²-²³ that illustrates the hydrophilic and hydrophobic surfaces on PEP1 is shown in the appendix.

5.2.3. Preparation of Microcantilever Surface

Each microcantilever chip contains eight rectangular silicon cantilevers, each with a spring constant of 0.026 N/m. To prevent binding preferentially to the gold side of the cantilever beam, polyethylene glycol (PEG) polymers were used. A dithiolaromatic–PEG molecule (C₂₅H₄₄O₆S₂, MW=504.74 g/mol, *Sensopath Technologies*, Bozeman, MT) was used to prevent vesicle adsorption to the gold surface of the cantilever. A blocking agent, bovine casein (*Alfa Aesar*, MA) solution, was used to prevent lipid bilayer and peptide adsorption on the silicon dioxide surface of the cantilever. Aqueous solution saturated with casein was filtered with a syringe filter of 0.22 μm pore size to remove undissolved protein aggregates. A reference cantilever was functionalized with both the dithiolaromatic–PEG and casein. The microcantilever will be used to probe the interactions between the peptide and the lipid membrane, as illustrated in Figure 5.1.
Figure 5.1. Schematic of the use of the lipid membrane–coated microcantilever for sensing the peptide adsorption and insertion. These interactions generate compressive stress on the microcantilevers and lead the microcantilever to bend toward the gold side.

5.2.4. Microcantilever Assay to Measure Peptide-Lipid Interactions

The cantilever deflection is a result of the changes in the surface free energy associated with physisorption or chemisorption of molecules to cantilever surface. Preferential molecular adsorption on either side of cantilever surfaces induces a mismatch in the surface stress between the front surface and back surfaces of the cantilever, causing the cantilever to bend. The relationship between cantilever deflection, $\Delta z$ (m), and the change in surface stress, $\Delta \sigma$ (N/m), is described by Stoney’s equation\textsuperscript{24}:

$$\Delta \sigma = \frac{Et^2}{3(1-\nu)L^2} \Delta z$$ \hspace{1cm} (5.1)

where $\nu$ is Poisson’s ratio of the cantilever material, $E$ is the Young’s modulus, $L$ is the cantilever length, and $t$ is the cantilever thickness. A solution of POPC vesicles was
injected at 0.42 µL/sec at 25 °C into the measurement chamber to form the SLB on the silicon dioxide surface of the microcantilever. PEP1 peptide solution in PBS at various concentrations was then injected to study peptide interaction with SLB. Small variations in the material properties of the cantilevers, such as stiffness or thickness of the gold layer, result in different signals. Thus the deflection of the microcantilevers was normalized by each cantilever’s thermo-mechanical sensitivity, which uses the change in deflection due to a 1 °C change in temperature. Each experiment was repeated at least three times on either the same or different chips, with a minimum of five cantilevers per chip.

5.3. Results

5.3.1. Lipid Deposition onto Functionalized Microcantilever Surfaces

Upon injection of the POPC vesicle solution into the flow chamber, the cantilevers undergo a surface stress change and deflect. Because the gold surface was previously made inert by dithiolaromatic-PEG, the vesicles are attracted only by the negatively charged SiO₂ surface of the microcantilevers and fuse onto the SiO₂ surface to form SLBs. This surface stress change is compressive, leading to the cantilevers bending away from membrane coated surface. The microcantilever bends to a deflection of 73 ± 6.0 nm. Thus, the measured surface stress, \( \Delta \sigma_{\text{ads,SLB}} \), for SLB formation on SiO₂ is 17 ± 1.4 mN/m. Note that the vesicle solution flowed past the cantilever for 7 min (as indicated with shaded area in Figure 5.2), after which, the valve was switched back to the PBS buffer port. Since the bilayer’s adsorption is irreversible over this time scale, the cantilevers remain deflected. The SLBs are stable for at least two hours of observation.
time and do not desorb after the solution is switched to buffer. A control cantilever (dashed line in Figure 5.2), in which both the gold and silicon surfaces are blocked with PEG and casein, respectively, shows minimum surface stress change upon vesicle addition.

**Figure 5.2.** PEP1s at a concentration of 5 µM insert into POPC SLBs. Experimental cantilevers (solid lines) on the same chip are shown. The SiO$_2$ surface of the reference cantilever (dashed line) is blocked by casein, thus no lipid membrane adsorbs on it. The shaded areas indicate the time when lipid vesicles (blue) or peptides (green) are introduced into the measurement chamber. Note that after injecting the PEP1 solution, there is a slight surface stress change on the reference cantilever, caused by a minute PEP1 adsorption on casein.

5.3.2. **PEP1 binds to POPC SLBs at Low Concentration**

After the SLBs are prepared on the SiO$_2$ surfaces of the microcantilevers, PEP1 is then introduced onto the SLBs. The adsorption curve of PEP1 at low concentration on the
SLB coated microcantilevers is compared with that of a casein coated cantilever in Figure 5.2. Upon PEP1 addition, there is an obvious increase in surface stress, indicative of PEP1 insertion into the lipid bilayer. On the control microcantilever (dashed line), the surface stress remains small after the introduction of PEP1 onto the surface, likely due to non-specific binding of PEP1 peptides to the casein coated surface through hydrophobic interactions. Additionally, after the system is rinsed with buffer, there is no changes in surface stress observed, which indicates the PEP1 adsorption is irreversible, unlike amphiphilic surfactants that have been previously studied.\textsuperscript{20} This is likely due to the peptide system having a much strong binding affinity to the membrane.

5.3.3. High Concentration of PEP1 Leads to POPC SLBs Lysis

At higher concentrations, the introduction of PEP1 leads to a precipitous drop in surface stress, indicative of the solubilization of the membrane from the sensor surface. I monitor the real-time surface process of the solubilization of a SLB when in contact with higher PEP1 concentrations. The surface stress drops from \(17 \pm 1.4\) mN/m to zero, suggesting that the SLB is perturbed and readily desorbs after contacting with PEP1 at 26 \(\mu\)M, as shown in Figure 5.3. This disruption of the supported bilayer was also previously observed using AFM for PEP1 at concentrations larger than 26 \(\mu\)M.\textsuperscript{6} Note that for the control cantilever (dashed line) in Figure 5.3, the adsorption of PEP1 on casein is larger than that in Figure 5.2, because this adsorption is nonspecific and is largely affected by PEP1 concentration.
Figure 5.3. POPC SLB is solubilized readily after contacting with a high concentration of PEP1s (26 µM). Both the experimental cantilevers (solid lines) on the same chip and the reference cantilever (dashed line, the SiO₂ surface is blocked by casein) are shown. The shaded areas indicate the time when lipid vesicles (blue) or peptides (green) are introduced into the measurement chamber. After injecting the PEP1 solution, there is a surface stress change on the reference cantilever, caused by PEP1 adsorption on casein.

5.3.4. Implication for Mechanism of PEP1 Interaction with SLBs

PEP1 at various concentrations are introduced onto the POPC SLBs, to study its interaction with lipid membrane. As shown in Figure 5.4A, this interaction is divided into three phases, based on the measured surface stress of the SLB. The bilayer structure of POPC lipids on the SiO₂ surface before the addition of PEP1 solution is illustrated in Figure 5.4B[0]. At bulk PEP1 concentrations lower than 4 µM, the PEP1 peptides initially adsorb to the membrane surfaces parallel to the membrane plane because of its α-helical structure which squeezes the membrane altitudinally, leading to additional lateral
internal stress,\textsuperscript{27} and incremental increases in the area of the outer leaflet of the membrane (Figure 5.4B[1]). Surface bound PEP1 then continues to accumulate at higher concentration in a Langmuir adsorption manner, further disrupting lipid packing and thinning the membrane. For this concentration range, the surface stress readily increases with the concentration in a linear fashion, indicating that the peptide adsorption is proportional to the concentration.

At bulk concentrations higher than 4 µM, the surface stress does not show any apparent increase with PEP1 concentration and remains at 25–28 mN/m, as shown in Figure 5.4A. The adsorbed PEP1 peptides saturate the lipid membrane at 4–5 µM bulk concentrations, so additional peptide concentration does not lead to surface stress changes. At a high enough concentration, PEP1 peptides begin to aggregate on the SLB surface which drives interaction across the membrane, leading to pore formation\textsuperscript{6}. The lateral internal stress generated by these PEP1 aggregates is sufficient enough for membrane disruption,\textsuperscript{13} and consequently pore formation in the SLB membrane, as shown in Figure 5.4B[2]. The stability and size of the pores are determined by the relative magnitudes of membrane tension and line tension at the edge of pores.\textsuperscript{12} Other studies with lipid vesicles have shown that there exists a critical concentration when PEP1 interacting leads to vesicle swelling,\textsuperscript{5} which suggests the pore formation\textsuperscript{28,29}. Even though the inner leaflet in a SLB membrane is near a solid support, the PEP1 interaction with SLBs is similar to that which is observed with free membranes.
Figure 5.4. (A) Surface stress generated on microcantilevers after the PEP1 solution is introduced to the SLBs. Three regions are separated by dashed lines. At concentration < 4 µM, the increasing trend of the surface stress is linear with PEP1 concentration; at concentration > 4 µM, the PEP1 peptides saturate the membrane surface, leading to constant surface stress. At concentrations ≥ 26 µM, the membrane is solubilized, eventually leaving to a blank SiO2 surface and a zero surface stress. (B) Schematic representation of PEP1 interaction with the SLB on microcantilever surface. Four diagrams illustrate the possible conformations of the SLB before [0] and after [1-3] interacting with PEP1 at various concentrations.
At PEP1 concentrations greater than 20 µM, there is a drop in the surface stress. It is thought that after a sufficient number of peptides have aggregated and formed pores in the SLB, the membrane internal stress accumulates and reaches a value where it cannot be counterbalanced by the membrane tension so the membrane solubilizes. The observed drop in surface stress elucidates the removal of lipids from the cantilever surface, likely resulting in patchy SLBs (20 µM) and complete membrane solubilization at concentrations greater than 26 µM, as shown in Figure 5.4B[3]. It has been previously reported using QCM-D and SPR that lipid vesicles rupture upon PEP1 exposure at a concentration of ~13 µM and a supported membrane remains adsorbed onto the gold or TiO₂ surface. The PEP1 induced lysis of lipid vesicles is a vesicle size-dependent process in which smaller vesicle can be more readily disrupted by the peptide. Compared with the PEP1 induced lysis of SLBs, the behavior of PEP1 when interacting with lipids is very likely related to the lipid curvature, which will be discussed in detail in the subsequent sections.

5.3.5. Surface Stress based Analysis

The total surface area of the membrane–peptide complex expands by the total area of adsorbed peptides and results in an internal stress in the lipid membranes. SLBs can be elastically deformed, thus an increase in the membrane surface area (or membrane strain in surface area) can be related to the internal stress by the membrane’s stretch modulus $K_A$. The membrane internal stress, $\Delta \sigma_{\text{mem}}$, accounts for the lateral interactions between the lipid-lipid and lipid-peptide in the SLB$^{12-13}$.

$$\Delta \sigma_{\text{mem}} = K_A \frac{A_p}{A_L} \frac{P}{L}$$  \hspace{1cm} (5.2)
where \( A_P \) and \( A_L \) represent the molecular area of the peptide and the lipid, respectively. \( P/L \) is the number ratio of membrane bound peptide to lipid. Other source of surface stress, \( \Delta \sigma_{ads} \), comes from the lipid–surface interactions, which is the chemical potential gained from adsorption. Thus the total surface stress, \( \Delta \sigma_{total} \), measured on the microcantilevers is comprised of the contributions from lipid–lipid and lipid-peptide (\( \Delta \sigma_{mem} \)) and lipid–surface interactions (\( \Delta \sigma_{ads} \)):

\[
\Delta \sigma_{total} = \Delta \sigma_{ads} + \Delta \sigma_{mem} = \Delta \sigma_{ads} + K_A \frac{A_P}{A_L} P \frac{P}{L} 
\]

(5.3)

The membrane internal stress is estimated from Equation 5.2 by taking \( K_A \) to be 240 mN/m for a POPC membrane,\(^{30} \) \( A_P \) be 360 Å\(^2\) and \( A_L \) be 65 Å\(^2\) for lipid membranes in a fluid state\(^6\). Huang et al. predicted that at concentrations when pore formation occurs, the typical value for \( \Delta \sigma_{mem} \) to be 5 ~ 15mN/m, which is close to the surface stress measurement derived from the microcantilevers, ~ 13 mN/m. By excluding the PEP1 adsorption term (\( \Delta \sigma_{ads} \)) and only accounting for the membrane internal stress term (\( \Delta \sigma_{mem} \)) for the calculation of the total measured surface stress, the \( P/L \) value can be estimated to be 1/102, which indicates that one PEP1 is surrounded by 102 lipid molecules. The actual \( P/L \) ratio may be smaller because the adsorption term (\( \Delta \sigma_{ads} \)) is not taken into consideration.

5.4. Discussion

The interaction of PEP1 peptides with lipid vesicles has been investigated and is currently used to rupture lipid vesicles and form the supported membrane under unfavorable conditions.\(^8,10,31\) The mechanism is as follows: PEP1 peptides first bind to
the lipid membrane, induce the pore formation which leads to vesicle swelling, and eventually rupture the vesicles.5 The results show that this mechanism also takes place on SLBs: the peptides adsorb, destabilize, and solubilize the lipid bilayer. There are interesting differences between free membranes and supported membranes. Mainly, the concentration threshold causing vesicle rupture is lower than that inducing SLB solubilization. Also, for the same peptide concentration, smaller vesicles are easier to disrupt.4 This indicates that the PEP1-lipid interaction is likely dependent on membrane curvature. It has been also reported that amphipathic α-helical peptides can be used as a sensing motif toward the membrane curvature.32-33 A membrane with higher curvature is found to possess more binding sites,34 and thus facilitate the pore formation and lipid lysis.35-36 Supported lipid membranes, with very small curvatures need a much higher critical concentration of PEP1 peptides to induce membrane solubilization. Additionally the underlying solid support hinders lipid mobility, making it more difficult to solubilize. Therefore, I hypothesize that the PEP1 interactions with different membrane models, either lipid vesicles or SLBs, are governed by the same process, in which the peptide concentration and membrane curvature synergistically determine the adsorption, destabilization, or solubilization of the lipid membrane.

The interaction of amphipathic surfactants, lysolipids, with SLBs was previously studied, which also involves the lysolipid adsorption and eventual membrane solubilization.20 Although the lysolipid behaves similarly to the PEP1 peptide when interacting with SLB, the PEP1-SLB interaction is stronger because the adsorbed PEP1 does not desorb from the SLB after a buffer rinse while the lysolipid easily desorbs. Additionally, PEP1 disrupts the SLBs at a much lower concentration, so it is more
efficient in solubilizing the lipid bilayers and removing them from the solid surface compared to the lysolipids. The lipid interaction with PEP1 is also comparable to that with antimicrobial peptides since they both have amphipathic α-helical and membrane active characteristics. The membrane interaction with antimicrobial peptides is determined by the molar peptide-to-lipid ratio \((P/L)\) in a two-state model.\(^3\) The peptides bind to the membrane at a ratio lower than a critical \(P/L\), but cause pore formation when the ratio is higher than a critical \(P/L\). With the presence of pores, more peptides do not give rise to the additional membrane thinning effect, and thus no more internal stress is generated.\(^1\) This also explains the plateau in Figure 5.4A where surface stress does not increase with PEP1 concentration. The amphipathic α-helical property is essential for peptides to interact with lipid membrane, so the model developed for antimicrobial peptides can be generally applied to the PEP1-SLB interaction.\(^6\)\(^,\)\(^28\) Because the peptide concentration is proportionally related to the \(P/L\), the mechanism of PEP1-SLB interaction can be presented by a three-state model: at \(P/L\) below the first critical ratio \((P/L<P/L^*)\), PEP1s adsorb onto the SLB in parallel; at \(P/L\) between two critical ratios \((P/L^*<P/L<P/L^{**})\), PEP1s insert into the SLB and form pores across the membrane; at \(P/L\) above the second critical ratio \((P/L>P/L^{**})\), PEP1s solubilize the SLB and lipids are removed from the surface. The main difference between the two systems is that the critical \(P/L\) for PEP1 is lower than most antimicrobial peptides, indicating the efficiency in which the PEP1 peptide can disrupt lipid membranes.\(^3\)\(^5\)

Additionally, comparing the response of PEP1 adsorption onto POPC SLBs using the microcantilever sensors with that of other sensors, such as QCM-D, the peptides partitioning into a SLB is more responsive to surface stress changes rather than mass
changes. For the adsorption of PEP1 to lipid membranes at PEP1 concentration higher than the critical concentration, the frequency change measured on QCM-D will be estimated to be ~3.5 Hz, while frequency change for the SLB formation is ~25 Hz. On microcantilevers, the maximum surface stress change induced by the PEP1 is 13 mN/m, while that induced by the SLB formation is 17 mN/m. Thus, the microcantilever sensor is more sensitive to small molecule adsorption to membranes and to interactions that are involved with lateral membrane internal stress change.

5.5. Conclusion

The bending motion of lipid membrane-coated microcantilevers is a direct surface measurement of surface stress change as PEP1 peptides bind to the membrane. Interactions between PEP1 and the lipid membranes are measured at different peptide concentrations. From the plot of surface stress versus PEP1 concentration, three phases of interactions are observed. At concentration < 4 µM \((P/L<P/L^*)\), the amount of the PEP1 peptides adsorbed to the SLB is linear with concentration. At a critical concentration of 4 µM \((P/L^*<P/L<P/L^{**})\), the surface stress change plateaus as PEP1 starts to aggregate and begin to form pores across the membrane. At concentration \(\geq 20\) µM \((P/L>P/L^{**})\), there is a significant drop of surface stress, indicating damage to the membrane integrity, as well as membrane solubilization. Compared to other surface-sensitive tools, such as QCM-D\(^7\) and SPR\(^8\), the microcantilever sensor is much more sensitive at probing small molecules adsorption and interaction because the membrane internal stress change can also be detected as a surface stress change. Thus the lipid membrane-coated microcantilever sensor is capable of characterizing the kinetics and dynamics of
membrane-peptide interactions with high sensitivity.

5.6. References

Chapter 6

Probing the Association of Triblock Copolymers with Supported Lipid Membranes using Microcantilevers

6.1. Introduction

The use of microcantilevers on sensitive measurements of the interactions between supported lipid membranes and amphipathic peptides is previously reported. Pluronics are another class of amphipathic molecules that are composed of hydrophilic poly(ethylene oxide) (PEO) and lipophilic poly(propylene oxide) (PPO) in a $\text{PEO}_m$-$\text{PPO}_n$-$\text{PEO}_m$ structure, where $m$ and $n$ represent the number of monomers in a block. The asymmetric feature of supported lipid membranes could influence the association of these
triblock copolymers with membranes. Thus the technique I utilize to study Pluronic interactions with supported lipid membranes is microcantilever sensor, which has the ability to sensitively measure surface stress changes associated with liquid-solid systems.\(^1\)

The Pluronics have found to be useful in a number of applications, such as detergency, dispersion stabilization, foaming, and lubrication.\(^2\) These triblock copolymers have also been able to interact with cell membranes. Pluronics have been reported to seal damaged cell membranes\(^3\)–\(^5\) and protect lipid membranes from peroxidation\(^6\). Pluronics have also been shown to permeabilize cell membranes, which has led to their applications in drug delivery\(^7\)–\(^8\) and gene and cancer therapies\(^9\). The ratio of the number of hydrophilic PEO monomers to the number of lipophilic PPO monomers determines the hydrophilic/lipophilic balance (HLB) of the copolymer. This affects the solubility of the copolymer with lipid membranes: higher HLBS can cross cell membranes while lower HLBS lead to insertion into the lipid bilayer\(^10\)–\(^12\). Additionally, temperature can be used to change the solubility of the copolymer with lipid membranes\(^13\)–\(^14\). Therefore, properties such as copolymer aggregation and phase behavior, as well as interaction with cell membranes are highly dependent on temperature\(^15\). Another important factor influencing the polymer-lipid association is the length of lipophilic PPO block. The conformation of the copolymer with the membrane greatly varies depending on its relative length compared to lipid membrane thickness\(^16\)–\(^17\).

There have been several interesting studies aimed at understanding the interactions of these copolymers with cellular membranes. These include probing the interactions with model lipid membranes, such as lipid monolayers at an air/water interface\(^18\) and lipid vesicles\(^16, 19\)–\(^20\). Interestingly, this polymer-lipid interaction remains
elusive because the results using one lipid system are inconsistent with that obtained from the other system,\textsuperscript{21} likely due to differences in the lipid configuration for these systems. For example, the incorporation of Pluronics into lipid vesicles was reported to impair lipid packing, leading to enhancement of lipid mobility and easier permeation of membrane.\textsuperscript{21-22} However, for a lipid monolayer or bilayer, the insertion of copolymers improves the lipid ordering by packing the lipids tightly.\textsuperscript{23-24}

In this Chapter, I investigate PEO-PPO-PEO triblock copolymer interactions with supported lipid bilayers (SLBs). SLBs serve as a usual model for cell membranes.\textsuperscript{25} An important feature of SLBs is their fluidity on a solid support. The main structural feature of SLBs is the asymmetry in the membrane environment: one membrane leaflet is exposed to an aqueous solution while the other is exposed to a solid support. This leaflet asymmetry is reported to cause differences in the surface tension, lipid diffusion, and phase transition temperature between SLBs and free membranes.\textsuperscript{26-32} The Pluronics studied here are F68 and F98, which are chosen because they have the same HLB value, but different lengths of lipophilic PPO blocks. Thus, the effect of length of lipophilic block on polymer-lipid association, as well as copolymer’s conformation can be studied.

The microcantilever sensor is applied to study Pluronic interactions with the SLBs. Surface coated films, either physisorbed or chemisorbed on a biomaterial cantilever cause a surface free energy change that results in bending of the cantilever,\textsuperscript{33} which can be readily detected using a position sensitive detector. Conformational changes in the adsorbed molecular films have been readily observed using microcantilevers.\textsuperscript{34-36} SLBs have been used to study lipid interaction with other amphiphilic molecules, such as diblock copolymers\textsuperscript{37} and lysolipids\textsuperscript{38}. Complementing the microcantilevers experiments,
I also study lipid diffusion and membrane fluidity in SLBs using fluorescence recovery after photobleaching (FRAP). A Langmuir adsorption based model was developed to illustrate the relative affinity of the copolymers towards SLBs, and a free area theory is used when analyzing lipid diffusion. The findings show that the solid support does indeed change the interactions of triblock copolymers with supported lipid membranes.

6.2. Materials and Methods

6.2.1. Materials

A zwitterionic lipid, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), was purchased from Avanti Polar Lipids (Alabaster, AL). A fluorescent lipid, Texas Red-1,2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine (TR-DHPE), was purchased from Invitrogen (Carlsbad, CA). Two poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) triblock copolymers (F68 and F98) were obtained from BASF Corporation (Mount Olive, NJ) under the name of Pluronic, Kolliphor, or Poloxamer, and their properties are summarized in Table 6.1. The dithiol-alkane-aromatic PEG3-OH (PEG-SH) was purchased from SensoPath Technologies (Bozeman, MT). All the lipids and chemicals were used as received without further purification.

6.2.2. Microcantilever Assay to Measure Triblock Copolymer Association with SLBs

The gold surface of the cantilever was then coated with PEG-SH monolayer to prevent vesicle binding. A solution of POPC vesicles was injected at a flow rate of 0.42 µL/sec into the measurement chamber to form the SLBs on silicon dioxide surface of microcantilever. Then the triblock copolymer (F68 or F98) solution at a concentration of
10, 50, 100 or 500 µM in PBS was injected at various temperatures (25, 30, 35 or 40 ºC). Because of small variations in the material properties of the cantilevers, such as stiffness or thickness of the gold layer, the deflections of microcantilevers were normalized by each cantilever’s change in deflection due to a 1 ºC change in temperature. Each experiment was repeated at least three times on either the same or different chip, with a minimum of five cantilevers on one chip.

Table 6.1. Characteristics of PEO-PPO-PEO triblock copolymers.

<table>
<thead>
<tr>
<th>Pluronic</th>
<th>MW a, g/mol</th>
<th>PO units</th>
<th>EO units</th>
<th>PO/EO</th>
<th>CP b, ºC</th>
</tr>
</thead>
<tbody>
<tr>
<td>F68</td>
<td>8400</td>
<td>30</td>
<td>2 × 75</td>
<td>0.2</td>
<td>&gt;100</td>
</tr>
<tr>
<td>F98</td>
<td>13000</td>
<td>47</td>
<td>2 × 117</td>
<td>0.2</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

a Molecular weight;  
b Cloud point (corresponding to phase separation temperature) in 10% aqueous solution.

6.2.3. Fluorescence Recovery After Photobleaching (FRAP)

Fluorescence recovery after photobleaching (FRAP) is a well-established technology for the measurement of the fluidity and lateral mobility of lipids\textsuperscript{40}, as well as proteins within lipid bilayer\textsuperscript{41}. For FRAP experiments, a simple microfluidic flow cell was created to generate SLBs. The simple rectangular microfluidic channel of 2 cm in length, 0.5 mm in width, and 50 µm in height, was fabricated in polydimethyl siloxane (PDMS) using standard soft lithography techniques\textsuperscript{42-44} and bonded to a glass coverslip. The glass coverslip in microfluidic device was cleaned with a mixture of hydrogen
peroxide and ammonia hydroxide at 75 °C, and oxygen plasma (Harrick Plasma). Initially, the SLB was formed on the coverslip by injecting a fluorescent LUVs solution (POPC with 0.5 mol% TR-DHPE) at a rate of 10 µL/min for 10 min into the microfluidic channel using a syringe pump followed by PBS buffer to remove excess vesicles. Then the triblock copolymer (F68 or F98) solution at a concentration of 100 µM was injected at various temperatures (25, 30, 35 or 40 °C), followed by PBS buffer.

The fluidity of lipids with and without the triblock copolymers was characterized by FRAP using confocal microscopy (Olympus IX81). A 23 µm spot was photobleached by a mercury lamp light source at 405 nm for 60 s. The fluorescence intensities of this spot and surrounding area (used as control) were monitored with time using a 40X objective. The fluorescence fraction is defined as:

$$f(t) = \frac{F(t) - F(0)}{F(\infty) - F(0)}$$  \hspace{1cm} (6.1)

where $F(t)$ is the fluorescence intensity as a function of time, $F(0)$ is the fluorescence intensity before bleaching, and $F(\infty)$ is the final recovered intensity. Thus, the recovery half time, $\tau_{1/2}$, is determined as the time where $f(t) = \frac{1}{2}$; $r$ is the radius of bleached area and $\gamma_D$ is the factor considering both the beam shape and bleaching extent. The lateral diffusion coefficient of lipids was calculated by the following equation:

$$D_f = \frac{r^2}{4\tau_{1/2}} \gamma_D$$  \hspace{1cm} (6.2)

6.2.4. Mathematical Modeling of Polymer-Lipid Interaction on Microcantilevers
The association of triblock copolymers with POPC SLBs on the cantilever surface was theoretically studied using a Langmuir isotherm model. The association process is shown as:

\[ S_{POPC} + Polymer \xleftrightarrow{K_A} S_{POPC} - Polymer \]  \hspace{1cm} (6.3)

In Equation 6.3, \( S_{POPC} \) represents the POPC bilayer, polymer represents the Pluronic, and \( K_A \) is the association constant, which allows us to compare the relative affinities of the different Pluronics towards the SLBs. At equilibrium, the polymer-SLB interaction can be written as a associating/disassociating reaction rate:

\[ k_A[Polymer](1 - \theta) = k_{-A}\theta \]  \hspace{1cm} (6.4)

where \( \theta \) is the fraction of the SLBs with attached polymer, and \( (1 - \theta) \) represents the sites available for further polymer association. Defining \( K_A \) as the ratio of \( k_A \) over \( k_{-A} \), the following equation describes the relationship between polymer concentration and the surface stress change measured on cantilever:

\[ \frac{[Polymer]}{\Delta Stress} = \frac{[Polymer]}{\Delta Stress_{max}} + \frac{1}{\Delta Stress_{max}K_A} \]  \hspace{1cm} (6.5)

where \([Polymer]\) is the molar concentration of triblock copolymers. \( \Delta Stress \) is the change of surface stress caused by polymer and \( \Delta Stress_{max} \) is the maximum change in surface stress when the polymer saturates the surface. \( K_A \) is obtained from the plot of \([Polymer]/\Delta Stress\) with respect to \([Polymer]\).

**6.2.5. Free Area Model for Lipid Diffusion in FRAP**

A free area model is used to characterize the lipid diffusion in a SLB. In this model, the lateral diffusion of molecules was considered to be a two-dimensional random
motion. To move, a lipid needs to meet two requirements: a minimum empty surrounding area and a sufficient activation energy.\textsuperscript{51,52} Therefore, the diffusion coefficient can be affected by two possibilities:

\[ D = D' p(a) p(E) \]  \hspace{1cm} (6.6)

with \[ p(a) = \exp \left( -\frac{a_0}{a(T)-a_0} \right) \] \& \[ p(E) = \exp \left( -\frac{E_a}{kT} \right) \]

where \( p(a) \) is the Boltzmann distribution of a lipid with minimum free surrounding area \( a_f \) and \( p(E) \) is the probability that the activation energy, \( E_a \), normalized to thermal energy, \( kT \), is sufficient. \( k \) is Boltzmann constant, \( a(T) \) is average lipid area, and \( a_0 \) is critical area of lipid when it is closely packed. Thus the average free area of lipids is \( a_f = a(T) - a_0 \).

Detailed derivation of this model has been previously reported by Reits \textit{et al.}\textsuperscript{53} In a two-dimensional SLB, the diffusion coefficient of lipids can be expressed as:

\[ D = \sqrt{\frac{kN_a}{8}} \sqrt{\frac{T a(T)}{M}} \exp \left( -\frac{a_0}{a(T)-a_0} - \frac{E_a}{kT} \right) \]  \hspace{1cm} (6.7)

where \( Na \) is Avogadro’s constant, and \( M \) is average molecular weight. The equation above is only valid for pure lipids. However, with the association of the triblock copolymer to the SLBs, the effect of the polymer on lipid diffusion must be considered. There is a decrease in the free area and change of activation energy. Two parameters, average polymer area \( a_{o poly} \) and molar ratio of polymer to lipid \( n \), are introduced into the following equation:

\[ D = \sqrt{\frac{kN_a}{8}} \sqrt{\frac{T a(T)}{M}} \exp \left( -\frac{a_0}{a(T)-a_0} - na_{o poly} - \frac{E_a}{kT} \right) \]  \hspace{1cm} (6.8)
The activation energy $E_a$ and molar ratio of polymer to lipid $n$ are fitting parameters, obtained from nonlinear least square fitting. More details about this model are described in the appendix.

6.3. Results and Discussion

6.3.1. Triblock Copolymer Interaction with SLBs using Microcantilevers

To form supported lipid bilayers (SLBs) on microcantilever surface, the gold side of cantilever is previously inactivated by PEG-SH. Then upon the addition of LUVs, the vesicles fuse onto the clean hydrophilic silicon dioxide side of cantilever and rupture to form a planar lipid bilayer. A representative result of how cantilever deflection changes with introduction of various components is shown in Figure 6.1. As indicated with blue shaded area, a compressive surface stress is induced by SLBs formation, causing the cantilever to bend toward the gold side. After switching back to PBS buffer, the microcantilever remains deflected, confirming that a stable SLBs has formed. The solution of triblock copolymer F98 or F68 is later introduced to the SLBs, causing a further compressive surface stress in microcantilever (shown by green shaded area in Figure 6.1). The change of surface stress after switching back to buffer flow indicates the association of the copolymer with the supported lipid bilayer.
Figure 6.1. Measurement of triblock copolymer interaction with SLBs using microcantilevers. The SLB is formed on PEG-coated cantilever, prior to the introduction of F98 (dashed lines) or F68 (solid lines) solution at 50 µM at 35 ºC. The lines with different colors represent the various cantilevers on one chip. The shaded areas indicate the time when lipid vesicles (blue) or copolymers (green) are introduced into the measurement chamber. The top diagrams illustrate the bending of microcantilevers.

One important parameter influencing copolymer interaction with lipid bilayer has been reported to be the length of lipophilic block.\textsuperscript{16} Thus two triblock copolymers with different length of lipophilic PPO block are studied. F98, with the longer PPO, has a stronger association with the POPC SLBs compared to F68 (Figure 6.1). The two copolymers are further investigated at various concentrations and temperatures, as shown in Figure 6.2. The microcantilevers’ signal is normalized by each cantilever’s thermo-
mechanical sensitivity, to offset the difference among cantilevers. At a certain temperature, as the copolymer concentration increases, the change of surface stress is increased, corresponding to a stronger association between the triblock copolymer and SLBs. The association of either F98 or F68 is also found to be enhanced for increasing temperatures. The extent of association which is directly proportional to the magnitude of surface stress change, reflects the relative solubility of each copolymer with the SLBs at a specific temperature. Both F98 and F68 show increased solubility with the SLBs with increasing temperature. The reason for the increased solubility is that triblock copolymer becomes more hydrophobic at higher temperature.\textsuperscript{13-14} The effect of temperature on this solubility in SLBs is in agreement with interaction measured in lipid monolayer\textsuperscript{15} and vesicle\textsuperscript{20}. Although concentration and temperature have the similar influence on F98 and F68, they display different solubilities in POPC SLBs. At the same temperature, the association of triblock copolymer with longer lipophilic block (F98) is much stronger than shorter copolymer (F68). The polymer-lipid interaction is highly dependent on the temperature, as well as the length of lipophilic PPO block of the Pluronic.
Investigate the association of triblock copolymer F68 (A) and F98 (B) at a concentration of 10, 50, 100 or 500 μM onto POPC SLBs under various conditions. F68 has shorter PPO block while F98 has longer PPO block. Four temperatures are tested: 25 °C (purple), 30 °C (green), 35 °C (red), and 40 °C (blue).

6.3.2. Mathematical Modeling and Calculation of the Association Constants

The association affinity between the triblock copolymers and the SLBs on microcantilevers can be determined by modeling the process using a Langmuir isotherm
model\(^{19}\). This model has been previously used to describe binding between antigen-antibody systems on microcantilevers.\(^{54-55}\) The association constant \(K_A\) is determined based on Equation 6.5. The plots of \([\text{Polymer}] / \Delta \text{Stress}\) with respect to \([\text{Polymer}]\) for F68 and F98 at 40\(^\circ\)C are selectively shown in Figure 6.3, because the two copolymers display the biggest difference in the association to SLBs at this temperature. In each plot four data points are used to optimize the linear data fitting. From the slope and intercept of each plot, the association constant \(K_A\) is calculated (Table 6.2). The values of \(K_A\) represent the relative solubility of each copolymer with the POPC SLBs, with the largest value corresponding to the highest solubility. It is important to notice that the relative solubility calculated here refers to the ability of each copolymer to associate with the POPC SLBs at a specific temperature. Thus, as shown in Table 6.2, the association constants become larger with increasing temperature, which is consistent with the experimental results. Because F98 has longer lipophilic block than F68, it displays larger association constants than F68 at corresponding temperature, confirming its higher solubility in SLBs, particularly at high temperature. Meanwhile, the change of association constant \(K_A\) from 25 to 40 \(^\circ\)C is larger for F98 than F68, so the solubility of F98 in SLBs is more sensitive to temperature\(^{16}\). This sensitivity is likely a result of the longer PPO block of F98. Fitting the experimental data to the derived mathematical equation (Equation 6.5) allows us to quantitatively determine the relative solubilities of copolymers with POPC SLBs.
Figure 6.3. Determining the association constant $K_A$ for the copolymers F68 (A) and F98 (B) at 40 °C. Experimental results for $[\text{Polymer}]/\Delta\text{Stress}$ is plotted with respect to $[\text{Polymer}]$ and fit to Equation 6.5. The equations for trend lines and R-squared values are shown.

Table 6.2. The value of association constants $K_A$ ($\mu$M$^{-1}$) at various temperatures.

<table>
<thead>
<tr>
<th>Constant $K_A$</th>
<th>$F68$</th>
<th>$F98$</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 °C</td>
<td>0.009011</td>
<td>0.009796</td>
</tr>
<tr>
<td>30 °C</td>
<td>0.009488</td>
<td>0.011837</td>
</tr>
<tr>
<td>35 °C</td>
<td>0.010351</td>
<td>0.013471</td>
</tr>
<tr>
<td>40 °C</td>
<td>0.011519</td>
<td>0.020052</td>
</tr>
</tbody>
</table>

6.3.3. Effect of Triblock Copolymers on Lipid Diffusion in FRAP

A typical FRAP experiment for a POPC SLB is shown in Figure 6.4. After photobleaching, the fluorescent intensity of bleached area increases with respect to time
(Image b-f), and finally reaches the value comparable to that before bleaching (Image a). The lipid diffusion coefficient is quantitatively determined by the rate of fluorescence recovery using Equation 6.2. Measurements of lipid diffusion coefficients $D$ with or without triblock copolymers are reported in Figure 6.5A. For the FRAP experiments, a copolymer concentration of 100 µM is chosen, because this concentration induced the sharpest increase in surface stress for the microcantilevers (Figure 6.2). For the POPC SLBs with or without copolymers, the lipid diffusion is always enhanced at increasing temperature. But the increase of diffusion coefficient for SLBs with copolymers is obviously smaller than the pure SLB system, indicating the inhibition of lipid diffusion caused by copolymer association. And this inhibition effect is stronger for F98 compared to F68. To better illustrate the copolymer induced hindered diffusion, a normalized diffusion coefficient is defined as the ratio of the diffusion coefficient of lipids in a SLB with copolymer over the diffusion coefficient of lipids in a pure POPC SLB at corresponding temperatures (Figure 6.5B). Because the temperature effect is eliminated, the normalized $D$ is the same for pure POPC at various temperatures, and is reduced at increasing temperature for POPC with copolymers. But the decreasing extent of normalized $D$ for POPC with F98 is much faster than F68. Therefore, the lipid diffusion is slightly hindered by F68, and temperature has a little impact on this inhibition. But based on the microcantilever results in Figure 6.2, the higher temperature increases the hydrophobicity of copolymer, and further improves the copolymer association with the SLBs. As a result, the inconsistency between temperature’s little impact on F68 induced diffusion inhibition and its large influence on the association of F68 on SLBs indicates a small effect of F68 association on lipid fluidity, which further suggests that the
adsorption of F68 occurs on the outer leaflet of the SLBs. F98 has a different effect on the lipid diffusion, causing more hindered lipid diffusion at higher temperature. Thus the temperature effect on diffusion inhibition is consistent with copolymer association, suggesting a deeper insertion of F98 into the SLB. The different effects of F68 and F98 results from the length difference of lipophilic PPO block. The PPO block of F68 is too short to insert into the hydrophobic region of the lipid bilayer, so its adsorption only slightly hinders lipid diffusion and more association does not further hinder diffusion. But for F98, its long PPO block allows for the deeper insertion, which highly hinders lipid diffusion and this effect increases with increasing temperature.

Figure 6.4. Recovery of fluorescence for POPC SLB with DHPE-TR as an indicator. The SLB was formed on surface of microfluidic device at 25°C. The images show the FRAP data measured by confocal microscopy. a: SLB before photobleaching; b-f: fluorescence recovery with respect to time.
Figure 6.5. Diffusion coefficient $D$ measured by FRAP for POPC with and without Pluronics (A). The concentration of either F68 or F98 is 100 µM. To better illustrate the effect of polymer on lipid diffusion, normalized diffusion coefficient is obtained by normalizing data using diffusion coefficient of lipids in a pure POPC SLB (B).
6.3.4. Modeling of Lipid Diffusion using A Free Area Theory

The effect of triblock copolymers on lateral diffusion of lipid molecules is investigated by a free area theory, which has been applied for quantitative study of lipid diffusion.\textsuperscript{51, 57-59} The free area theory takes into account both activation energy and free area. The diffusion coefficient can be calculated from Equation 6.8. The fitting of the free area model to the experimental data is shown in Figure 6.6. The calculated diffusion coefficients are expressed as a function of temperature, and accurately reproduce the variation trend of lipid diffusion: the diffusion is hindered more strongly by F98 compared to F68 and the inhibition increases with increasing temperature. Additionally, the molar ratio of polymer over lipid $n$ is also calculated from the free area model by the nonlinear least square fitting, as shown in Table 6.3. The ratio $n$ for F98 is larger and more affected by temperature, compared to F68, demonstrating larger solubility of F98 in the SLBs, which is also confirmed by the cantilever and FRAP data. Furthermore, the relative values of $n$ agree well with the variation trend of surface stress change from microcantilevers in Figure 6.2 at corresponding conditions, indicating that $n$ also reflects the amount of associated copolymers in the SLBs on cantilever surface.
Figure 6.6. Diffusion coefficient $D$ as a function of temperature for three systems: POPC only (blue), POPC with F68 (red), and POPC with F98 (green). The bar graph gives experimental data while lines show calculated diffusion coefficient from free area model, according to Equation 6.7 and 6.8.

Table 6.3. The molar ratio of polymer to lipids $n$ (%) at various temperatures.

<table>
<thead>
<tr>
<th>Ratio $n$</th>
<th>$F68$</th>
<th>$F98$</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 °C</td>
<td>4.74</td>
<td>5.18</td>
</tr>
<tr>
<td>30 °C</td>
<td>5.43</td>
<td>6.86</td>
</tr>
<tr>
<td>35 °C</td>
<td>6.11</td>
<td>8.54</td>
</tr>
<tr>
<td>40 °C</td>
<td>6.79</td>
<td>10.22</td>
</tr>
</tbody>
</table>

To better illustrate the solubility of triblock copolymers in supported lipid bilayers, the possible conformations of Pluronics within the lipid model SLBs with respect to
temperature is illustrated in Figure 6.7. At low temperatures, F68 or F98 adsorbs weakly to the outer leaflet of the SLBs and slightly hinder lipid diffusion. Although two copolymers are different, the adsorption or inhibition effect of F98 is just slightly higher than F68. However, with increasing temperature the difference between F68 and F98 becomes larger. Although the association of F68 is increased at higher temperature, the inhibition of lipid diffusion does not change much because F68 only partially inserts into SLBs due to its short PPO block. As for F98, with the long lipophilic PPO block, the higher temperature not only increases its association with the SLBs, but also allows for greater insertion into the hydrophobic region of the SLBs, which highly hinders the lipid diffusion. In this study, though the F98 has the potential to span the SLB, as reported previously for lipid bilayers\cite{16,17}, it would prefer to have the PEO ends remain on the outer leaflet. The reason lies in the asymmetric environment for the SLBs.\cite{26} The proximal leaflet of SLB is confined by a thin water layer and the solid support. The energy penalty to disrupt this confinement is too great. The inhibition effect of triblock copolymers on lipid diffusion in this study is consistent with the studies using lipid monolayer,\cite{23,24} but different from the studies using vesicles\cite{21,22} where lipid fluidity is enhanced. Additionally, for a lipid monolayer prepared on a Langmuir trough, or supported lipid bilayer on solid surface, the packing of lipids is either controlled at steady surface pressure\cite{60} or confined by a solid support\cite{61}, so the insertion of copolymers tightens the lipid packing and reduce the membrane permeability and lipid diffusion.\cite{23} In contrast, the lipid packing is not confined in lipid vesicles, where the presence of copolymers during vesiculation increases the size of the lipid vesicles.\cite{19} Therefore, the incorporation of copolymers disturbs the lipid packing and accelerate the leakage and mobility of membrane.\cite{22}
Figure 6.7. Schematic of possible SLB interaction with different Pluronics with respect to temperature.

6.4. Conclusion

The solubility of tribock copolymers with SLBs has been studied using microcantilevers and FRAP. To better understand the polymer-lipid interactions, a Langmuir isotherm model and a free mean area theory have been applied to explore the association of the copolymers with the SLBs and hindered lipid diffusion, respectively. The SLB is interesting in that it has an asymmetry with one leaflet exposed to solution while the other is confined by a solid support. This asymmetry can greatly affect the polymer-lipid interactions. The microcantilever results indicate that the association of triblock copolymers with a POPC SLB is enhanced with increasing temperature, where F98 is more sensitive to temperature compared to F68 due to its longer lipophilic block. FRAP data further demonstrates the possible conformation of soluble Pluronic
SLBs by monitoring copolymer induced inhibition of lipid diffusion. I suggest that a greater inhibition in lipid diffusion indicates a greater copolymer insertion into the SLBs. Therefore, both F68 and F98 adsorb onto the bilayer surface at low temperatures without affecting lipid diffusion. But at higher temperatures, F98 inserts into the SLBs revealed by its high inhibition on lipid diffusion, whereas F68 just partially inserts into the SLBs still with low inhibition. In summary, although F68’s and F98’s solubilities in SLBs are both increased by temperature, the solubility of F98 with longer PPO block is always higher and more sensitive than that of F68. Therefore, the microcantilever sensor is capable of not only sensitively detecting molecular association, but also measuring the conformational change of molecular structure at the interfaces.

6.5. References


Chapter 7

Conclusion and Future Directions

7.1. Summary of Results

This dissertation has presented the application of surface-sensitive sensors to better understand the influence of solid surfaces on molecular structure and molecular interactions at the liquid-solid interfaces. Two sensor platforms are specifically studied: microcantilever and quartz crystal microbalance (QCM), sharing several advantages, such as high sensitivity and specificity, high-throughput signals, label-free and real-time measurements. By monitoring and analyzing the change of surface stress or bound mass, the intermolecular interactions and association affinity are investigated, as well as the conformational structures of molecules of interest.
The microcantilever sensor is first applied to the detection of pathogenic bacteria, *Salmonella*. In Chapter 2, among various recognition elements, a phage-derived peptide MSal 020417 was found to have the highest binding affinity and specificity compared to a commercially available anti-*Salmonella* antibody. A multiplexed screening system to quickly determine the binding affinities of various peptides highly improves the efficiency of the peptide screening process. Furthermore, by immobilizing a collection of peptides that present high selectivity for different bacterial pathogens, a multiplexed detection system could be generated to detect multiple pathogens simultaneously, largely improving the detection efficiency of the microcantilever. Thus a rapid and specific microcantilever-based sensor has been constructed for the pathogen detection. This sensor has the potential to be further developed as a screening method to identify pathogen-specific recognition elements. In addition, QCM has also been developed for the sensitive detection of 2,4,6-trinitrotoluene (TNT) in Chapter 3. Because of the small size of TNT molecule, a displacement strategy was used for the detection of TNT, resulting in a higher sensitivity and specificity than conventional binding. ELISA, which is the conventional method, was used as a control to evaluate the effectiveness of the displacement assay for TNT detection, and calculate the relative affinity of anti-TNT antibody. The robustness of this method was confirmed by using a fertilizer solution and artificial seawater as crowded, dirty environments, in which are typically difficult to detect low concentrations. The detection time was considerably reduced by introducing the slope of signal change as a parameter to determine the concentration of unknown TNT samples. In both Chapter 2 and 3, a Langmuir adsorption isotherm model was used to describe the molecular interactions at the sensor surface, as well as to evaluate the
binding properties of the various recognition elements to the different targets. Thus this QCM based displacement assay also provides a method for rapid detection of TNT with high sensitivity and specificity. As a result, both microcantilever and QCM provide novel techniques to rapidly and accurately detect target analytes. As a result, these two surface-sensitive sensors have been proved to be a useful technique to characterize molecules at the liquid-solid interface.

Besides the detection of target analytes, the microcantilever has also been adapted for the characterization of lipid membrane and its interaction with other molecules. Supported lipid bilayers (SLBs) have served as a useful model for complex cell membranes. The main structural feature of SLBs is the asymmetry in the membrane environment: one membrane leaflet is exposed to an aqueous solution while the other is exposed to a solid support. A membrane property that is affected by this leaflet asymmetry is the temperature induced lipid phase transition, as discussed in Chapter 4. With increasing temperature, the lipid acyl chains transition from an ordered solid state to a disordered liquid state, accompanied by a free energy change, which is coupled to changes in the surface stress in the underlying solid support layer. This surface stress change can be readily detected using microcantilever. The shift of this phase transition temperature for a monolayer indicates the influence of the solid support on the monolayer structure. The existence of cholesterol reduces the phase transition temperature. By considering the bimetallic effect, the thermal expansion coefficients of lipid bilayers were calculated and used to explain the cantilever deflection. This coefficient can also be used to describe the physical structure of the lipid bilayer. Thus in addition to the binding detection, the microcantilever has been proven to accurately probe the conformational
changes of macromolecular assemblies confined on solid surfaces. Also, treating the SLB as a thin film is shown to be a reasonable way to characterize the bilayer. I offer this microcantilever based technique as a promising tool for future studies on the lipid interactions with other molecules at liquid-solid interface.

After characterizing the phase transition of a SLB, its interaction with the membrane-active molecules is further studied using the SLB-coated microcantilevers. A synthetic amphipathic peptide from the Hepatitis C Virus’s nonstructural protein NS5A, PEP1, is investigated in Chapter 5. Interactions between PEP1 and the SLBs were measured at different peptide concentrations. Three phases of interactions were found from the plot of surface stress versus PEP1 concentration. At low concentrations, PEP1 simply adsorbs onto the SLBs and the amount of adsorbed PEP1 is linear with the PEP1 concentration. At a critical concentration, the PEP1 peptides aggregate causing membrane pore formation. PEP1 at higher concentrations damaged membrane integrity and induced solubilization of SLBs. The microcantilever shows high sensitivity in probing small molecule association with SLBs because the microcantilever measures the membrane internal stress change. Besides peptides, the association of an amphiphilic triblock copolymer with the SLBs is also reported in Chapter 6. The solubility of Plurorons with SLBs was studied using microcantilevers and further characterized using FRAP measurements, as a function of temperature and the length of lipophilic block. A Langmuir isotherm model and a free mean area theory were also applied to explore the association of the copolymers with the SLBs and hindered lipid diffusion, respectively. Both F68 and F98 adsorbed onto the bilayer surface at low temperatures without affecting lipid diffusion. With increasing temperature, F98 inserted into the SLBs leading
to inhibition of lipid diffusion, whereas F68 just partially inserted into the SLBs with low inhibition. Compared with free membranes, the asymmetry of the SLBs greatly affected the polymer-lipid interactions. Therefore, the bending motion of lipid membrane-coated microcantilever is capable of measuring the lipid association with other molecules, as well as the conformations of these molecules within the SLBs.

In summary, two surface-sensitive sensors, microcantilever and QCM, are discussed in this dissertation. They have been successfully applied to the sensitive detection of analytes and the characterization of supported lipid membrane. The utilization of these sensors helps to understand the effect of solid surface on molecular structure and function, as well as the intermolecular interactions at the liquid-solid interfaces.

7.2. Recommendations for Future Work

7.2.1. Characterizing Interactions between Lipid Membranes using GUV Chains

Besides the supported lipid bilayers, giant unilamellar vesicle (GUV) is another common artificial model for cell membrane. Previously, the interactions between lipid membranes have been studied using either surface forces apparatus (SFA)\(^4\) or atomic force microscopy (AFM)\(^5\). However, these techniques have been limited to the supported lipid bilayer. To better mimic the behavior of cell membranes and understand the interactions between lipid bilayers in vesicles, it would be useful to construct a model system that is better representative of cell membranes. I have constructed GUV based system to measure lipid interactions because of the similar size of GUVs to cells.
Monodisperse GUVs were synthesized by depositing patterned lipid films via microcontact printing technique using a PDMS stamp, which is prepared by photolithography. To force the GUVs to approach each other, a uniform magnetic field was applied to the GUVs suspended in the ferro-fluid (a dispersion of ferromagnetic nanoparticles), which generated magnetic dipoles. The GUVs were aligned in the direction of the magnetic field and formed vesicle chains. The behavior of GUVs under magnetic field at different intensities was recorded using video microscope, and analyzed in Matlab. The vesicle center-to-center distance was reduced with increasing magnetic field intensity (Figure 7.1). The forces between lipid bilayers could be calculated using corresponding model in future. The objective of this work is to construct a novel platform to characterize interactions between lipid bilayers.

![Figure 7.1. Vesicle distance (center-to-center) measurements at various intensities of magnetic field. The GUV chains at corresponding magnetic intensity are shown in small images inside that were obtained from confocal microscope.](image)

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7.2.2. Probing the Association of Antimicrobial Peptides with SLBs

Antimicrobial peptides are involved in the defense system for most of organisms, including humans. Amphipathicity is a key feature of these peptides, which enables them soluble in the intercellular matrix, as well as to attach on the cell surface. For these systems, the surface immobilized peptides can further insert into the membrane, either forming pores or accumulating like a carpet, both of which can break the cells and cause the cell death. Antimicrobial peptides receive increasing attention because of their therapeutic potential as new antibiotics for the bacteria with resistance. The peptide-to-lipid ratio (P/L) is commonly used to determine the concentration threshold. In this study, alamethicin is chosen, because it follows a typical barrel-stave model, in which peptide helices form a bundle with a central lumen and peptides do not always interact with the lipid headgroups. The interaction of alamethicin with DOPC SLBs on QCM is shown in Figure 7.2A. At low concentrations (~1 µM), the mass increased (signal decrease), indicating the binding of peptide on bilayer surface. When the concentration reaches 50 µM, the mass decreases (signal increase), indicating disruption of bilayer. This disruption was also observed under confocal microscope using a supported lipid bilayer with fluorescently labeled lipids (Figure 7.2B). There were a few bright circles, representing lipid vesicles or micelles, while no circle was shown for the intact bilayer without adding peptide. Thus the formation of vesicles or micelles indicates the disruption of bilayer due to alamethicin. To better understand the interaction between alamethicin and lipid bilayer, a microfluidic device can be constructed to form fluorescent lipid bilayer on glass substrate, and then flow in peptide solution. Thus the entire process can be monitored under confocal microscope.
7.2. Investigating the Catalytic Activity of Phospholipase A2 with SLBs

Phospholipase A2 is the other biomolecule that is interesting to study with SLBs. It is the enzyme that hydrolyzes the second acyl bond of phospholipids, releasing fatty acid and lysophospholipids. Phospholipase A2 (PLA2) exists widely in mammalian tissues and bee or snake venom. It has been reported that PLA2 activities are related with neural trauma, head injury, and some neurodegenerative diseases, such as Alzheimer’s disease and Parkinson’s disease. Thus the study of PLA2 could help to determine the biological roles of phospholipases A2 in complex cellular processes and might also have a therapeutic potential. Several methods have been used to study the interaction between...
PLA2 and lipids, including a pH assay, fluorescence microscopy, ellipsometry, and quartz crystal microbalance. The PLA2 used in this study is secreted PLA2, requiring the presence of calcium to be catalytic. PLA2 was first added to SLBs without calcium, so the enzyme is non-catalytic, as shown in Figure 7.3. After addition of calcium, PLA2 became catalytic, and the signal is increased, indicating the solubilization of the bilayer. The PLA2 effect was not activated immediately, the lag phase is about 8 min. Since the observed mass loss from the frequency measurement decreases by one half, I hypothesize that only the outer layer of bilayer on sensor surface is hydrolyzed. In addition, the activity of PLA2, as well as peptide-lipid interactions, could be also studied through membrane leakage experiment.

![Figure 7.3](image)

**Figure 7.3.** QCM data for the interaction between PLA2 and lipid bilayer. PLA2 at 10μg/mL was tested. DOPC was used to form bilayer on the sensor surface. After binding of PLA2 on DOPC, calcium at 1μM was added to activate PLA2 (red line). Buffer without calcium was added as control (blue line).
7.2.4. Study of Conformational Change of Amyloid-beta and its Interaction with SLBs

I have studied the conformational change (fibrillation) of amyloid-beta (Aβ), whose deposition in brain is believed to be primary cause of Alzheimer’s disease. The fibrillation of Aβ was first monitored by fluorescence assay using Thioflavin T (ThT) in Figure 7.4A. The increased fluorescence indicates aggregation of Aβ. The soluble oligomers are considered the most toxic species among Aβ conformers.\textsuperscript{20} Though the goal was to form the Aβ oligomer, the dot blot results shown in Figure 7.4B indicate difficulty in forming the oligomer. Because the formation of Aβ oligomer has been proved to be sensitive to several factors, such as buffer composition, temperature, and vibration, the optimization of these conditions might help to form Aβ oligomer. Meanwhile, because of the possible importance of the interactions between Aβ conformers and cell membrane in Aβ toxicity,\textsuperscript{21} the interaction of Aβ with the SLBs were studied in both QCM and microcantilevers, with two conformers tested, monomer and fibril (Figure 7.4C). Although neither monomer nor fibril showed obvious interaction with SLBs, this agreed with the hypothesis that the oligomer might be the primary pathologic species. Future studies with Aβ oligomer using QCM and microcantilevers could detect the interaction between Aβ oligomer and SLBs.
Figure 7.4. A, Fluorescence assay for Aβ fibrillation. Aβ at two concentrations were tested: 10 (green line) and 60μM (red line). Buffer without any Aβ was used as control (blue line). The sample was monitored for one day to observe the change of fluorescence. B, Dot blot for the detection of oligomer formed by Aβ. Two antibodies were used: anti-Aβ (generally sensitive to all the Aβ conformers) and anti-oligomer (only sensitive for oligomer). PBS buffer only was used as negative control, while Aβ monomer is used as positive control. C, QCM (left) and microcantilever (right) results for the interaction between Aβ and SLBs. Two Aβ conformers were available and tested: monomer (blue line) and fibril (red line). POPC was used to form bilayer on both sensors. And the conditions, such as temperature and buffer composition, were the same in two cases.

7.2.5. Other Studies of Molecular Interactions using Microcantilever and QCM

In addition to the SLB interactions with amphipathic peptides and polymers reported in previous chapters, lipidated peptides, which are able to incorporate into lipid membranes and induce liposome fusion, are of interest. The lipid structure of lipidated peptide enables this molecule to controllably interact with lipid membranes, while a pair
of complementary peptides could mimic protein-protein recognition\textsuperscript{23} and induce membrane fusion. The incorporation of lipidated peptides into the SLBs can be investigated using QCM, by monitoring the change of bound mass on crystal surface. The association between the complementary peptides can be also studied in QCM, as well as the further membrane fusion between the SLB and lipid vesicle.

Another interesting and important family of chemicals is glycome, whose diversity is larger than that of proteome and genome\textsuperscript{24}. The carbohydrate interactions with proteins play important roles in various biological processes, such as cell signaling, bacterial pathogenesis, and fertilization\textsuperscript{25}. The multiplex measurement in microcantilevers can be used to efficiently detect the carbohydrate-protein binding. This rapid screening of binding affinity between carbohydrates and proteins help understand the role of glycan in the biological processes.

Finally, the binding of the transcription factor to the operator DNA can be probed in vitro using microcantilever and QCM\textsuperscript{26}. The tetracycline repressor protein and the tetracycline operator DNA are chosen as models because the tetracycline can be used to tune the gene expression that may be further applied to detect the activity of a certain biological system\textsuperscript{27}. The tetracycline repressor protein (\textit{TetR}) binds to the tetracycline operator DNA (\textit{To}) and blocks the following transcription, serving as a negative transcription factor. The inducer tetracycline (\textit{Tc}) is able to bind to the \textit{TetR} at a higher affinity, so the \textit{TetR} is displaced from the \textit{To} and the transcription is resumed. Therefore, the microcantilever and QCM can be used to probe the binding of \textit{TetR} to \textit{To}, the displacement of \textit{TetR} induced by \textit{Tc}, as well as the conformational change of \textit{TetR} upon the bindings. The comparison of results from both in-vitro biosensors and other in-vivo
studies could explain the binding kinetics in the biological process and determine the relative binding affinities. In end, this thesis provided an experimental and theoretical framework in which biomolecular systems on surfaces can be studied.

7.3. References

5. Pera, I.; Stark, R.; Kappl, M.; Butt, H. J.; Benfenati, F., Using the atomic force microscope to study the interaction between two solid supported lipid bilayers and the influence of synapsin I. *Biophysical Journal* 2004, 87 (4), 2446-2455.


A.1. Helical Wheel Diagram for PEP1 Peptide

Figure A.1. The helical wheel diagram of the 31 residue alpha helix peptide, PEP1, with the amino acid sequence: SGSWLRDVWDWICTVLTDFKTWLQSKLDYKD-NH₂. The diagram is used to illustrate the hydrophilicity of the peptide. (See Table A.1 for the color code of this diagram) The PEP1 shows a hydrophobic face as indicated by the arrow and a hydrophilic face on the other side. The plot is generated from the program on the website: http://rzlab.ucr.edu/scripts/wheel/wheel.cgi

A.2. Note on the Helical Wheel Diagram

In Figure A.1, the helical wheel diagram is coded by shape and color. The greenish colors indicate more hydrophobic residues on the amino acids and the reddish colors indicate more hydrophilic residues. The program to generate the plot is created by
Don Armstrong and Raphael Zidovetzki (Department of Cell Biology and Neuroscience, University of California, Riverside)

**Table A.1.** The representative codes of the helical wheel diagram used in Figure A.1.

<table>
<thead>
<tr>
<th>Shapes</th>
<th>Hydrophilic: circle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hydrophobic: diamond</td>
</tr>
<tr>
<td></td>
<td>Potentially negatively charged: triangle</td>
</tr>
<tr>
<td></td>
<td>Potentially positively charged: pentagon</td>
</tr>
<tr>
<td>Colors</td>
<td>Hydrophobic residue: green</td>
</tr>
<tr>
<td></td>
<td>Zero hydrophobicity: yellow.</td>
</tr>
<tr>
<td></td>
<td>Hydrophilic: red (uncharged) and light blue (charged)</td>
</tr>
</tbody>
</table>
Appendix B

B.1. Deduction Steps to Construct Model for Calculation of Association

**Constant \( K_A \).**

The polymer-lipid interaction is actually the association of triblock copolymer with the SLBs on the cantilever surface, which is theoretically studied using a Langmuir isotherm model. The association process is shown as:

\[
S_{POPC} + \text{Polymer} \overset{K_A}{\leftrightarrow} S_{POPC} - \text{Polymer}
\]  

(6.3)

In equation above, \( S_{POPC} \) represents POPC bilayer, polymer represents the Pluronic, and \( K_A \) is the association constant, which allows us to compare the relative affinities of the different Pluronics towards the SLBs. The following assumptions are made in the model: the cantilever surface is totally covered by SLBs before the addition of copolymers; the association of copolymers with the SLBs is monolayer adsorption; the association process reaches equilibrium at a certain time. As a result, the polymer-SLB interaction can be written as a associating/disassociating reaction rate:

\[
k_A[\text{Polymer}](1 - \theta) = k_{-A}\theta
\]  

(6.4)

where \( \theta \) is the fraction of the SLBs with attached polymer, and \( (1 - \theta) \) represents the sites available for further polymer association. Defining \( K_A \) as the ratio of \( k_A \) over \( k_{-A} \), the expression for \( \theta \) is given as:
\[ K_A = \frac{k_A}{k_{-A}} = \frac{\theta}{(1-\theta)[\text{Polymer}]} \rightarrow \theta = \frac{K_A[\text{Polymer}]}{1+K_A[\text{Polymer}]} \]  
(B.1)

Since \( \theta \) is defined as the fraction of the SLB attached with polymer, two expressions for \( \theta \) can be correlated, as follows:

\[ \theta = \frac{\Delta \text{Stress}}{\Delta \text{Stress}_{\text{max}}} \rightarrow \frac{K_A[\text{Polymer}]}{1+K_A[\text{Polymer}]} = \frac{\Delta \text{Stress}}{\Delta \text{Stress}_{\text{max}}} \]  
(B.2)

The following equation describes the relationship between polymer concentration and the surface stress change measured on cantilever:

\[ \frac{1}{\Delta \text{Stress}} = \frac{1}{\Delta \text{Stress}_{\text{max}}} + \frac{1}{\Delta \text{Stress}_{\text{max}}K_A[\text{Polymer}]} \]  
(B.3)

\[ \rightarrow \frac{[\text{Polymer}]}{\Delta \text{Stress}} = \frac{[\text{Polymer}]}{\Delta \text{Stress}_{\text{max}}} + \frac{1}{\Delta \text{Stress}_{\text{max}}K_A} \]  
(6.5)

where \([\text{Polymer}]\) is the molar concentration of triblock copolymers. \(\Delta \text{Stress}\) is the change of surface stress caused by polymer and \(\Delta \text{Stress}_{\text{max}}\) is the maximum change in surface stress when the polymer saturates the surface. \(K_A\) is obtained from the plot of \([\text{Polymer}] / \Delta \text{Stress}\) with respect to \([\text{Polymer}]\).

Figure B.1 shows the plots of \([\text{Polymer}] / \Delta \text{Stress}\) with respect to \([\text{Polymer}]\) for F68 and F98 at various temperatures. In each plot four data points are used to optimize the linear data fitting. From the slope and intercept of each plot, the association constant \(K_A\) is calculated (Table 6.2). The values of \(K_A\) represent the relative solubility of each copolymer with the POPC SLBs, with the largest value corresponding to the highest solubility.
B.2. The Deduction Steps in Free Area Model.

A free area model is used to characterize the lipid diffusion in a SLB.\(^{5-6}\) In this model, the lateral diffusion of molecules was considered to be a two-dimensional random motion. To move, a lipid needs to meet two requirements: a minimum empty surrounding area and a sufficient activation energy.\(^{7-8}\) Therefore, the diffusion coefficient can be affected by two possibilities:

\[
D = D'p(a)p(E)
\]  \hspace{1cm} (6.6)

In Equation 6.6, \(p(a)\) is the Boltzmann distribution of a lipid with minimum free surrounding area \(a_f\) and \(p(E)\) is the probability that the activation energy is sufficient.

\[
p(a) = \exp\left(-\frac{a_0}{a(T) - a_0}\right)
\]  \hspace{1cm} (B.4)

\[
p(E) = \exp\left(-\frac{E_a}{kT}\right)
\]  \hspace{1cm} (B.5)

In the equations above, \(a(T)\) is average lipid area, \(a_0\) is critical area of lipid when it is closely packed, \(E_a\) is activation energy, and \(k\) is Boltzmann constant. Thus the average free area of lipids is \(a_f = a(T) - a_0\). Detailed derivation of this model has been previously reported by Reits \textit{et al}.\(^9\) In two dimensions, the preexponential factor \(D'\) is shown as:

\[
D' = \frac{\delta^2}{4\tau}
\]  \hspace{1cm} (B.6)
where $\delta$ is the distance between molecules and $\tau$ is the time for each movement. Therefore, the average velocity of molecular movement is $v = \delta / \tau$. The velocity $v$ can be further related to the thermal energy through following equation.

$$E_k = \frac{1}{2} m v^2$$

(B.7)

The mass $m$ can also be expressed by the average molecular weight $M$ via $m = M / N_a$, where $N_a$ is Avogadro’s constant. The distance $\delta$ is calculated through $\delta = [a(T)]^{1/2}$. Thus the preexponential factor $D'$ is finally expressed as:

$$D' = \frac{\delta}{2\sqrt{2'}} \sqrt{\frac{kT}{m}} = \frac{1}{\sqrt{8}} \sqrt{\frac{kN_a a(T) T}{M}}$$

(B.8)

In a two-dimensional SLB, the diffusion coefficient of lipids can be expressed as:

$$D = \sqrt{\frac{kN_a}{8}} \sqrt{\frac{T a(T)}{M}} \exp \left( \frac{-a_0}{kT} - \frac{E_a}{kT} \right)$$

(6.7)

The equation above is only valid for pure lipids. However, with the association of the triblock copolymer with the SLBs, the effects of the polymer on lipid diffusion must be considered, which are the decrease of free area and change of activation energy. Two parameters, average polymer area $a_{\text{poly}}$ and molar ratio of polymer to lipid $n$, are introduced. The expressions for the average free area of lipids $a_f$ and the average molecular weight $M$ are corrected, as follows:

$$a_f = a(T) - a_0 - n a_{\text{poly}}$$

(B.9)

$$M = M_{\text{lipid}} + n M_{\text{polymer}}$$

(B.10)
Substituting two equations above into Equation 6.7, the general expression for the diffusion coefficient of lipids, no matter whether polymer is present or not, is given by

\[
D = \sqrt{\frac{kN_a}{8}} \sqrt{\frac{T a(T)}{(1-n)M_{lipid} + n M_{polymer}}} \exp \left( \frac{-a_0}{a(T) - a_0 - na_{poly}^0} - \frac{E_a}{kT} \right)
\]  

(6.8)

The values of parameters in the equations above are estimated based on their values in literatures. The activation energy \( E_a \) and molar ratio of polymer to lipid \( n \) are fitting parameters, obtained from nonlinear least square fitting. Figure 6.6 shows the fitting of the free area model to the experimental data, and Table 6.3 gives the molar ratio of polymer over lipid \( n \).

![Graphs showing association constant Ki for copolymers F68 and F98 at various temperatures.](image)

**Figure B.1.** Determination of the association constant \( K_i \) for the copolymers F68 (top) and F98 (bottom) at various temperatures. Experimental results for \([\text{Polymer}] / \Delta \text{Stress}\) is plotted with respect to \([\text{Polymer}]\) and fit to Equation 6.5. The equations for trend lines and R-squared values are shown.
B.3. References