When the Nazis came for the communists, I remained silent;
I was not a communist.
When they locked up the social democrats, I remained silent;
I was not a social democrat.
When they came for the trade unionists, I did not speak out;
I was not a trade unionist.
When they came for the Jews, I remained silent;
I was not a Jew.
When they came for me, there was no one left to speak out.
- Martin Niemöller

Life is no brief candle to me. It is a sort of splendid torch which I have got a hold of for the moment, and I want to make it burn as brightly as possible before handing it on to future generations.
- George Bernard Shaw

University of Alberta

Use of surfaces functionalized with phage tailspike proteins to capture and detect bacteria in biosensors and bioassays

by

Sarang Dutt

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- Dr. Stephane Evoy
- Dr. Christine Szymanski
- Dr. Jie Chen

I dedicate this thesis to Shiv and Purnima Dutt, wonderful parents, whose endless love and support made this possible.

Abstract

The food safety and human diagnostics markets are in need of faster working, reliable, sensitive, specific, low cost bioassays and biosensors for bacterial detection. This thesis reports the use of P22 bacteriophage tailspike proteins (TSP) immobilized on silanized silicon surfaces, roughened at a nano-scale, for specific capture and detection of *Salmonella*.

Towards developing TSP biosensors, TSP immobilization characteristics were studied, and methods to improve bacterial capture were explored. Atomic force microscopy was used to count TSP immobilized on gold thin-films. Surface density counts are dependent on the immobilization scheme used.

TSP immobilized on flat silicon (Si), silanized with 3-aminopropyltriethoxysilane and activated with glutaraldehyde, showed half the bacterial capture of gold thin-films. To improve bacterial capture, roughened mountain-shaped ridge-covered silicon (MSRCS) surfaces were coated with TSP and tested. Measurements of their bacterial surface density show that such MSRCS surfaces can produce bacterial capture close to or better than TSP-coated gold thin-films.

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To all the people I mentioned in here, and those I missed from this list:
Thank you.

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List of Abbreviations and Elemental Names

AFM	Atomic force microscopy
APTES	3-aminopropyltriethoxysilane
Au	Gold
BCD	Bacterial capture density
BSA	Bovine serum album
CDC	Centers for Disease Control and Prevention
cDNA	Complementary DNA
cfu	Colony forming units
Co	Cobalt
DNA	Deoxyribonucleic acid
EDC	1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride
ELISA	Enzyme-linked immunosorbant assay
Fab	Antigen binding fragment
Fc	Crystallizable fragment
FRET	Fluorescence resonance energy transfer
GA	Glutaraldehyde
HOPG	Highly ordered pyrolytic graphite
Ig	Immunoglobulin
IMS	Immunomagnetic separation
LB	Lysogeny broth, also known as Luria Bertani broth
MEMS	Microelectromechanical systems
MPTES	Mercapto-propyl triethoxysilane

MSRCS	Mountain-shaped ridge-covered silicon
MSRV	Modified semi-solid Rappaport-Vassiliadis medium
NB	Nutrient broth
NHS	*N*-hydroxysulfosuccinimide
Ni	Nickel
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
QCM	Quartz crystal microbalance
RNA	Ribonucleic acid
RT-PCR	Reverse Transcriptase polymerase chain reaction
SAM	Self assembled monolayer
SAW	Surface acoustic wave
SEM	Scanning electron microscopy
Si	Silicon
SPR	Surface plasmon resonance
ssDNA	Single stranded DNA
Sulfo-NHS	N-hydroxysulfosuccinimide
TSP	Tailspike protein
TCEP	*tris*(2-carboxyethyl)phosphine

1

Bacteria and their detection

1.1. Introduction

Bacteria can be found almost everywhere on Earth, in places where oxygen is present and even where it is not. Not all are pathogenic (infectious disease causing) and they can even be found in human beings. Though many are yet to be identified, the most common residing in the human colon include bacteria of genera: *bacteroides*, *bifidobacterium*, *eubacterium*, *clostridium*, *peptococcus*, *peptostreptococcus*, and *ruminococcus* [(1)]. They aid vital functions including fermentation of carbohydrates, synthesis of vitamins, and absorption of ions such as calcium, magnesium and iron.

There are also many bacteria that are disease causing. Some can be fatal to human beings. Famous examples are: *Yersinia pestis* which causes Bubonic Plague; *Mycobacterium tuberculosis* which causes Tuberculosis; *Clostridium tetani* which causes Tetanus; *Salmonella* Typhi which causes Typhoid Fever; and *Treponema pallidum* which is responsible for Syphilis.

From a medical treatment perspective, doctors want to prescribe medicines that target pathogenic bacteria while being harmless to those that are useful to the human body. Pharmaceutical companies try to develop drugs that only target pathogenic bacteria to address this need. In

the first step to this process, the pathogen affecting a patient needs to be identified. Techniques for bacterial identification are consequently important to public health and pharmaceutical industries.

Bacterial detection is also vital in the food industry. For example, Centers for Disease Control and Prevention (CDC) reported that between April 16th and August 11th, 2008, 1442 people in USA were infected with the rare Saintpaul serotype of *Salmonella enterica* [2]. The source of the infection was food produce: jalapeno and serrano peppers. *Salmonella*, *Listeria*, *Campylobacter*, and *E.coli* are important groups of microorganisms that are known for food-related outbreaks, illnesses and deaths [3; 4]. Testing of food products by manufacturers and distributors prior to distribution of food can prevent outbreaks. This is not always done, particularly at distribution centers. Testing programs are expensive and require costs of testing (incl. testing kits and labour) to be low. Testing tools need to be simple enough for users to accept them, for policy makers to implement them in testing programs, and for state/provincial inspectors to enforce their use. Long wait times for test results (to measure food quality and to see if safety standards are met) can also cause loss of product and raw material, product recalls, and heavy financial burdens for food product manufacturers and distributors.

From military and law enforcement perspectives, biological weapons and bioterrorism raise many applications for bacterial detection. In 1984, 751 people were infected with *Salmonella enterica* subspecies *enterica* serovar Typhimurium (also referred to as *Salmonella* Typhimurium or *S*. Typhimurium) due to intentional contamination of food at restaurant salad bars [5]. Additionally, in 2001, 10 cases of inhalational anthrax were caused by intentional delivery of *Bacillus anthracis* through mailed letters or packages [6]. Bacteria are easy to culture, grow, and mass produce. This increases the risk of similar bio-terror attacks. These examples provide ample reason for authorities to be concerned. The CDC

reported "that the most critical component for bioterrorism outbreak detection and reporting is the frontline healthcare professional and the local health departments. Bioterrorism preparedness should emphasize education and support of this frontline as well as methods to shorten the time between outbreak and reporting." [7] However, support to this frontline must include the tools used to diagnose and detect bacterial pathogens. These tools need to produce quick, accurate, and repeatable results to allow timely and reliable reporting. There are major implications for public health if certain pathogens are not detected accurately or quickly enough. This need for pathogen detection methods that provide fast results, with high sensitivity, specificity, and accuracy, arouses much interest in this line of research.

1.2. Established pathogen detection methods

Currently there are few pathogen screening methods and they often have many steps for sample preparation and analysis. These methods can be time consuming, complex, and/ or expensive. Such disadvantages are commonly accepted as tradeoffs for higher sensitivity and selectivity.

1.2.1. Culturing methods & colony counting techniques

Bacterial culturing and plate counting is the oldest technique for detecting pathogenic bacteria. It involves culturing bacteria in a sample by incubating it under optimum growth conditions for a period of time in a growth medium. A small volume of the enriched bacterial solution is diluted several times, and measured droplets of each of the dilutions are placed on culture plates. These plates are also pre-coated with selective and differential growth media [8; 9].

Depending on the goals of a culture technique, a chosen growth medium can be non-selective, selective, or differential. Non-selective media equally enrich all bacteria in a sample. Selective media allow targeted bacteria to be grown, while others are killed or their growth will be inhibited. Differential agar plating media allows targeted bacteria to be

visually detected upon plating. For example, when a *Salmonella* species is cultured on a bismuth sulfite agar coated plate, the *Salmonella* colonies become black because bismuth sulfate reduces to a sulfide [9].

After exposure to the placed droplets, culture plates are incubated so bacteria from the droplets reproduce until single colonies are visible. Colonies of predicted colour at the placed droplet sites are counted to measure the concentration of the enriched solution. Each colony is called a colony forming unit (CFU) and is assumed to have originated from a single bacterium. Since the volume of the placed droplet was measured, units for measuring bacterial concentration are: colony forming units per unit volume (e.g. cfu/ml). This unit, together with colony colour, are measurable indicators of bacterial contamination of the source or sample.

The plating medium's selectivity also affects accuracy of test results and time taken to acquire them [10]. In tests for *Salmonella*, modified semi-solid Rappaport-Vassiliadis (MSRV) medium can be used to obtain results within 48 hours. This is an improvement over use of LJ media for isolation and detection of *Salmonella* [9; 11]. DIASALM agar, an alternative to MSRV medium, also provides faster results than LJ media [9; 12].

An enrichment step is usually performed prior to plating. Though such steps raise the technique's sensitivity, speed is traded off in return. Culturing methods can take several weeks depending on the species being isolated and detected. Also, improvements in time taken to get positive results in culturing methods often come with loss in sensitivity and increased risk of false-negative results. Due to issues with speed, genetic and immunological methods are often preferred over culturing techniques.

1.2.2. Polymerase Chain Reaction (PCR) and related methods

PCR-based methods first appeared in the '80s and are now often used for bacterial detection [3; 13]. They are popular for their exquisite sensitivity and speed. Several versions of PCR have been developed. This includes real-time PCR [14], multiplex PCR [15], and reverse transcriptase

PCR (RT-PCR) [16]. These can also be combined with other techniques such as surface acoustic wave (SAW) sensors [16] and evanescent wave biosensors [17].

In PCR target portion(s) of DNA material taken from a biological sample are amplified. Tools such as agarose gel electrophoresis and microarrays allow the amplified DNA to be detected and identified. Time taken to acquire a detection result is dependent upon the version of PCR being used. The process usually takes less than 24 hours [3]. This is a significant improvement over plating and culturing methods.

DNA microarrays are typically glass or silicon substrates with patterned arrays of square or circular spots. Patterning of the arrays of spots is usually done using photolithography. Each spot has a different type of probe designed to capture a target DNA sequence [18]. This technology can be used for genetic sequence comparisons and checking gene expression [19].

PCR requires thermal cycling of ingredients: heat-stable DNA polymerase enzymes, free nucleotides, and small strands of DNA called primers [20]. Nucleotides are the building blocks of DNA. Primers act as ‘starting points’ from which the newly copied DNA chains are built. The polymerase enzymatically assembles new identical DNA strands from free nucleotides and primers during heat cycling. The new DNA strands are identical copies of a template single-stranded DNA acquired from the sample being amplified.

This method also has issues. Food and environmental samples require sample preparation since PCR’s sensitivity to impurities can generate false positive results. A few procedures can be applied to reduce contamination such as physical isolation of PCR equipment, and use of negative controls and clean sample preparation spaces in laboratories housing PCR equipment [21; 22; 23]. In setups where several different PCR reactions and simultaneous running of gels are being conducted, costs and laboratory space occupied by reagents, disposable materials (e.g.

PCR tubes and pipettor tips), and gel electrophoresis equipment for PCR can add up [22; 23]. Lower cost and more consistently reliable techniques are desired for pathogen detection applications.

1.2.3. Immunology-based approaches

A number of current methods involve labelling of chemical markers or proteins (including enzymes and antibodies) as part of sample preparation and/ or analysis [3; 24]. Immunoassays and protein microarrays are two types of established immunology-based detection methods that apply labelling schemes [25]. Use of immunomagnetic separation (IMS) as a pre-treatment &/or pre-concentration step [26], followed by methods such as optical and magnetic force microscopy, has been reported.

Immunoassays work by taking advantage of the affinity that antibodies have for specific target antigens in a sample. For details on structure of antibodies, refer to section 2.3.

ELISA (enzyme-linked immunosorbent assay) [27] is an example of an immunoassay. There are three basic configurations of ELISA: Direct ELISA, Indirect ELISA, and Sandwich ELISA. Each has many variations.

In Direct ELISA, the antigen is first immobilized onto a polystyrene microtiter plate by passive adsorption and incubation [27]. These plastic microtiter plates typically contain 96 wells in a 12 x 8 format. Each well is approximately 5 mm deep x 8 mm diameter. The wells are rinsed to remove unattached antigen. Antibody solution is poured into each well followed by incubation. The antibody is selected to target only to the desired antigen and is labelled with an enzyme. The antibody solution also contains a high concentration of blocking protein.

The blocking reagent or protein is selected such that it has no affinity to the antibody, the enzyme, its substrate (the molecule or substance that an enzyme acts upon), or the antigen. Bovine serum albumin (BSA) is an example of a blocking protein. The wells are washed again and a specific substrate that changes colour due to enzymatic

catalysis is added. Alternatively, a substrate and a colourless chromophore (dye solution) that changes colour owing to enzymatic catalysis can be added to the well.

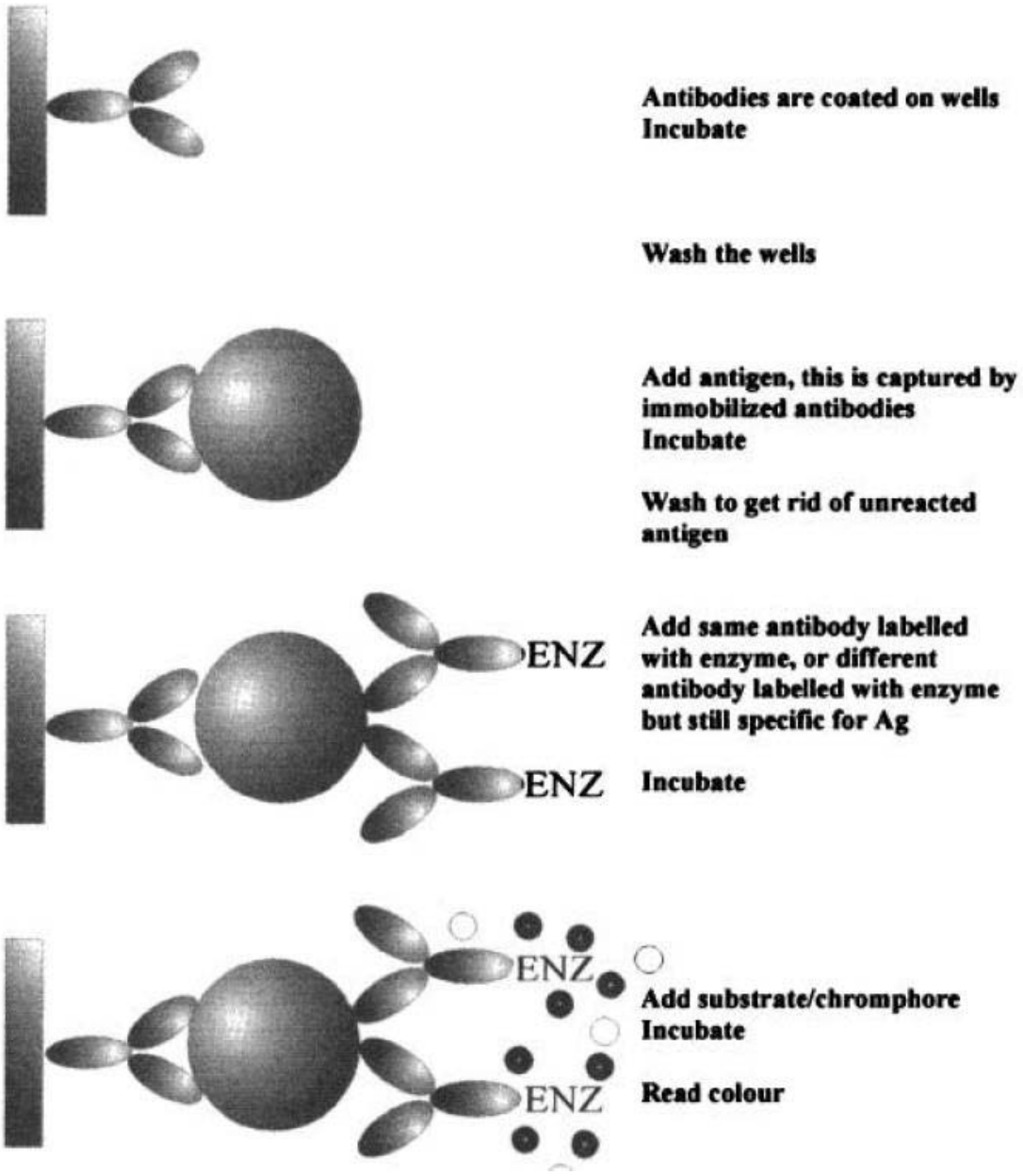

Figure 1: Sandwich ELISA – direct [27]. *Molecular Biomethods Handbook (1998), pg. 601, Chapter 8 – Enzyme-Linked Immunosorbent Assay (ELISA), John R. Crowther, Fig. 4, Copyright © Humana Press Inc. Printed with kind permission of Springer Science+Business Media.*

Washing or rinsing steps are sometimes done using detergents such as Tween20. The colour change is measured with a spectrophotometer.

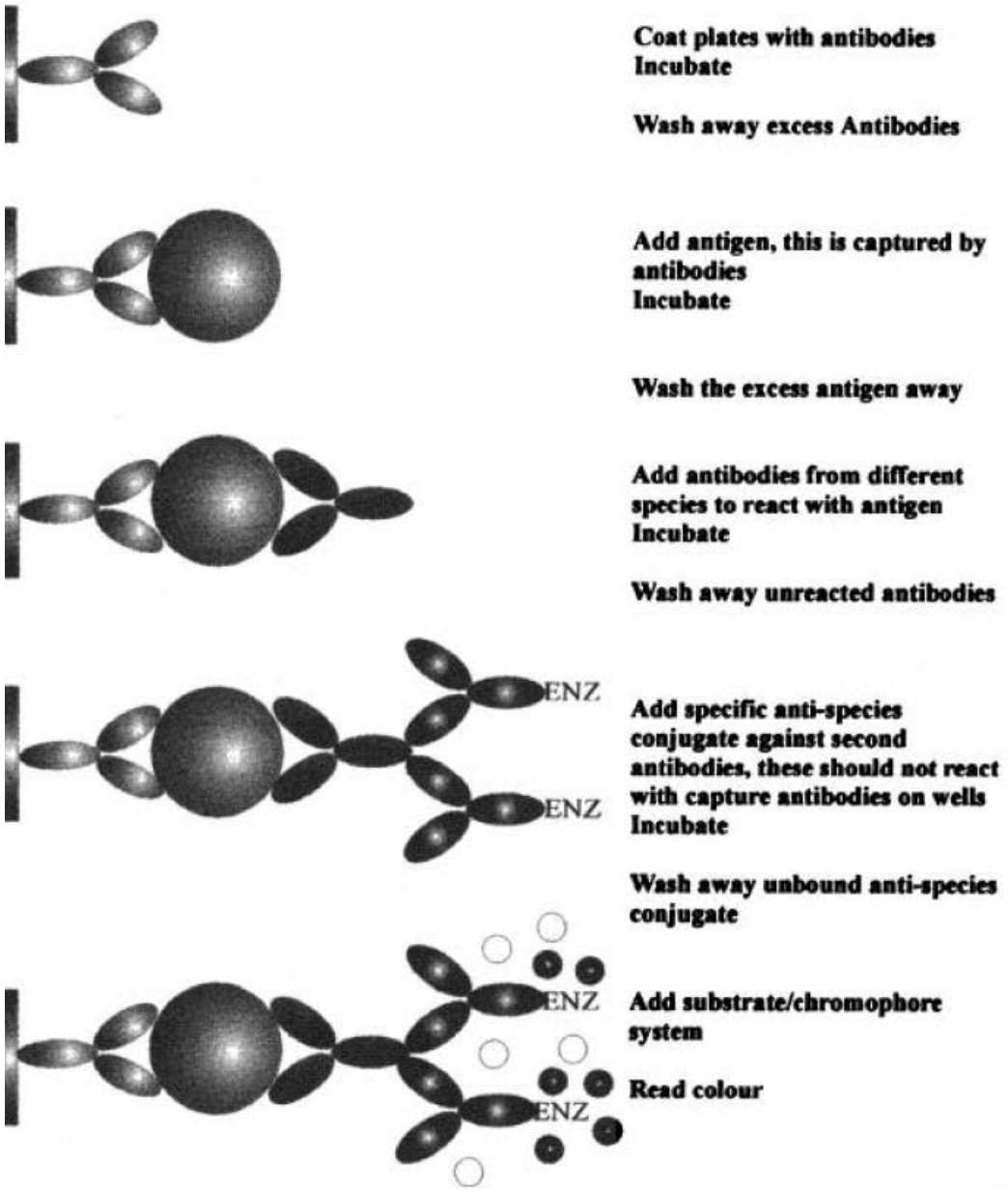

Figure 2: Sandwich ELISA – indirect [27]. *Molecular Biomethods Handbook (1998), pg. 602, Chapter 8 – Enzyme-Linked Immunosorbent Assay (ELISA), John R. Crowther, Fig. 5, Copyright © Humana Press Inc. Printed with kind permission of Springer Science+Business Media.*

Indirect and direct ELISAs have similar steps until the point where the antibody solution is added. The antibodies are not labelled with an enzyme. After adding the antibody (in solution with a buffer protein) the microtiter plate is incubated. Next, an anti-species antibody labelled with an enzyme is added. The antispecies and enzyme are together known as an antispecies conjugate. The antispecies antibody is selected or created such that it is only specificity to the antibody already bound to the antigen. The plate is again incubated and washed. A colour-changing substrate, or a substrate and chromophore are added, and the colour change is measured with a spectrophotometer.

Figures 1 and 2 show two types of sandwich ELISAs: direct and indirect. The difference between them also shows the difference between direct and indirect ELISAs discussed earlier. ELISA often suffers from issues relating to antibody stability and specificity [(9)].

Protein microarrays are useful for high throughput study of protein functions. They are similar to DNA microarrays. However, instead of DNA each spot has a different type of protein printed into it in high spatial density [(25)].

Immunoprecipitation (IP) and yeast two-hybrid (YTH) systems are other examples of biosensing techniques that use labelling schemes [(25)]. The western blot is an example of an IP-based technique used to identify proteins in a sample. In it gel electrophoresis is performed on the sample. The gel and its contents are exposed to target-specific antibodies on a membrane. A colour change occurs on the membrane upon exposure to the target protein [(28)].

Immunomagnetic separation (IMS) is a method where antibody-coated or enzyme-coated magnetic particles are suspended in a solution containing mixed cells. If the protein or chemical layer coating the particles has an affinity or specificity to the target cell type’s receptors, the magnetic particles in the suspension will likely bind to the target cells during incubation. Most types of particles used for this technique are super-

paramagnetic, i.e. they only exhibit magnetic properties when exposed to an external magnetic field. A magnetic separator can be used for removing the magnetic particles and bound target cells from the suspension [24] (29).

As discussed, techniques such as ELISA and IMS require immunolabelling. Labelling increases time taken and cost for analysis. Labels are also often bound to antigens or antibodies in a random manner. Depending on the site the label is bound to, it can interfere with chemical functioning of the protein or antigen being analysed. This can reduce one's ability to perform certain studies or experiments on a sample. To ensure specific binding, a labelling scheme should only label the specific desired target and not other molecules that are also present in the sample. Therefore, it may be necessary to create new labels and new labelling methods for newly discovered proteins, molecules or antigens (30). This can require significant time, labour and funding. For these reasons, techniques relying on biosensors are considered the future for pathogen detection.

1.2.4. Commercially available products

There are a number of products currently available on the market for bacterial pathogen detection. Majority of them fall under the categories of: classical cell culture and plating methods, immunoassays, and PCR or genetic methods. Manufacturers include *3M*, *Dupont*, *Neogen*, *Applied Biosystems* (a division of *Life Technologies Corp*.), and Qualicon. Examples of commercial portable test systems for the detection of *Salmonella* in food include: the TECRA Unique test (by *3M*), and the TaqMan system (by *Qiagen*). Available products and current manufacturers are by no means limited to this list.

The Tecra system is based on ELISA-like approach where a dipstick exposed to anti-*Salmonella* antibodies is used to capture bacteria and provide a colour change to indicate the presence of the *Salmonella* strain or lack thereof (31; 32). The TaqMan system is a PCR-based product (33; 34).

Although such dipstick-based products are simple, colour changes require the presence of sufficient antigen to produce them. This results in low test sensitivity. The low sensitivity can be compensated for by using a bacterial pre-concentration step. However, pre-concentration steps typically require the sample to be incubated in a growth medium, thereby trading off speed and simplicity for sensitivity. This problem is pervasive among pathogen detection methods.

For pathogen detection applications, new products that are faster, lower cost, simpler, and more sensitive than those currently available are necessary.

1.3. Summary

Although there are many products available presently, they are based on a limited number of detection methods. Those methods are also accompanied by various challenges and issues. Plating and colony counting methods are unsuitable for applications where speed is of the essence. Use of PCR-based techniques for extensive testing purposes can be expensive; the technology is also highly sensitive to impurities. Immunoassays and immunology-based techniques often require labelling and involve several steps to the procedure. Furthermore, development of new assays often requires development of new labels. For high-throughput and time-critical applications, ELISA is not a suitable solution. As a result of these issues, an alternative technology is necessary – one that is portable, simple to use, sensitive, offers high specificity to the target antigen(s), and provides reliable and accurate results within a couple of hours. Biosensors are one of the research areas currently being explored for a more ideal solution.

2

Biosensors

2.1. Definition

A biosensor has two parts: a transduction platform and a biological recognition element. The transduction platform or method translates a measurable property into data and outputs it as a meaningful electrical signal. Various transduction methods have been developed to detect bacteria. On their own, transduction methods lack the ability to distinguish between or identify pathogens. The recognition element or probe provides the ability to distinguish between the target and other contaminants in a sample. It ensures that the transducer only measures or detects a target that it 'recognizes'. This concept also applies to many immuno and bioassays.

Similar to the above explanation, the Biosensors and Bioelectronics journal defines biosensors as "analytical devices incorporating a biological material (e.g. tissue, microorganisms, organelles, cell receptors, enzymes, antibodies, nucleic acids, natural products etc.), a biologically derived material (e.g. recombinant antibodies, engineered proteins, aptamers etc) or a biomimic (e.g. synthetic receptors, biomimetic catalysts, combinatorial ligands, imprinted polymers etc) intimately associated with or integrated within a physicochemical transducer or transducing microsystem, which may be optical, electrochemical, thermometric, piezoelectric, magnetic or micromechanical." [35]

2.2. Transduction methods and biosensor platforms

Various types of transduction methods exist for biosensors. They include: optical methods such as fluorescence detection and SPR; piezoelectric methods such as QCM; electrochemical methods such as amperometric and potentiometric biosensors, and electrochemical impedance spectroscopy (EIS) [3]. Microelectromechanical system (MEMS) based devices such as cantilever-based biosensors and microfluidic lab-on-a-chip type sensors are newer research trends. Fluorescence detection, SPR and QCM are further explained below.

2.2.1. Fluorescence-based methods

A valence electron is elevated from its ground state into an excited state when it absorbs heat or light of sufficient energy. Excess energy can be released in the form of photons. The valence electron loses its excess energy and returns to its ground state by giving off a photon. Emitted light is of a longer wavelength than the absorbed light. This phenomenon is called fluorescence. Fluorescence detection exploits this behaviour to detect biomolecules and can be combined with many different platforms, such as flow-cytometry, DNA microarrays, and fluorescence resonance energy transfer (FRET) based biosensors [36]. Fluorescence microscopy is one of the most well known applications of this detection method. Labelling compounds used with these platforms can have various wavelengths of absorption and emission. Fluorescein isothiocyanate (FITC) [37] is one of many such compounds available for purchase.

2.2.2. Surface Plasmon Resonance (SPR)

Surface plasmon resonance (SPR) is a tool for measuring adsorption of substances onto flat surfaces such as gold thin-films. The research community has great interest in SPR as a foundation for biosensor applications.

Surface plasmons are surface electromagnetic waves that propagate parallel to the surface of the substrate [38]. When excited by a

beam of electrons or light in a resonant manner, adsorption of molecules such as proteins to the metal surface induces a change in the local index of refraction. This in turn causes a change in the angle of reflection at which reflection intensity is minimal. The angle of reflection minimum is also known as the SPR minimum. Change in the SPR minimum is recorded and is a measure of amount of adsorption of molecules on a substrate. Various biosensors applying this transduction platform have been researched. Some of this research is focused on the detection of S*almonella* [39; 40; 41]. A disadvantage of SPR is that only conductive surfaces such as metals or metal-like substrates can be used, e.g. gold thin-films on silicon (Si). Plain Si wafers can consequently not be used as SPR sample substrates.

2.2.3. Quartz Crystal Microbalance (QCM)

Quartz Crystal Microbalance (QCM) is a commercially available piezoelectric technology. It measures changes in mass on the transducer's surface as a function of change in resonance frequency [42]. Numerous sensors with varying antibody immobilization strategies have been demonstrated for QCM and similar techniques [43; 44]. For example, use of thiolated anti-*Salmonella* antibodies to coat a QCM gold electrode for the specific detection of *Salmonella* has been reported by Park and Kim (1998) [45]. However, detection capability of QCM in this application was limited to a cell concentration ranging from $9.9x10^5$ to $1.8x10^8$ CFU/ml. Typical materials safety data sheets (MSDS) on *S.* Typhimurium tell users that consumption of 100 *Salmonella* Typhimurium bacteria are sufficient to induce illness. The detection limit of QCM was clearly insufficient in this application since lower concentrations of *Salmonella* would be missed without a pre-amplification or pre-concentration step. The addition of such a step slows the testing process and carries potential for errors and false results. Using IMS for pre-concentration, Su and Li (2005) demonstrated a passable detection limit of 10^2 CFU/ml [44].

2.3. Specificity: Common recognition elements and their immobilization

To recap: Transduction methods do not have the ability to distinguish pathogens. They are used to measure a property with which presence of a target antigen can be measured or detected. Specificity, the ability to distinguish the target pathogen from other pathogens or substances present in the sample, is provided by the recognition element used.

2.3.1. Common recognition elements

Specificity is an important aspect of pathogen detection. If a biosensor were used on a sample containing multiple species, it should only detect the desired target(s) and ignore other contaminating species.

To ensure specificity one or more recognition elements that 'recognize' some aspect or property of the target bacteria must be used. The most common recognition elements used in biosensors are: nucleic acids (DNA or RNA), and antibodies [(3)]. They can be used separately or in combination as part of the pathogen detection and identification process. Although enzymes are sometimes used as recognition elements, they are more often used as labels or as part of the recognition mechanism.

(a) DNA as a recognition element

DNA microarrays (introduced in section 1.2.2), also known as "Gene chips", are a popular technology and are an excellent example of a biosensor where the recognition element or probe is a DNA or nucleotide sequence. Gene chips take advantage of the affinity that complementary DNA (cDNA) strands have for one another. The probes can be long (500-1500bp) single stranded DNA (ssDNA) sequences or shorter (25-70 mer) oligonucleotide sequences. They are covalently bonded to the surface [(18)]. Probes bind to their complementary nucleic acid sequences when they are exposed to the sample. Microarrays can be imaged after exposure to the target DNA sequence (followed by washing to eliminate nonspecific binding) using florescent or radioactive dyes.

This tool is more expensive than other alternatives such as ELISA test kits when used for small numbers of experiments. However, it becomes a lower cost and more appropriate solution for applications needing high throughput screening.

(b) Antibodies as recognition elements

To understand how antibodies can be used as recognition elements what they are, how they are made, their function and structure should be better understood. Figure 3 shows the structure of one type of immunoglobulin (Ig) or antibody, called immunoglobulin G (IgG) [46].

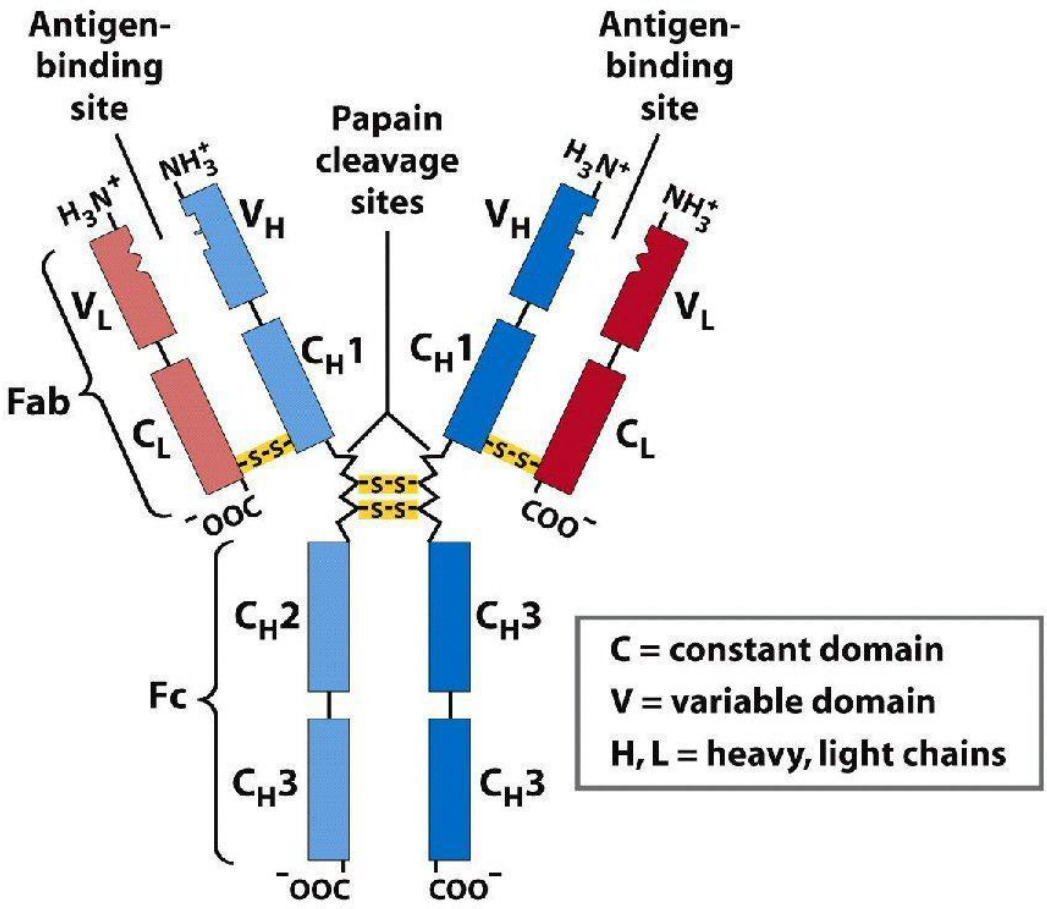

Figure 3: Immunoglobulin G (IgG) antibody [46].

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Antibodies are produced in animals in reaction to the presence of molecules recognized as ‘foreign’ by the animal’s immune system. Their function is to bind to these foreign molecules and facilitate their removal from the animal’s body. B lymphocytes that interact with immunogens (molecules that cause the immune system to react) secrete antibodies into the plasma [47]. Antibodies are manufactured by repeatedly exposing host animals (e.g. cats, chickens, dogs, goats, horses, rabbits, etc.) to immunogens. The animal’s serum is collected and purified to separate the desired antibodies. Antibodies produced from serum are polyclonal – i.e. they are produced by multiple B lymphocyte cell lines. Consequently, polyclonal antibodies can have specificity for multiple immunogens. In contrast, monoclonal antibodies are produced by a single B lymphocyte cultured cell line and have higher target specificity.

The monomeric Ig molecule is a Y shaped glycoprotein with a molecular weight around 150 kDA. Most antibodies typically have a similar structure. They have two identical halves, bound by two disulphide bonds. Each half is composed of a light chain, weighing approximately 25 kDa, and a heavy chain, approximately 50 kDa. Igs have two types of domains: Fab, the antigen binding fragment; and Fc, the crystallisable fragment. The two Fab domains each provide an antigen binding site and are composed of both a light and heavy chain. The Fc domain is composed of only heavy chains and provides two carboxyl groups for binding purposes. Antibodies are also divided into classes such as IgA, IgD, IgE, IgG, IgM, and IgY [47]. The class is identified by specific amino acid sequences in an antibody’s Fc domain.

The antibody should preferably be immobilized onto surfaces using the carboxyl groups on the Fc domain in biosensing applications. This will orient the antigen binding sites away from the surface and give bacteria in the sample steric access to the binding sites [48]. Multiple orientations reduce antigen binding capacity.

A novel FRET-based biosensor using antibodies as a recognition element was developed in 2003 [36]. The FRET biosensor used enzymatic proteins such as proteins A & G (tagged with acceptor fluorophores). The proteins were bound to the Fc domains of anti-*Salmonella* and anti-*listeria* IgG antibodies. These antibodies are also tagged with donor fluorophores on their Fab domains. These proteins were bound to the antibodies to form conjugate antibodies. When exposed to the target antigen a conformational change occurred in the protein complex which caused the donor and acceptor fluorophores to move closer to one another. The change in distance between the fluorophores caused a change in fluorescence wavelength between the acceptor and donor fluorophores. This change in wavelength was used to indicate the presence of the target pathogen.

Another report of the use of antibodies as recognition elements was an immunoelectrochemical biosensor coupled with IMS for detecting *S.* Typhimurium. The method involved mixing samples with: magnetic beads coated with *S.* Typhimurium antibodies for separation of *Salmonella* from the sample; and alkaline phosphatase-labelled *S.* Typhimurium antibodies [49]. Use of *S.* Typhimurium antibodies ensured specificity of testing to *Salmonella* Typhimurium.

Protein and antibody microarrays have applications in autoantibody profiling, cancer research and signal pathway characterization. They are not a mature technology [50]. These microarrays are expensive to manufacture in comparison to gene chips. Producing proteins and antibodies is not as simple as amplifying DNA using PCR [48]. Antibodies cannot be ‘grown’ or replicated in the same manner that conventional proteins can be (by inserting the genes coding the protein into a bacterium and selectively amplifying it). This makes antibody production the slowest and most expensive.

2.3.2. Immobilization of recognition elements

Recognition elements typically need to be immobilized on a substrate. Broadly defined, there are three approaches that can be used for immobilization: physical immobilization, covalent immobilization, and immobilization using bio-affinity [51]. These approaches can be implemented using the following modification strategies: (i) modifying the probe to allow/improve immobilization chemistry, (ii) modifying chemistry of the surface onto which the probe will be adsorbed, (iii) modifying material properties of the surface (such as increasing surface area, roughness, and applying patterning techniques to influence molecular force related interactions) [52].

Physical immobilization involves exploiting intermolecular forces, such as ionic bonds, van der Waals forces, hydrogen bonds, and hydrophobic and polar interactions to induce binding of probes onto surfaces. In any physical immobilization protocol, the types of intermolecular forces that influence adsorption depend on the probe's properties and those of the surface it is adsorbing onto. The disadvantages of this approach are that probes are randomly oriented and weakly attached. Washing with buffers or detergents can remove proteins causing poor, non-uniform probe distribution on the surface [51].

Another issue with physical immobilization is nonspecific binding. Since binding in physical immobilization is not specific, nearly any substance or impurity present in the sample can adsorb onto the surface. If nonspecific binding is not minimized during experiments, results can include false positives for the presence of the target. Non-specific binding can be reduced by exposing the surface to a blocking protein or reagent (defined in section 1.2.3).

Blocking protein is usually used after covalent immobilization of a probe such as an antibody or ss-DNA onto a surface. The surface would then be washed to remove physically immobilized or loose probes and impurities, followed by exposure to a blocking protein such as BSA. The

blocking protein should ideally be covalently immobilized in gaps and regions where recognition element coverage is not present. This should be followed by another washing step to remove physically immobilized blocking protein and impurities.

Covalent and bio-affinity-based immobilization approaches are preferred because they achieve more uniform probe surface coverage. Covalent immobilization involves covalently bonding the probe to the surface. This can be done by either modifying the surface's chemistry, by modifying the probe, or by modifying both to allow covalent binding to the surface. Table 1 lists the common chemical groups found in proteins. A detailed assessment of the amino acids where these functional groups can be found can be made from looking at Figure 4, a 2D illustration of each of the twenty amino acids. Table 2 provides a list of different chemistries that can be used for covalent immobilization.

Table 1: Common functional groups in proteins [51]

Side groups
$-NH_2$
-SH (Only in found in cysteine. Figure 4)
-COOH
-OH

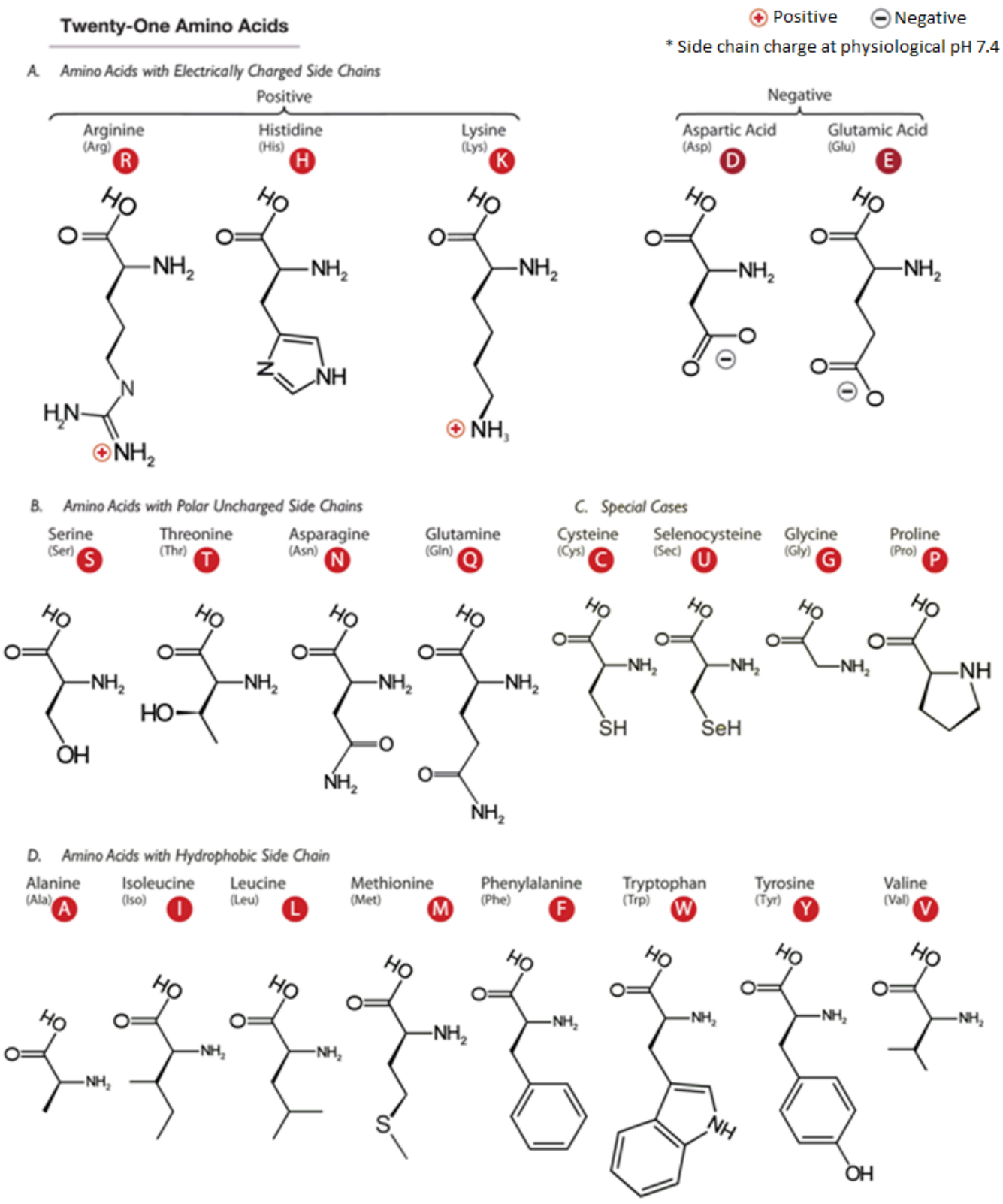

Figure 4: Structure of the twenty amino acids [53]**.**

Original image by Dan Cojocari, University of Toronto. Modified by Sarang Dutt, University of Alberta, and printed under terms of GNU Free Documentation license.

Table 2: Common Methods for Covalent Immobilization [48; 50; 51; 54]

#	Target	Common Techniques
1	Proteins (antibodies, enzymes, etc)	Silanize Si wafer with 3-aminopropyltriethoxysilane (APTES) then activate with glutaraldehyde (GA). This method causes multiple locations of the protein to be covalently bound to the surface, so the protein loses flexibility. Bound probes have no specific orientation.
2	Antibodies	Coat the surface with protein A or G. Antibodies bind to protein A or G via Fc domain. IgG-Protein A/G complex can be stabilized with a cross-linker such as dimethyl pimelemidate. • Requires Protein A to be bound to surface first – this can be done by above method or via bio-affinity. • Protein G has a higher affinity for mammalian Ig G. Wild type protein G has an affinity for albumin, so a recombinant version of protein G, lacking albumin affinity, must be used.
3	DNA	Stage 1: Sputter a silicon surface with a thin film of gold or silanize it with a mercaptosilane (silane with a sulphur group that is sterically accessible). Stage 2: Label the DNA strand with a thiol-linker [55] to use thiol or disulphide chemistry for DNA immobilization on gold thin-film or silicon respectively. • This method provides an advantage of allowing some DNA coated surfaces to be reused. To reuse, simply heat the surface to the DNA melting point. This will break the bonds holding the complementary DNA sequence to the DNA immobilized on the surface.
4	Proteins (antibodies, enzymes, etc)	Stage 1: Modify surface and apply thin film of Au or silanize with a mercaptosilane. Stage 2: Use cysteine tag's sulphur group to create a thiol bond with the Au thin-film or disulphide bond with the mercaptosilane.

		• If the protein does not have a sterically accessible cysteine tag (cys-tag), then genetic engineering can be used to create a recombinant version of the protein with a single extra cysteine residue or tag for the purpose of immobilization. • This scheme produces oriented covalent immobilization.
5	DNA & Proteins	Modify surface chemistry to provide carboxyl groups, then expose to EDC followed by Sulfo-NHS. • Method 1 (silanizing Si with APTES) can be used to modify surface to provide carboxyl groups. • 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (Sulfo-NHS) together are used to couple carboxyl groups to amine groups to form stable amide bonds.

Table 3 provides a list of bio-affinity based immobilization techniques. In bio-affinity-based techniques bonding is non-covalent. Consequently, surfaces can be cleaned and reused. Using these techniques only makes economic sense if the cost of replacing surfaces outweighs the cost of the reagents and supplies required for manufacture, cleaning and reuse.

Table 3: Techniques for Bioaffinity-based Immobilization [48; 51]

#	Target	Technique
1	DNA & Proteins	DNA and proteins are immobilized by exploiting biotin-avidin affinity. The probe must be biotinylated, and the surface must be coated with avidin or vice versa. • Biotin is commonly known as vitamin H or B_7. • Avidin, a water-soluble glycoprotein, remains stable under a wide pH range & has strong affinity for biotin. Each avidin can bind up to four biotin molecules. • The binding of biotin to nitro-avidin (a chemically modified version of avidin) can be dissociated by raising the pH > 10. This allows the surface to be reused without denaturing the protein [56].
2	DNA & Proteins	DNA and proteins are immobilized by exploiting biotin-streptavidin affinity. • Similar to biotin-avidin affinity. • Streptavidin's affinity to biotin is not as strong as avidin's, but it aggregates less, and has a lower tendency than biotin to bind non-specifically. • Nitro-streptavidin can also be created [56].
3	Recombinant proteins or DNA cross-linked to metal ions	Part 1: Use a recombinant protein probe with a His-tag™ exposed to give steric access to its active site(s). Part 2: Modify surface to bind with nitrilotriacetic acid, expose to Zn^{2+}, Cu^{2+}, Ni^{2+} or Co^{2+} ions, followed by exposure to the probe. • The bond can be broken using imidazole [57]. The His-tag™ is a proprietary tag. It consists of 2 to 6 histidine amino acid residues which have been together have a strong affinity for Zn^{2+}, Cu^{2+}, Ni^{2+} and Co^{2+} ions. Genetic modification is used to tag the protein.

There are many types of cross-linkers, chemicals, tags, and surface chemistries that have been explored. This list of techniques for covalent immobilization is by no means exhaustive. SAMs using various silanes (other than APTES) and thiols have also been used to functionalize Si and Au substrates for the immobilization of antibodies for *E. coli* [58; 59]. Permutations and combinations of available options or steps provide a large number of available protocols that can be used for protein and DNA immobilization.

2.4. Summary

From an engineering perspective, simplicity, ease, minimum number of steps involved in executing the procedure, cost, steric access to the probe, probe binding density (number of probes immobilized per unit area), and the effectiveness of the scheme in producing accurate results are all aspects to be considered in the search for a viable immobilization scheme.

Antibodies tend to be difficult and expensive to produce, phages can have issues with reliability and bacterial capture ability, and PCR methods can be expensive, particularly in high throughput applications. Genetic and PCR-based methods can also be affected by contaminants.

It is intuitively predictable that the type of recognition element, immobilization method, and surface used can significantly impact data gathered from a biosensor. Transduction methods can merely provide qualitative or quantitative information about how many bacteria were captured on a biosensor's surface. The bacterial capture capability of the surface is determined by the above properties. It is necessary to explore alternative recognition elements, immobilization schemes, and surfaces to develop biosensors that offer sensitive, specific, and reliable results quickly.

3

Use of tailspike protein for bacterial capture

3.1. Bacteriophages and their use as recognition elements

The use of immobilized monolayers of bacteriophages to capture bacteria is a recent development toward improving biosensor specificity and cost effectiveness. Bacteriophages (casually called ‘phages’) are a type of virus that target bacteria. They bind to specific receptors and inject their genetic material into the bacteria as a means to reproduce. This genetic material integrates itself into the host, causing the host to replicate new phages. The host dies when the new virions are released. New virions target other nearby bacteria with receptors identical to those of the dead host. Bacteriophages have specificity toward certain species of bacteria – e.g. T4 targets *E. coli*, and the P22 bacteriophage targets *Salmonella*. The P22 bacteriophage has been characterized very well [60; 61; 62].

The phage is composed primarily of two segments: the sphere-like ‘head’ and the ‘tail’ or ‘tail machine’. The head is an icosahedral protein capsid that contains its double-stranded DNA. The tail machine possesses a collar, a base plate with tailspike proteins, and tail fibers. Bacteriophages range in size from approximately 100nm to 250nm in length, depending on the species.

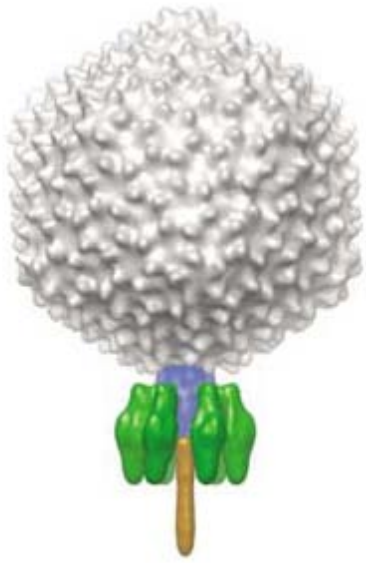

Figure 5: Model of a P22 bacteriophage [62]

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P22 typically has 6 tailspike proteins (TSPs) on its tail machine, though this number can vary. The P22's TSPs gives this phage its ability to bind to *Salmonella* bacteria [61; 62; 63]. The P22 tailspike protein will be discussed in more detail in section 3.2.

Use of phages for pathogen detection has been reported for QCM, SPR, flow cytometry, and complementary metal-oxide semiconductor (CMOS) chips [64; 65; 66; 67; 68]. The use of immobilized P22 and T4 phage monolayers as recognition elements for the identification of *Salmonella* and *E. coli* has also been reported [69; 70].

Using bacteriophages as recognition elements has two big advantages: (1) high specificity, and (2) ease of amplification resulting in low manufacturing cost (unlike antibodies).

The use of phages also comes with disadvantages. Firstly, due to the size of bacteriophages, the numbers of phages that can bind to a surface are reduced in comparison to antibodies. This reduces the bacterial capture capability of the surface. Secondly, the enzymatic endorhamnosidase activity of the phage TSP cleaves lipopolysaccharides

(a carbohydrate chain) known as “O-antigen receptors” found on the surface of *Salmonella* bacteria. This cleaving behaviour causes the phages to temporarily release binding with the bacterium. This rapid release and rebinding function evolved in the P22 to allow the virus to find a suitable site on the bacterial surface from which to inject its DNA. Consequently, it is possible that some bacteria will disengage from the surface when using tools that apply an active flow of liquid over the surface. This is a relevant problem because less bacterial capture will result in decreased assay/ biosensor sensitivity to bacterial presence. Multiple phages must non-simultaneously bind to each bacterium that comes in contact with the surface to prevent this disengagement. To compensate for this problem, the immobilization method must bind a number of phages to the surface while making the TSPs sterically accessible.

The lytic cycle of the bacteriophage also offers a challenge. Lytic cycles are the reproductive processes of the bacteriophages. The cycle starts after the P22 phage injects its DNA into the bacterium. The DNA merges itself into the bacterium’s chromosome, and is over-expressed causing the creation of new virions. These new phages accumulate in the cell until digestive enzymes coded in the phage DNA are translated and cause the bacterium to lyse and release the newly created virions. The amount of time taken from the time of infection to cellular lysis varies by viral type, and is related to bacterial doubling time. Among T4 and P22 this duration is approximately 20 minutes at 37°C. In biosensor applications, exposure of the phage-coated surface to bacteria to should be limited to less than 20 minutes and followed by fixation in glutaraldehyde (GA), to ensure that the number of bacteria and phages on the surface do not change. To ensure capture in the presence of low bacterial concentration in the sample longer exposure times may be required. In such conditions, this 20 minute time limit can be an issue.

3.2. The P22 TSP as a recognition element

Singh *et al*. (2010) recently showed that the TSP from the P22 bacteriophage can be used as a molecular probe or recognition element for the detection of *Salmonella* [71]. To explain advantages of using TSP as a recognition element, the P22 TSP, and recent research will be reviewed.

P22 tailspike proteins have two important functions: (i) the binding of O-antigen receptors on the surface of *S.* Typhimurium bacteria and, (ii) endorhamnosidase activity resulting in cleavage of the O-antigen receptor. The binding function facilitates DNA injection into *Salmonella* bacteria. The endorhamnosidase activity cleaves the α(1,3)-O-glycosidic bond between rhamnose and galactose of the O-antigenic repeating units at a rate of 2 bonds per minute [63]. The function of this cleaving behaviour is to allow the phage to find a suitable location on the bacterial cell wall for DNA insertion. Figure 6 shows the chemical structure of the O-antigen receptor's repeating units, which form chains that varying in length from 19 to 34 repeating units [63; 72]. The arrow marks the cleaving site.

The TSP gives the P22 bacteriophage its bacterial recognition capability. TSP protein could be manufactured recombinantly by isolating its gene and expressing it in *E. coli* through genetic engineering methods.

TSP can be used as a probe or recognition element for biosensor and pathogen detection applications (specific to *Salmonella* in this case). Research discussed below suggests that although this idea may be intuitively obvious, the method for producing and using TSP is not. By identifying the TSP genes for other bacteriophages, it may be possible to produce TSPs specific to *Escherichia coli*, *Campylobacter jejuni*, *Listeria monocytogenes*, *Bacillus subtilis*, and other bacteria. The process of identifying the 'TSP' gene in bacteriophages is not trivial.

Collaborators at the NRC Institute for Biological Sciences (NRC-IBS, Ottawa) cloned the gene that encodes the P22 phage's TSP. The gene was modified to deactivate the endorhamnosidase activity and remove the head-binding domain that connects the TSP to the P22 phage

(this did not affect thermostability of the TSP). Codons for cysteine and polyhistidine residues (cys-tag and His-tag™ respectively) were also added. Recombinant mutant TSP was successfully produced by adding and over-expressing this modified gene in E.coli. Removal of the endorhamnosidase activity eliminated the rapid release and rebinding behaviour that was previously a disadvantage of using whole phages. Inserting the cys-tag and His-tag™ allowed the protein to be purified and, more importantly, provided binding sites that can be used for oriented covalent immobilization.

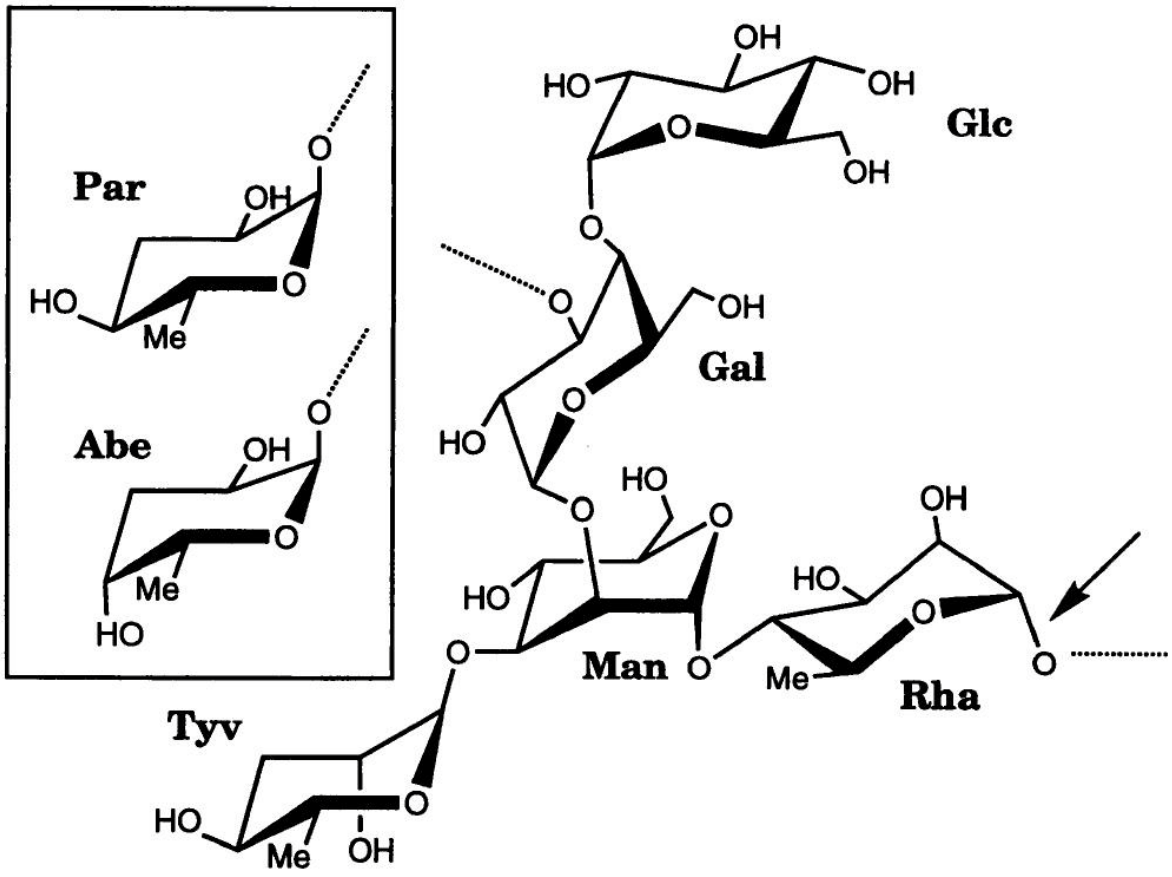

Figure 6: Structure of Salmonella O-antigenic repeating unit. [63]

Steinbacher *et al*., PNAS, 93 (20), 10584-10588.

Two variants of mutant TSP were created, named Mut9 and Mut3. The distinction between them lies in the location of the cys-tag. Naturally synthesized proteins typically have an N-terminus (the protein's starting point possessing an exposed amine group) and a C-terminus (ending point possessing a carboxyl group). Mut9 TSP, also called N-Cys TSP, is a homo-trimer with a cysteine tag at the N-terminus, away from the bacterial binding site. The Mut3 TSP, also called C-Cys TSP, is a homo-trimer with a cysteine tag at the C-terminus, near the bacterial binding site. without its head-binding domain, the TSP is 133 Å in length, and between 35 and 80 Å in diameter [(72)].

Using the TSP as a recognition element in biosensors and assays has many advantages. For example, recombinant over-expression of this mutant TSP in *E. coli* provides a manufacturing cost advantage over antibodies. The protein is more stable to temperature and pH variations than antibodies, allowing a longer inventory shelf-life. Unlike bacteriophages, TSP can also survive desiccation after immobilization on a surface.

3.3. Recent work by Singh *et al*. [(71)]

As mentioned earlier, biosensors are composed of a transduction mechanism and a recognition element. Consequently, sensitivity of the biosensor or assay is impacted by the sensitivity of the transduction mechanism, *and the effectiveness of the recognition element*. This concept also applies to many immunoassays and bioassays. An intuitive parametric analysis can tell one that, assuming the transduction platform of an assay has ideal or sufficient sensitivity, an assay's overall sensitivity will be determined by the effectiveness of the assay's recognition element at capturing the antigen. Therefore, in biosensors or assays for bacterial detection, the recognition element's ability to 'capture' and hold target bacteria in the vicinity of the transduction mechanism is important. In work discussed in chapters 3 and 4, this capture ability is evaluated by

measuring bacteria captured onto the surface per unit area, using the units: bacteria/100μm^2.

In recent work by Singh *et al*. (2010), BCDs for immobilized TSP were compared to those of immobilized whole phages [71]. TSP was immobilized using multiple chemistries and the viability of its use as a molecular probe or recognition element was demonstrated. The oriented immobilization schemes included: use of thiol linkage of TSP to gold thin-films, on silicon using disulphide linkage, and on silicon using the TSP's His-tag. Optimized protocols for the first two schemes are provided below.

3.3.1. Protocol 1: TSP immobilization using thiol linkage

Gold thin-film coated substrates were first sonicated for 5 min in acetone, and washed in isopropyl alcohol (IPA) and ethanol. This was followed by rinsing in Milli-Q water for 5 min each prior to surface functionalization. The TSP solution was reduced with 50mM *tris*(2-carboxyethyl)phosphine (TCEP) at 60 ˚C for 15 min, prior to exposure to the cleaned surfaces. Reduced TSPs were immobilized onto the cleaned surfaces in a shaker overnight at 40 ˚C. Previous work included experimenting with immobilization at room temperature, and 60 ˚C.

After TSP immobilization, surfaces to be exposed to bacteria were washed in PBS buffer followed by washing in 0.05% PBS-Tween20 solution to remove non-covalently bound TSP and other impurities, and twice again with PBS for 5 min each to remove the Tween20 detergent. The surfaces were then blocked using 1mg/ml of bovine serum albumin (BSA) in PBS for 30 min to prevent non-specific binding. This was followed by washing in 0.05% PBS-Tween20 solution followed by PBS for 5 min each prior to exposure to the bacterial suspension for 20 min. Surfaces were rinsed in 0.05% PBS-Tween20 solution and twice again with deionized water for 5 min each to remove unbound bacteria. Bacteria on surfaces were fixed using glutaraldehyde (GA) exposure for 30 min, and

the bacterial capture density was measured using scanning electron microscopy (SEM).

3.3.2. Protocol 2: TSP immobilization using disulfide linkage

Flat silicon substrates were cleaned in piranha solution (3:1 H_2SO_4:H_2O_2) for 15 minutes followed by two washes in deionized water for 5 min each. Cleaned surfaces were silanized in a 2% solution of mercapto-propyl triethoxysilane (MPTES) in acetone for 2h. The silanized surfaces were washed twice in acetone for 5 min each, followed by drying with nitrogen. Silanized substrates were incubated overnight in TCEP-reduced 1µg/ml solution of TSP in PBS. The sulphur group in the TSP cys-tag binds to the sulphur group of the silane to form disulphide bonds. Surfaces were then washed in 0.05% PBS-Tween20 solution followed by two washes of 5min duration each in PBS. The surfaces were blocked with BSA in PBS for 30 min to prevent non-specific binding of bacteria. This is followed by the same washing and bacterial exposure procedure as in protocol 1.

3.3.3. Bacterial Solution Preparation Method

Two colonies of S. Typhimurium were inserted into a glass culture tube containing 3 ml of NB medium. The solution was incubated at 37 ˚C and shaken at 180 RPM for 19 hrs. When multiple culture tubes were made simultaneously for experiments, the contents of the culture tubes were thoroughly mixed, and then redistributed into their original tubes. To allow exposure of the surfaces to the bacterial solution, 1ml of NB medium containing S. Typhimurium was distributed into an eppendorf tube. The tube was then centrifuged at 13,000 RPM for 1 minute. The supernatant was removed and carefully replaced with 1ml of phosphate buffer saline (PBS). After thoroughly mixing the bacteria into the new PBS medium, the suspension was used in the experiment protocols. Consequent dilution and culturing of the bacterial solution revealed that the average bacterial count in the solutions used was $3x10^9$ cfu/ml.

A similar procedure was followed for the preparation of *E.coli* (*E.C. 12* strain) solution, however, LB medium was used instead of NB. Dilution and culturing of the bacterial solution revealed that the average bacterial count in the solutions used was $3x10^9$cfu/ml.

3.3.4. Comparison of bacterial capture densities (BCDs)

Unreduced N-Cys (Mut9) TSP immobilized on gold at room temperature, and at 60 ˚C using a TSP solution concentration of 5µg/ml showed *Salmonella* BCDs of 12.64 ±0.49, and 25.65 ± 0.74 bacteria/100µm^2 respectively. Unreduced C-Cys (Mut3) TSP showed respective BCDs of 5.8 ± 1.1 and 8.57 ± 0.19 bacteria/100µm^2. This intuitively suggests that orientation of C-Cys (with the binding site closer to the Au surface) is unfavourable for bacterial capture, and that orientation of the immobilized TSP causes significant impact to bacterial capture density.

The efficiency of bacterial capture increased with reduction using TCEP. When reduced N-Cys TSP was immobilized using solution concentrations of 1µg/ml at room temperature and 40 ˚C, capture densities were 6.03 ± 0.57, and 23.28 ± 0.45 bacteria/100µm^2 respectively.

Use of disulphide linkage with MPTES to immobilize N-Cys TSP (in TCEP reduced 1µg/ml solution) at onto silicon substrates showed capture density of 12.21 ± 0.19 bacteria/100µm^2. Use of the (poly)his-tag to bind C-Cys TSP (in TCEP reduced 1µg/ml solution) onto silicon substrates showed capture density of 7.03 ± 0.34 bacteria/100µm^2. Protocol and BCDs for the TSP immobilized on surfaces using amide linkage is discussed in chapter 4.

Wild type TSP (wtTSP), possessing endorhamnosidase activity, immobilized on Au thin-films at 40 ˚C showed bacterial capture density of 3.52 ± 0.34 bacteria /100µm^2.

Similarly, whole phages (activated using EDC and NHS, followed by 19hr immobilization overnight on cysteamine functionalized gold substrates) demonstrated host bacterial capture density of 4.4±0.26

bacteria/100μm^2. It is likely that the endorhamnosidase activity, related to the rapid release and rebinding behaviour mentioned earlier, results in low bacterial capture.

The data suggested that: (i) the genetically engineered TSPs, particularly N-Cys (Mut9) type, function as better recognition elements or molecular probes than whole P22 phages; (ii) there are many ways to immobilize TSP; and (iii) immobilization methods that increase steric accessibility of the TSP binding site to bacteria in the sample improve bacterial capture. Research done also showed that bacterial capture increased as a function of increasing concentration of TSP solution that was used for surface preparation. Chapter 4 discusses results for TSP immobilization using amide linkage, and surface optimization work.

3.4. Atomic Force Microscopy as a characterization tool

Atomic Force Microscopy (AFM), invented by Binnig *et al*. [73] in 1986, is a microscopy technique that was initially designed for imaging the surface topography of samples at high resolution and for measuring forces at atomic scales. Since its invention, this characterization technique has been studied in detail, and thoroughly modeled. As a result, it has matured over the past decade into a popular characterization technique. An AFM was even built into the suite of instruments carried onboard the Phoenix Mars Lander [74; 75] to study the structure of soil and ice grains on the surface of Mars.

AFM imaging works by using a cantilever with a sharp tip to raster scan (raster scan: drag or tap the tip from left to right and back, in a pattern across the surface) the surface of a sample (Fig. 7 & 8) [76]. Variations in height of the surface cause the cantilever to correspondingly move up or down. The cantilever's motion is amplified by a laser beam reflecting off the rear of the cantilever onto a photo detector. The distance between the tip and the sample is constantly corrected by a feedback loop between the AFM's sample/tip positioning system and a computer controlled piezo-element. By plotting the height/vertical correction signal

produced by the feedback loop on the z-axis against the [x, y] position of the cantilever tip, we can produce a 3D topography of a sample's surface (77). The resolution of the method is primarily limited by properties of the tip (e.g. tip radius, tip inclination angle & aspect ratio), and we can expect an apparent vertical resolution of about 0.1nm. Lateral dimensions measured are typically enlarged due to the size of the tip. Typically this broadening of features is known as the "tip convolution" or "tip broadening" effect. It can be compensated for using software correction or using calculations taking into account an estimate of the tip radius [78].

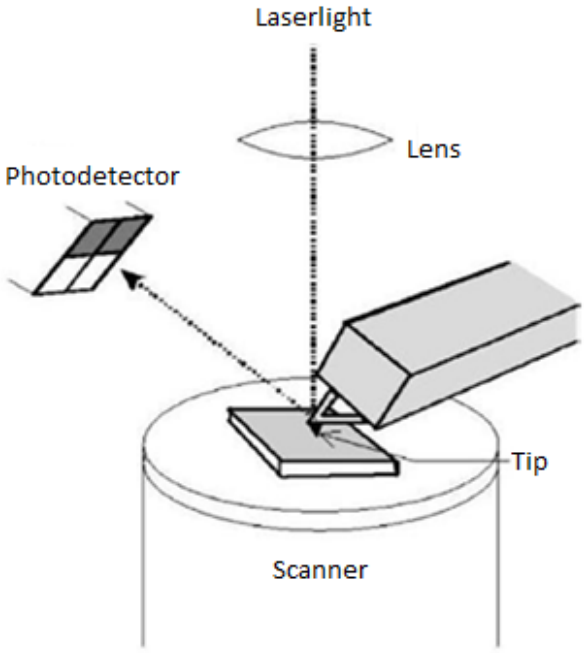

Figure 7: Schematic of an Atomic Force Microscope (AFM) [76]

The above mentioned AFM method is commonly known as "contact mode", and occasionally called "static AFM". It relies on the static deflection of the cantilever. The disadvantage of contact mode AFM is that the tip is in direct contact with the substrate being imaged. Raster scanning in contact mode creates lateral forces that damage sensitive samples. Dynamic AFM methods were developed to image surfaces in a mechanically non-invasive way.

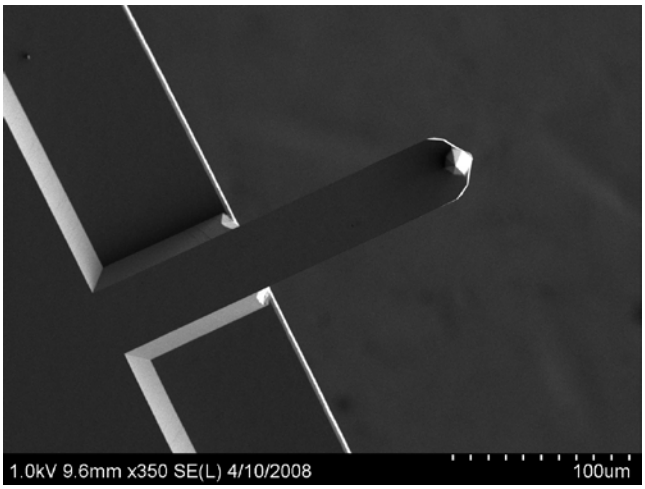

Figure 8: SEM image of underside of a cantilever showing a conical tip with a radius less than 10nm

Dynamic AFM can be categorized into two methods: amplitude modulated AFM (AM-AFM), where the cantilever is oscillated at constant frequency and amplitude is allowed to vary, and frequency modulated AFM (FM-AFM), where the cantilever is oscillated at constant amplitude and frequency is allowed to vary. AM-AFM is more commonly known as "tapping mode" or "intermittent contact mode" and FM-AFM is more commonly known as "non-contact mode" [79]. Although the technique in its simplest form provides an ability to image surface topography, we can also use it to produce force versus distance curves (force curves), from which we can study localized material properties in samples, such as elasticity, hardness, viscosity and adhesion. [76; 77]

3.5. AFM characterization of TSP functionalized surfaces

Although the viability of TSP as a molecular probe or recognition element is now demonstrated with the above research, there are two aspects of this topic for which further characterization and development should be shown before the TSP is used in a commercial product. Firstly, the relationship of number of TSP immobilized on surfaces to bacterial capture density needs to be further explored. This section discusses experiments conducted to explore this relationship. The second and more

important aspect is related to maximizing bacterial capture. This is further discussed in Chapter 4.

3.5.1. Surface density measurements for immobilized TSP

AFM was used to characterize the TSP surface density on substrates where TSP was immobilized using thiol linkage and amide linkage. Following the thiol linkage immobilization protocol, surfaces were washed in deionized water, blow-dried under a stream of dry nitrogen, and then imaged using AFM.

Surfaces were imaged using a Digital Instruments (DI) Multimode AFM with NanoScope IV controller, E Scanner, and Nanoscope software (Versions. 5.12R and 6.03) by Veeco. AFM tips with different spring constants were purchased from MikroMasch USA. Image processing was also performed using the Nanoscope software. Image processing involved low pass filter, and median functions on all images. Height images were flattened using a 2nd degree flattening function.

A major limitation in work with AFM is the size of images that can be taken. The DI Multimode AFM together with the “E” scanner was only able to offer a maximum sampling of 512 samples per scan. This means that every time the AFM scanned from left to right or vice versa, a maximum of 512 samples of data were taken. Consequently, resolution decreases rapidly with increasing image size. To be able to image TSPs and count them, the maximum size images that could be taken were limited to an area of 1 μm^2.

Characterization of these surfaces also required the ability to differentiate between proteins and the surfaces on which they were immobilized. For TSP immobilized on gold thin-films, AFM height imaging could not be used to resolve TSP surface density. Figure 9 supports this conclusion with a graph of counts of height peaks/μm^2 measured over six images (from the same sample).

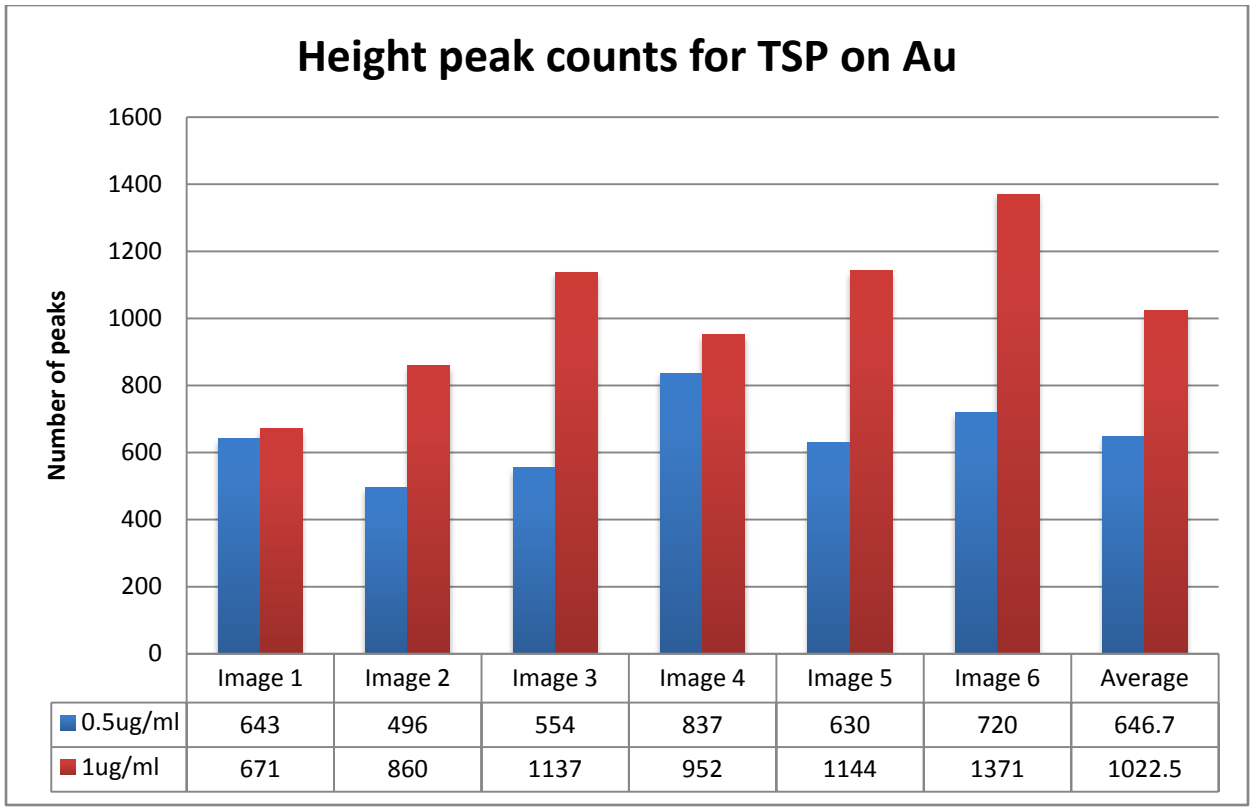

	Image 1	Image 2	Image 3	Image 4	Image 5	Image 6	Average
0.5ug/ml	643	496	554	837	630	720	646.7
1ug/ml	671	860	1137	952	1144	1371	1022.5

Figure 9: Height peak counts for mut9 mTSP immobilized on 20nm Au thin-film. Averages for the two samples, one exposed to 0.5 ug/ml and the other to 1ug/ml TSP solution, were 646.7 ± 49.4 and 1022.5 ± 93.9 peaks/μm^2 respectively.

It was hypothesized that the number of height peaks on imaged surfaces would increase as a function of the concentration of TSP solution to which the gold surfaces were exposed. In figure 9 there is a high variation in peak counts and there was no way to verify which peaks were caused by gold grain as opposed to TSP. Figure 10 provides an example of a typical AFM height image of TSP immobilized on gold thin-film. Height images lacked clear contrast between the immobilized P22 mTSP and grain on the Au thin-film surface. As a result, during initial results, it was only assumed that number of TSP on surfaces increased with increasing concentration of the TSP solution used to prepare the respective samples.

To improve the ability to image using AFM, different types of AFM cantilevers were tested, and lower spring constant cantilevers were tried. The procedure started with cantilevers with spring constants of 10 N/m,

followed by 5 N/m, 3.5 N/m, and concluded with 0.9 N/m (part # NSC19-ALBS). AFM phase imaging was also attempted with all of the above cantilever types. Only the last cantilever allowed the AFM to acquire clear phase images. The former three types provided noisy phase images where image noise decreased as a function of decreasing spring constant.

Colour contrast was maximized using the Nanoscope software. AFM phase images are made by mapping the phase lag of the AFM cantilever's oscillation (with respect to the cantilever's piezo driver) against the cantilever's position. Phase lag is affected by material properties, e.g. Young's modulus, and viscoelasticity. Phase images show contrast between materials of different Young's modulus, therefore showing a better contrast between gold, protein, and other substances. Figures 10 and 11 compare height and phase images of the same region on an Au thin-film with TSP immobilized using thiol linkage. The phase image shows better contrast between TSP and gold grain.

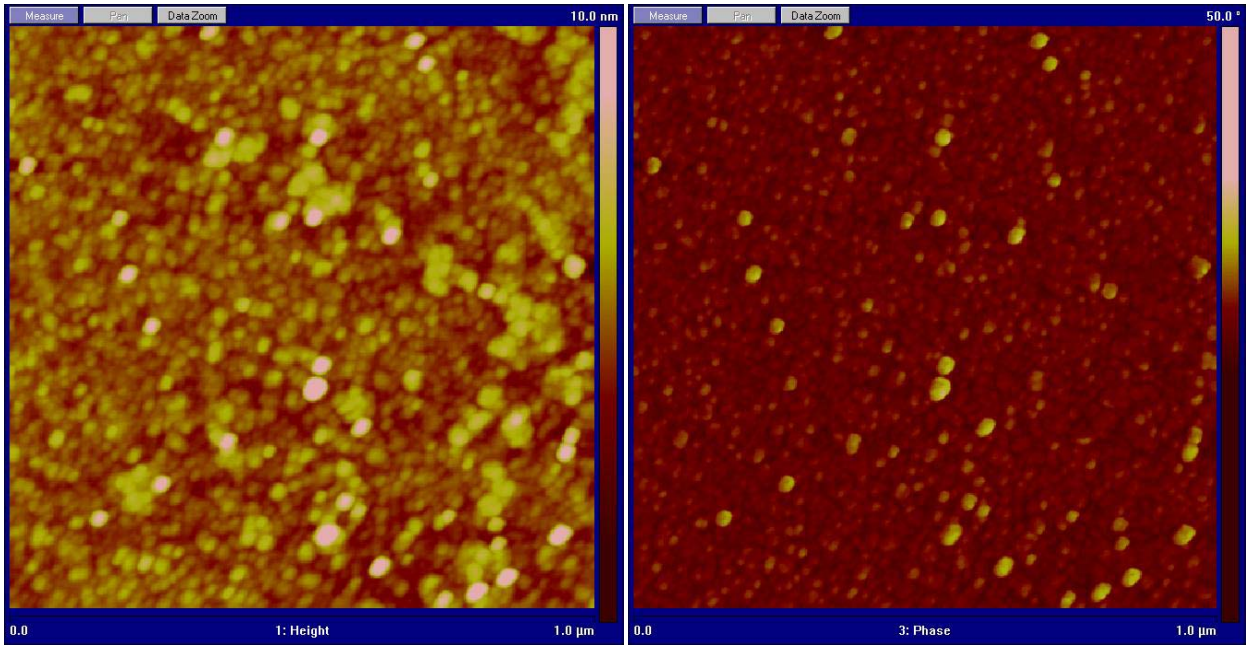

Figure 10 (left): Height image of TSP immobilized on 20 nm Au thin-film.

Figure 11 (right): Phase image corresponding to Figure 10.

TSP was fixed using glutaraldehyde (GA) to maximize contrast between gold thin-film and protein

Observations:

1) Firstly, to test whether the substance immobilized on to gold was really TSP, three mica surfaces were exposed to TSP solutions for one hour, washed in deionized water, dried with nitrogen, and imaged under AFM. Different batches of TSP solution were used for preparation of the surfaces. Height images showed that there were four differently sized structures adsorbed onto the mica surfaces (refer to Figure 12).

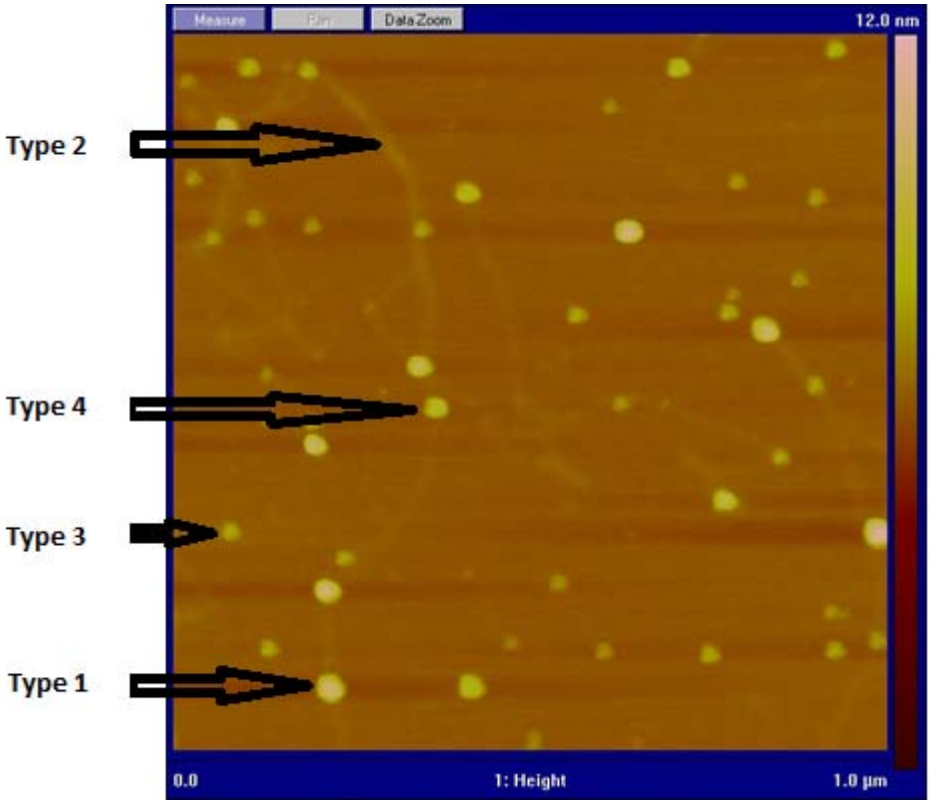

Figure 12: AFM image of TSP on Mica

Literature indicates that the TSP is cylindrical in shape, with length of 13.3nm and a diameter ranging from 3.5 to 8 nm [(72)]. Muir et al reported that height of DNA on mica (a hydrophilic surface) is lower than that on HOPG (Highly Ordered Pyrolytic Graphite, hydrophobic surface) [(80)]. Due to the strongly hydrophilic nature of mica, we can intuitively predict that most TSPs will be flatly oriented (i.e. the 'cylinder' will lie on its side).

The first type of structure (Type 1) had heights ranging between 4 and 5 nm. Assuming the heights were distorted due to substrate interaction, the measurements still lie within the range given by literature. It seems reasonable to conclude that the type 1 structures are P22 TSP.

The second type of structure (Type 2) looks like a “snail trail”. It is unclear what the structures are. They may be protein residue trails or salt trails left by proteins as the flow of TSP solution dragged the TSPs over the surface since images show that there is usually at least one TSP on each ‘trail’. Trails showed an average height of 0.212nm (height range from 0.091nm to 0.453nm) for 30 readings over 3 samples. This type of structure was not visible on the control mica sample.

The mean height of the third type of structure (Type 3) was approximately 1.1 nm for 30 readings over 3 samples (height range from 0.646 nm to 1.733 nm). These were also most likely contaminants remaining in the solution from TSP production process.

Heights of the fourth type of structure (Type 4) range from 2.5nm to 3.5nm. This height range is close the lower limit of the diameter of TSP listed in literature, so these may be TSP with reduced height due to surface interaction. Alternatively they may be contaminant proteins. It is unknown what they are. However, it is highly likely that these structures were responsible for earlier difficulties with counting of TSPs.

To resolve the concern about differentiating between gold grain and proteins of varying height AFM phase imaging was used together with maximization of contrast using image processing. However, it may be possible that counts will still be impacted by the presence of this type of structure.

As a positive control, height of TSP immobilized on gold thin-film was measured at 5.7nm. For negative control purposes, a freshly cleaved, flat mica surface (from the same batch as the experiment surfaces) was exposed to PBS solution for one hour each, washed in deionized water, nitrogen dried, and imaged under AFM. Nine images (three from each of

the three locations for each control sample) were taken. The control images only showed a clean flat mica surface with no features.

2) Using AFM phase imaging, the variation of TSP surface density was measured as a function of concentration of the TSP solution used to prepare the samples. Using a TSP solution concentrations of 0.125µg/ml, 0.5µg/ml, and 1µg/ml, samples were imaged and average TSP counts observed. Nine images were taken for each sample. Images were taken from random locations on each sample. Image processing was done on raw image files to maximize contrast as a function of phase. Images were compared to those of control surfaces, e.g. bare 20nm gold thin-film coated surfaces exposed to PBS for 19 hrs followed by AFM sample preparation and imaging. Only circular shapes looking similar to the size and brightness expected were counted as TSPs. For TSP immobilized by thiol linkage was approximately 2.8 ± 0.7 TSP/µm^2, 15.2 ± 1.0 TSP/µm^2, and 35.7 ± 3.7 TSP/µm^2 respectively (refer to Fig. 13).

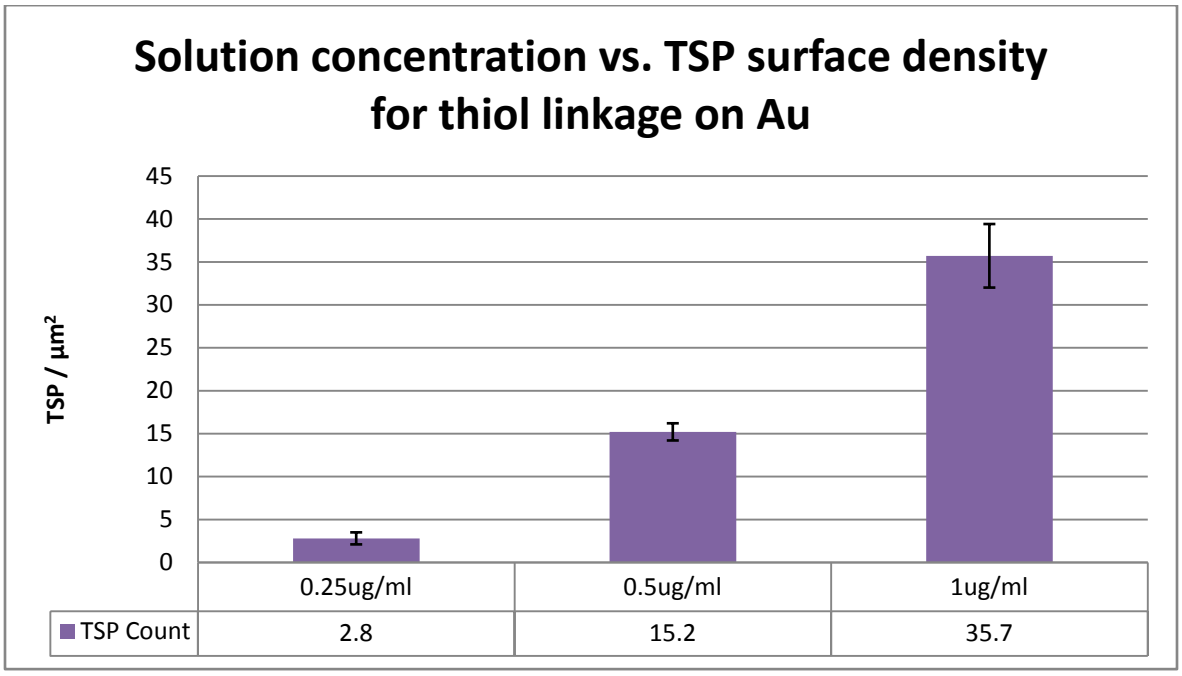

Figure 13: TSP surface density as a function of solution concentration

3) TSP immobilized, using amide linkage with exposure to APTES for 1 hour (without annealing the surface), on two 5mm x 7mm silicon chips were also counted. This procedure using a TSP solution concentration of 1µg/ml produced an average TSP surface density count of 4 TSP/µm^2. However, the surfaces when imaged under SEM showed almost no bacterial capture (refer to experiment 1, chapter 4). After attempting the silanization for 19 hrs (overnight) the TSP surface density count measured by AFM was 42.3 TSP/µm^2. AFM imaging of the samples showed odd structures covering the TSPs (Figure 14). SEM imaging showed negligible number of bacteria captured on the samples even though the TSP numbers had increased.

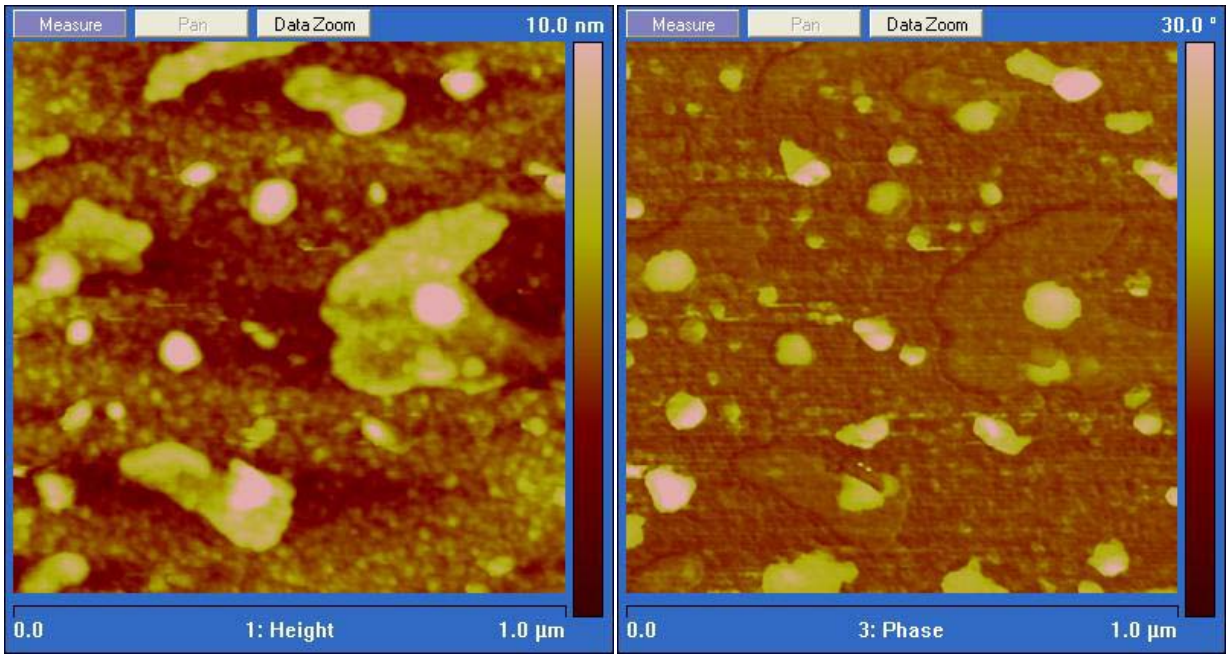

Figure 14: AFM height (left) and phase (right) images of TSP immobilized on Au with APTES exposure for 19 hrs

Figure 14's AFM phase image shows four contrasting materials: TSPs, coloured bright white; bright green 'halos' surrounding the TSP; puddle-shaped structures surrounding the green halos; and silicon surface in the background. It is likely that extended APTES exposure lead to the formation of multilayered polymeric structures that were not removed by

washing in acetone after exposure. This agrees with existing literature [81; 82]. It may also be likely that some of the excess APTES (with attached glutaraldehyde) may have dissociated during TSP immobilization and bound to TSP binding sites, thereby interfering with the bacterial capture abilities of the TSP coated surfaces. This hypothesis seems logical since TSP is present (as shown in Figure 14) yet negligible bacterial capture was attained.

3.6. Summary

Singh *et al*. showed that genetically engineered TSP, particularly N-Cys TSP (Mut9), function as better recognition elements or molecular probes than whole P22 bacteriophages and wild type TSP. There are many ways to immobilize TSP. Data also suggests that immobilization methods that that increase steric access to TSP binding sites cause improved bacterial capture.

AFM is a tool that provides detailed images of surfaces. Its use for imaging is accompanied by many challenges and can be very time consuming. Challenges encountered included the ability to eliminate noise, artefacts due to tip shape and damaged or dirty tips, limited image size due to small protein size, and limited resolution and sample rate.

It is evident from the TSP counts taken that adsorption of TSP is highly dependent on the immobilization scheme used. Gathered data allowed only for qualitative observations. It was observed that TSP counts increase as a function of increasing TSP solution concentration to which surfaces are exposed. This was most likely due to increased TSP immobilization as a function of increased concentration of the TSP solution.

TSP counts are also higher when immobilized using thiol linkage on gold than when using an amide linkage protocol on silicon. Increasing the amount of time for which silicon surfaces were exposed to APTES did increase the number of TSP immobilized on the surfaces. However, for those surfaces, increased TSP count did not correspond with increased

bacterial capture. Simply increasing APTES exposure time did not increase bacterial capture. This suggests that TSP function may have been impacted by the presence of excess APTES.

4

Optimization of bacterial capture

4.1. Experiment goals and design

The work discussed earlier shows that TSP can be used for the specific binding and immobilization of *S.* Typhimurium onto surfaces such as Au thin-film coated, and MPTES-coated Si surfaces. Comparison of bacterial capture densities from experiments showed that TSP immobilized on MPTES-coated flat Si surfaces capture less than half the number of bacteria that gold thin-films do. However, though the highest capture density was acquired on gold surfaces, gold is an expensive option, particularly for mass-manufacturing purposes. Although gold surfaces are suitable for transduction methods such as SPR and florescence/optical detection methods, silicon surfaces are preferable, particularly for making biosensors with integrated microelectronics. Bare silicon wafers are also available at lower cost and more easily available on the market than Au thin-film coated surfaces. Although this makes Si substrates an economically preferred alternative, the surface and immobilization scheme would need to be further optimized to ensure that the bacterial capture capability of the surface is equal to or higher than that of Au thin-film based capture.

Intuitively, when a recognition element is coupled with a transduction platform, improving target capture will increase the probability of detecting the pathogen. Therefore, the objective of the work discussed

below is to optimize bacterial capture on surfaces such as tsp-coated silanized silicon and demonstrate bacterial capture densities close to or greater than the numbers acquired for Au thin-film substrates.

The approach followed by Singh, *et al.* thus far has been to chemically modify the surface and genetically modify the P22 TSP to allow protein immobilization. The protein-coated surface would then be exposed to bacteria to test for effectiveness of capture (measured in units of *number of bacteria per 100μm*^{2}).

An alternative, not previously considered, was the approach to improve TSP immobilization, and in turn bacterial capture, by modification of the surface's physical structure to improve the surface's ability to capture/ immobilize bacteria.

An ideal surface for bacterial capture would

- Have a large surface area, allowing for more immobilized probes per unit area.
- Increase probability that the protein used for bacterial capture is oriented such that antigen binding sites are more likely to be exposed to the sample solution.
- Be sufficiently hydrophilic such that it has affinity for the protein and for bacteria.
- Be coated with an effective blocking layer (a monolayer of blocking protein or blocking reagent) that has protein resistant properties such that non-specific binding is prevented.

4.2 Protocols for surface preparation

To prepare the necessary surface, a set of specifications for the substrates (listed below) were provided to collaborators, the Buriak group. The collaborating group was asked to prepare these substrates using common and/or known methods and processes for applying block co-polymers to fabricate the required surfaces.

Specifications given:

- The surface should either have pits or bumps (with sides as vertical as possible) to maximize the probability that TSPs will be oriented vertically.
- Order is irrelevant; however, surface area needs to be maximized to ensure that the maximum amount of TSP can bind to the surface.
- The surfaces should be hydrophilic in nature.
- The dimension of the bumps or pits should be as follows:
    - Ideally around 10-15 nm deep.
    - Ideally spacing of bumps/ridges should be minimized.

The protocols below discuss the methods that were used for surface fabrication. The acquired surfaces were characterized before and after bacterial capture.

4.2.1. "Mountain-shaped ridge-covered" silicon surfaces

Bare silicon wafers were thoroughly cleaned using RCA cleaning procedures to remove any organic substances from the surfaces. The wafers were first cleaned in RCA 1 (1:1:5 mixture of NH_4OH:H_2O_2:H_2O) at 90^oC for 10 minutes and then subsequently in RCA 2 (1:1:6 mixture of HCl:H_2O_2:H_2O) at 90^oC for 10 minutes. The cleaned wafers were then spin-coated with block copolymer solutions (1% solutions of poly(styrene-b-2-vinyl pyridine) in toluene) to obtain thin films of polystyrene containing poly-2-vinyl pyridine nanostructures.

Following spin-coating, the films were used to template the formation of metallic nanostructures. To do this, wafers were immersed in metal salt solutions of 20mM $Na_2PtCl_{4(aq)}$ and 3% HCl for 3 hours. Metal salts bind to the 2-vinyl pyridine structures to create localized regions (masks) of high metal salt concentration wherever there are 2-vinyl pyridine structures. The wafers were then removed from the salt solutions and rinsed with deionized water.

Next, the wafers were placed in an oxygen plasma (55mTorr, 30W RF) for 35s and followed by an argon plasma (100mTorr, 50W RF) for 5s

to reduce the metal salts and to ash away the polymer matrix. The wafer was then placed in a 1:2.5 mixture of O_2 and SF_6 plasma (10mTorr, 20W RF) for 300s to etch patterns into the silicon wafer surface. The reduced metal nanostructures in the previous step were used as an etch mask. Once the etching process was completed, the wafers were exposed to an oxygen plasma to make them hydrophilic.

Four types of wafers, named B, M, S, and SM were made. The ratio of styrene chain length to vinyl pyridine chain length was different for each of the wafers:

Sample Name	**Ratio of Styrene: Vinyl pyridine chain lengths** (chain lengths were measured in number of monomers in the polymer chain)
B (“Big”)	125,000 : 58,500
M (“Medium”)	52,400 : 28,000
S (“Small”)	32,500 : 12,000
SM (“Smaller”)	25,500 : 23,500

The acquired surfaces were imaged before and after bacterial capture. SEM images (below) show mountain-shaped grains (either conical or pyramidal). Grain/ridge sizes in sample B were mostly larger than those in sample S. Maximum base widths of grains in sample B and S were approximately 180nm and 120nm respectively. Minimum base widths of grains in sample B and S were approximately 40nm and 20nm respectively. Images show their heights appeared to be around 60-80nm. The top of the bumps were either pointed or rounded off. The polymer chain lengths were in decreasing order and therefore the widths of grains on the four wafers, B, M, S and SM, were also expected to be in decreasing order.

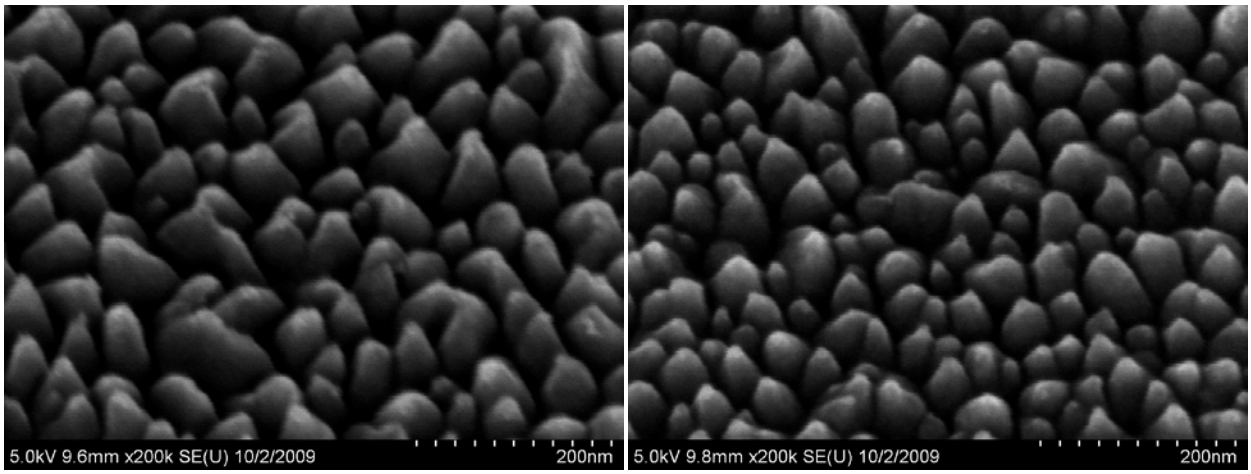

Figure 15 (left): SEM image of ridges on Sample B surface.

Figure 16 (right): SEM image of ridges on Sample S surface.

4.2.2. Protocol 3: TSP immobilization using amide linkage

The protocol below was used for modifying the surface chemistry on MSRCS surfaces to allow TSP immobilization. The protocol does not cause oriented binding.

In this protocol, the silicon surface is functionalized with carboxyl groups (-COOH) which bind to amine groups ($-NH_2$) on the TSP to form strong amide bonds. Surfaces were washed for 5 min each in acetone, IPA, and ethanol, followed by silanization. In initial experiments, silanization involved exposure to 2% 3-aminopropyltriethoxy silane (APTES) in acetone for 1 hour. Silanization for a duration of 19 hrs (overnight) was also attempted. The one hour silanization protocol also provided low bacterial capture densities on MSRCS surfaces as discussed in a later section named Experiment 1.

The amide linkage protocol was optimized by Dr. Amit Singh. Repetitions of the experiment and its results are discussed in a later section labelled Experiment 3. The optimized protocol's silanization step involved exposure of the washed surfaces to 2% 3-aminopropyltriethoxysilane (APTES) in 95% ethanol for 2 min. The surfaces were then washed in ethanol for 5 minutes, nitrogen blow-dried, and annealed at 110 ˚C for 10 min. This was followed by rinsing in

deionized water for 5 min and activation with glutaraldehyde (GA) for 30 min. Activated surfaces were rinsed twice in PBS for 5 min each, then incubated in TSP solution (previously reduced using TCEP at 60 ˚C for 15 min) for 19 hrs at 40 ˚C.

After TSP immobilization, the surfaces that were to be exposed to bacteria were washed in PBS buffer for 5 min followed by 10% ethanolamine for 10 min to remove non-covalently bound TSP and other impurities. The surfaces were then rinsed twice in PBS for 5 min each, and blocked using 1mg/ml of bovine serum albumin (BSA) in PBS for 30 min to prevent non-specific binding. This was followed by washing in 0.05% PBS-Tween20 solution followed by PBS for 5 min each prior to testing. Surfaces were tested for bacterial capture capability through exposure to bacterial solution for 20 min. Refer to Section 3.2.3 for notes on bacterial solution preparation.

Bacteria bound on surfaces were fixed using glutaraldehyde (GA) exposure for 30 min, and the bacterial surface density was measured using scanning electron microscopy (SEM). This optimized protocol is detailed in Table 4.

Table 4: Optimized protocol for TSP immobilization via amide linkage

#	**Activity**	**Additional details**	**Time**
1	Wash surface in Acetone		5 min
2	Isopropyl Alcohol (IPA) wash		5 min
3	Ethanol (95%) wash		5 min
4	Expose sample to 2% APTES	20µl of APTES + 980µl Ethanol (95%)	2 min
5	Ethanol (95%) wash		5 min
6	Dry in N_2 stream		
7	Anneal wafers at 110 ˚C		10 min

8	DI H_20 wash		5 min
9 & 10	Expose to glutaraldehyde (GA)	To activate surface	30 min
11	H_20 wash (x2)		5 min each
12	Phosphate Buffer Saline (PBS) wash	7.2 pH	5 min
13	Exposure to TSP protein solution (1µg/ml) at 40 ˚C & 180 rpm.	Surfaces were exposed to 1ml of TSP solution; solutions contained 100µl of TCEP and were incubated for 15 min at 60 ˚C prior to use.	19 hrs
14	PBS wash		5 min
15	Ethanolamine (10%) wash	100µl Ethanolamine + 900µl H_20	10 min
16	PBS wash		5 min
17	Exposure to bovine serum albumin (BSA)	BSA protein was used as a blocking layer to prevent non-specific binding	30 min
18	PBS wash		5 min
19	Exposure to bacterial solution	Refer to bacterial solution preparation notes	20 min
20	PBS wash		5 min
21	PBS Tween20 (0.05%) wash	Used to remove non-immobilized bacteria	5 min
22	PBS wash		5 min
23	H_20 wash		5 min
24 & 25	Expose to glutaraldehyde (GA)	To fix bacteria for SEM imaging	30 min
26	H_20 wash (2x)		5 min
27	Dry in N_2 stream		

4.3. Study conducted

4.3.1. Experiment 1

Experiment #1 was conducted with samples B & S and compared to results acquired with flat silicon. To measure the performance of MSRCS surfaces, the same protocol (refer to section 4.2.2 and 3.2.3) was applied to all samples. Additionally to test for specificity of the surface, a second pair of B and S surfaces were prepared with the same protocol, and exposed to *E. coli (EC 12)* bacterial strain instead of *S.* Typhimurium. Surfaces were then imaged using scanning electron microscopy (SEM). Five images or more were taken of each sample from random regions of their surface. After SEM images were taken of B & S experiment and control samples, the bacteria on the images were counted. The area of the images was measured using the scale bar on the respective SEM images, and the average number of bacteria per 100μm^2 was calculated for each sample (experiment and control).

Results:

Average bacterial count for Sample S was 8.14 ± 0.77 bacteria/ 100μm^2. Average bacterial count for Sample B was 2.51 ± 0.41 bacteria/ 100μm^2. In comparison the plain silicon wafer exposed to the same protocol as samples B and S, showed an average of 0.31 ± 0.35 bacteria/ 100μm^2. Although fewer SEM images were taken for the flat surface, it was clearly noted during imaging that for the protocol used, the surface modification provided a significant enhancement over flat silicon for which the same protocol was applied. For the *E. coli* specificity tests, the average bacterial count for samples S and B were negligible, at 0.31 ± 0.19 and 0.05 ± 0.07 bacteria/ 100μm^2 respectively.

When these bacterial capture densities are compared to earlier work where *S.* Typhimurium was captured using TSP immobilized using disulphide linkage, the capture density result of 12.21 ± 0.19 bacteria/100μm^2 was much higher. These results suggested that bacterial capture numbers were low overall, and that there was a need for

improvement of surface chemistry and preparation protocol to increase TSP and blocking protein immobilization.

4.3.2. Experiment 2:

Experiment #2 was only conducted with samples B & S. This experiment was designed to be a simple test of whether or not the optimized amide linkage protocol (which included an annealing step, as shown in Table 4) caused improvement to MSRCS surface bacterial capture.

Results:

Average bacterial count for Sample B was 13.2 ± 3.56 bacteria/ 100μm^2. Average bacterial count for Sample S was 25.5 ± 3.34 bacteria/ 100μm^2. The standard deviation is generated by variations in bacterial capture density counted in the images taken using SEM. It could be conjectured that the capture density variation is attributable to excessive blow-drying of the surface with nitrogen in the final steps of the protocol. Figures 17, 18, and 19 depict the visible difference in bacterial captures for surfaces B and S. Additionally, comparing this result to oriented binding attained using disulphide linkage, sample B appears to demonstrate similar bacterial capture density. Sample S appears to demonstrate similar bacterial capture densities as that of surfaces where N-Cys TSP was immobilized using thiol linkage.

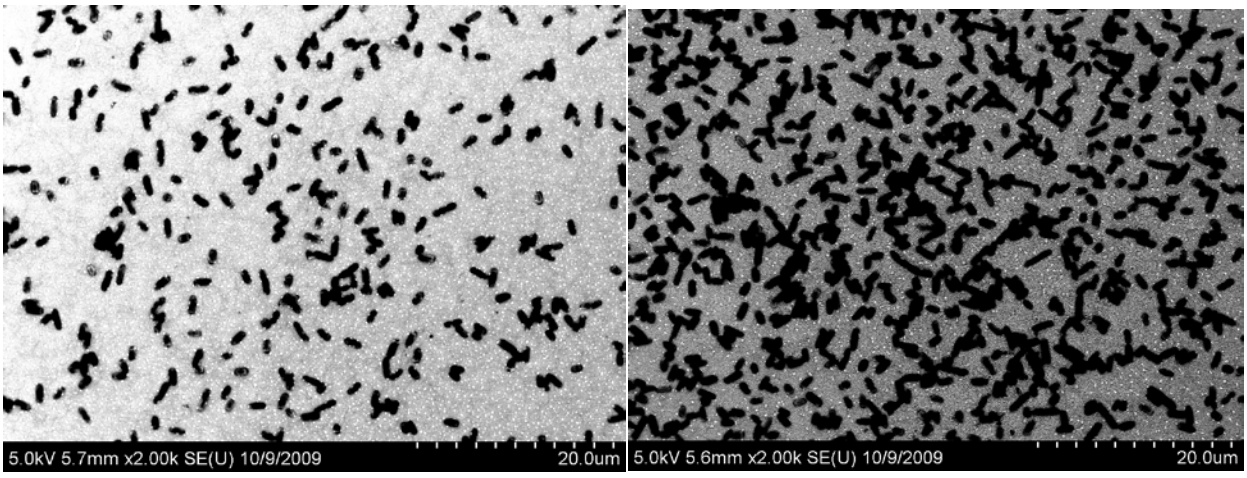

Figure 17 (left): Sample B with optimized protocol applied.

Figure 18 (right): Sample S with optimized protocol applied.

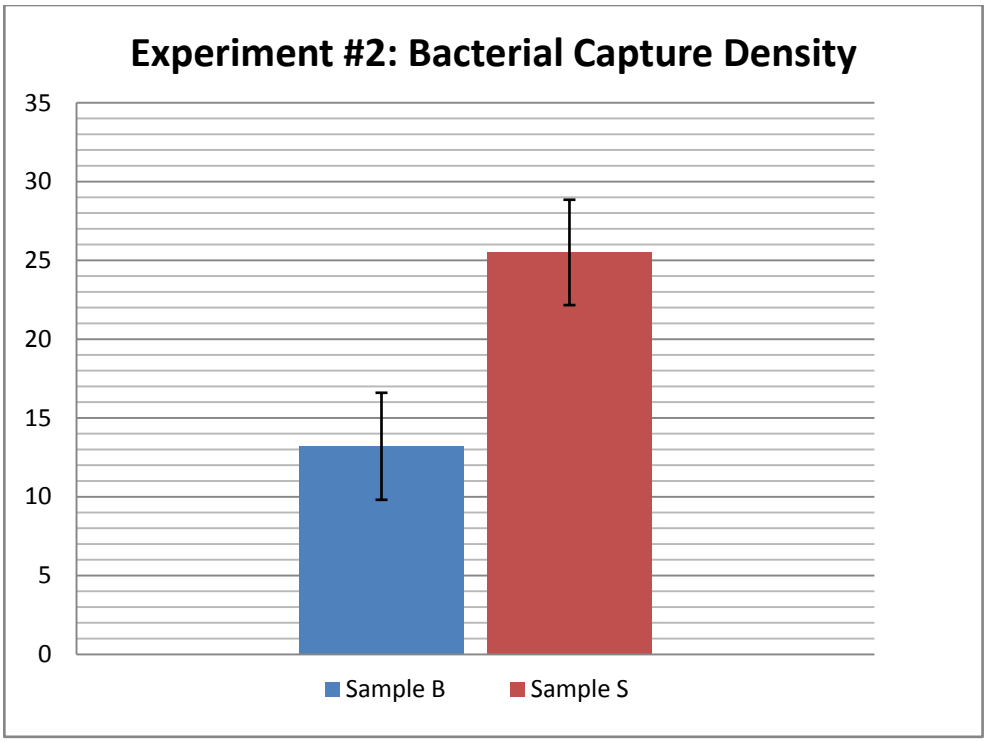

Figure 19: Bacterial capture for samples B & S

4.3.3. Experiment 3 and controls:

The objective of experiment 3 was to:

- Test all the surfaces, B, M, S, and SM, and verify that bacterial capture increases with decreasing grain/ridge-size.
- Perform control experiments to show that specificity of the MSRCS surfaces is retained even when using the optimized protocol.
- Compare bacterial capture densities of the MSRCS surfaces to flat silicon prepared with the optimized amide linkage protocol.

Table 5 shows a list of samples, sample names, the protocols used, and details of preparation for each. Once the samples were prepared, they were imaged using the same procedure

Table 5: List of samples prepared for Experiment #3

Sample #	Sample name	Preparation details
List of experiment samples		
1	B	B wafer sample was prepared using the optimized protocol.
2	M	M wafer sample was prepared using the optimized protocol.
3	S	S wafer sample was prepared using the optimized protocol.
4	SM1	SM1 and SM2 were SM wafer samples that had been fabricated in identical manner. The optimized protocol was used for their preparation. SM2 was made to test for repeatability since the result for SM1 was inconsistent with the expected result.
5	SM2	
List of control and comparison samples		
9	B.E.C	The optimized protocol was applied to B wafer sample. However, instead of exposure to S. Typhimurium bacteria, the B.E.C sample was exposed to *E. coli* (EC12).
10	S.E.C	The optimized protocol was applied to S wafer sample. However, instead of exposure to S. Typhimurium bacteria, the B.E.C sample was exposed to *E. coli* (EC12).
11	C1	The optimized protocol was applied to plain flat Si that had been cleaned using RCA1 and RCA2. However, the C1 sample was not exposed to any TSP – instead it was exposed to PBS for 19 hours. Sample C1 was a negative control for sample C2 (below) and was used to test the surface for its propensity for non-specific binding when a blocking layer of BSA is applied.
12	C2	Samples C2 were tests for bacterial capture density of flat Si. The optimized protocol was applied to plain flat Si, after

		cleaning using RCA1 and RCA2.
13	BC1	The optimized protocol was applied to B wafer sample. However, the sample was not exposed to any TSP – instead they were exposed to PBS for 19 hours. This was a negative control for B wafer samples.
14	SC1	The optimized protocol was applied to S wafer sample. However, the sample was not exposed to any TSP – instead they were exposed to PBS for 19 hours. This was a negative control for S wafer samples.

Results:

Controls C1, BC1, SC1, BEC and SEC show negligible bacterial capture in comparison to the experiment samples (0.12 ± 0.07, 0.07 ± 0.06, 0.14 ± 0.1, 0.22 ± 0.1, and 0.05 ± 0.04 bacteria/100μm^2 respectively). The C2 surface (flat silicon surface that underwent the optimized protocol) was tested twice, showing average bacterial capture of 12.29 ± 0.33 bacteria /100μm^2 respectively. Experiment and the above control bacterial capture densities are shown below in Table 6.

Table 6: List of bacterial capture densities for Experiment #3.

Sample Name	**Ave. Bacterial Capture (Bacteria/ 100μm^2)**	**Comments:**
B	13.75 ± 2.09	For both batches of sample B (B1 and B2), data is consistent with experiment 2.
M	16.97 ± 1.98	This result is consistent with the prediction that increased roughness and surface area improve TSP immobilization and in turn bacterial capture.

S	23.85 ± 1.32	This result is comparable to the one acquired in experiment#2.
SM1	0.84 ± 0.38	The results for SM1 and SM2 are surprising. They could be attributed to either repeated error during processing of the protocol (which is unlikely), or due to some property of the surface itself. To test this, the ridges on the surface of M and SM samples were imaged (Figures 20 and 21). Images showed that the slopes of peaks on SM samples were steeper than those in sample of type M. A plausible explanation of this result is: • Since the pin-like peaks are taller than 20nm, and due to the steeper slope, TSPs are immobilized between the pin-like peaks rather than on their sides. • As a result, the active sites of the immobilized TSP were not sterically accessible to bacteria, resulting in decreased capture.
SM2	1.78 ± 0.23	
BEC	0.22 ± 0.1	This result shows that the surface was effectively blocked using BSA, and that the P22 TSP does not bind to *E. coli* as expected.
SEC	0.05 ± 0.04	This result shows that the surface was effectively blocked using BSA, and that the P22 TSP does not bind to *E. coli* as expected.
C1	0.12 ± 0.07	This result shows that the flat Si surface was effectively blocked using BSA, and that non-specific binding was negligible.

C2	12.29 ± 0.33	This test was repeated twice to attain the shown average and error. This result shows that the flat Si surface was capable of bacterial capture. Bacterial capture was less than that of samples B, M, and S.
BC1	0.07 ± 0.06	This result shows that the sample B surface was effectively blocked using BSA, and that non-specific binding was negligible.
SC1	0.14 ± 0.1	This result shows that the sample S surface was effectively blocked using BSA, and that non-specific binding was negligible.

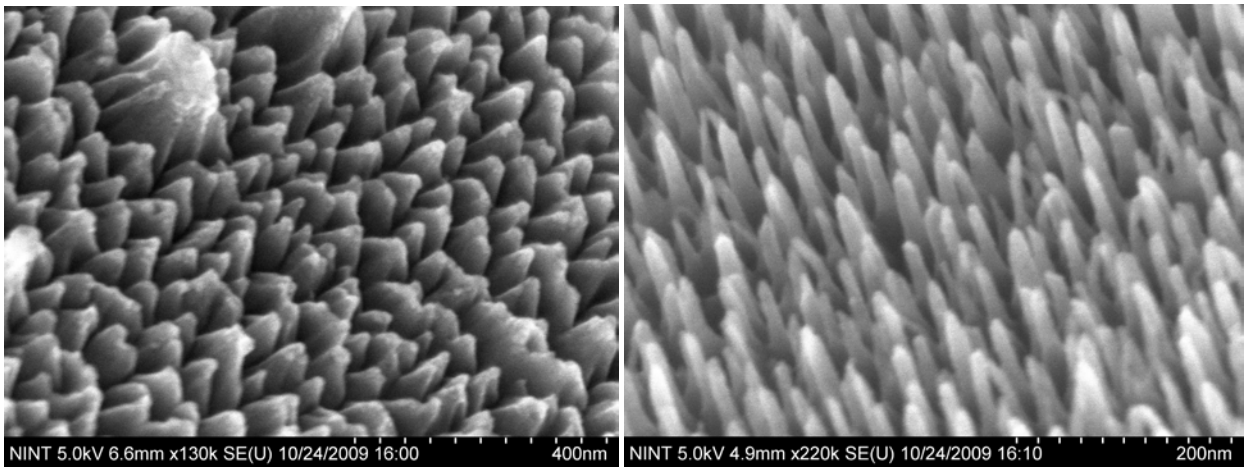

Figure 20 (left): SEM image of ridges on surface of sample M.
Figure 21 (right): SEM image of pin-shaped peaks on sample SM.

Increased surface contours or roughening results in increased surface area. In Table 6, surfaces B, M, S and SM are listed in order of increasing surface area. Figure 22 shows a graph clearly indicating a pattern of increased bacterial capture as a function of increased surface area. SM samples deviate from this pattern per the explanation hypothesized in Table 6.

Controls show that without TSP bound to the roughened MSRCS surfaces, bacterial capture is negligible. This indicates that the modified surfaces coated with BSA show high specificity. Specificity was enforced by using TSP in combination with a blocking protein such as BSA. One of the intentions in creating MSRCS surfaces was to increase the likelihood that TSP would be vertically immobilized and to improve steric accessibility of the binding sites of TSPs. However, there is no way to prove that the shape of the bumps caused TSP to be vertically immobilized. Since 'roughened' MSRCS surfaces have increased surface area, more TSP can be immobilized per unit surface area. Since non-specific binding was shown to be negligible for MSRCS surfaces, and since we know that the P22 TSPs allow capture of bacteria onto these surfaces, we can hypothesize that the increase in bacterial capture can only be attributed with certainty to increased TSP immobilization (rather than to improved TSP orientation). Comparison to control surfaces C2 suggests that this nano-scale surface 'roughening' increases TSP immobilization over that of flat silicon.

When comparing SEM images of samples B, M, S, we see that bacterial captures increase as a function of smaller domain size (sizes of mountain-shaped bumps). This suggests that as the slope of the bumps approaches 90° (vertical pin-shaped structures) the bacterial capture increases. Contradictory to the above pattern, although SM samples possess the narrowest domains or spine-shaped 'bumps', bacterial capture decreases. This suggests that there may be an ideal slope for the sides of the mountain-shaped bumps. However, there is insufficient data to ascertain what this ideal slope may be.

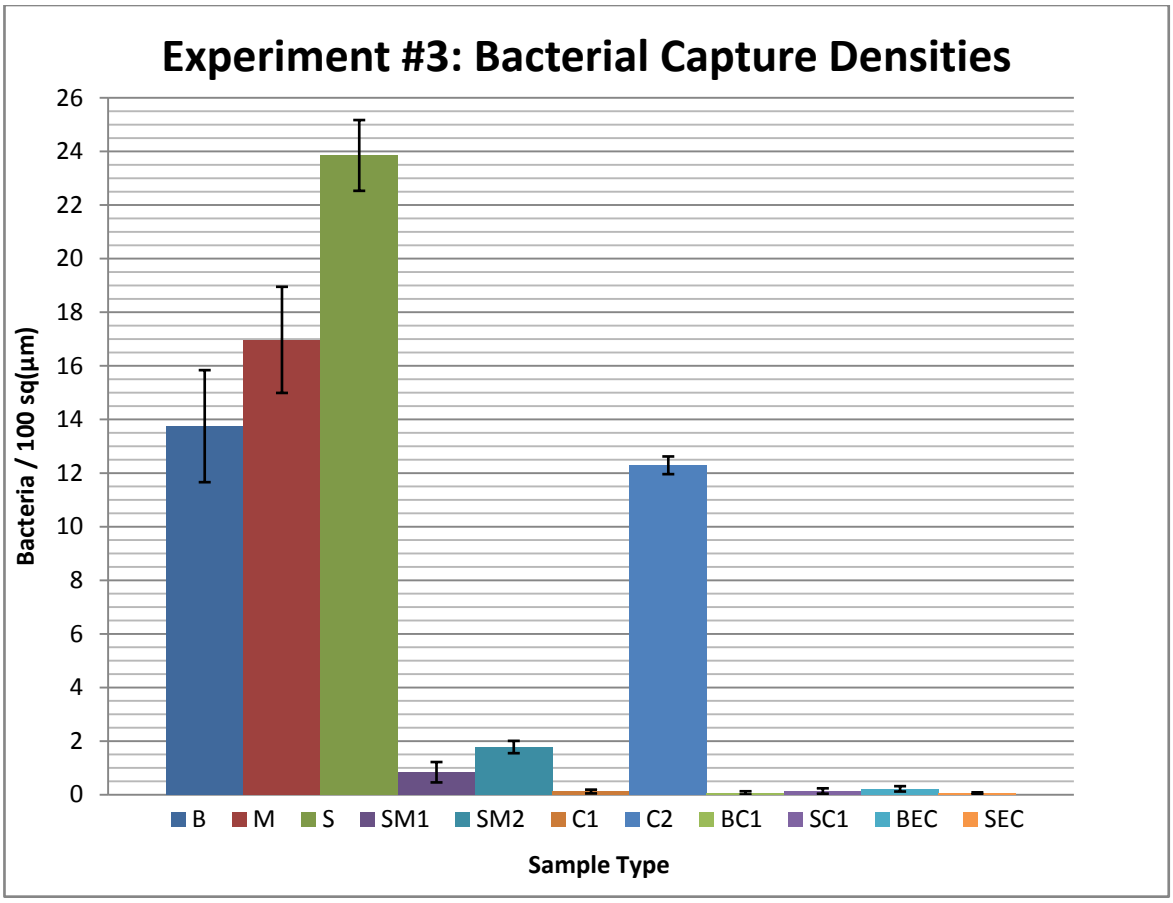

Figure 22: Bacterial capture densities in experiment #3

4.5. Summary

The bacterial capture capability of MSRCS surfaces is clearly higher than that of flat silicon. The MSRCS surfaces are highly specific to *S.* Typhimurium capture when P22 TSP and BSA are present on the surface. When the BCD results of MSRCS surfaces are compared to those of: gold thin-films (prepared with TCEP reduced 1µg/ml TSP solution at 40 ˚C: 23.28 ± 0.45 bacteria/100µm^2), disulphide linkage with MPTES-coated silicon (prepared in TCEP reduced 1µg/ml TSP solution at 40 ˚C: 12.21 ± 0.19 bacteria/100µm^2), wild type TSP (on gold thin-films at 40 ˚C: 3.52 ± 0.34 bacteria /100µm^2), and whole phages (immobilized on cysteamine functionalized gold substrates: 4.4±0.26 bacteria/100µm^2), we see that S type MSRCS samples attain the highest bacterial capture densities.

The above surface optimization experiments suggest that an increase in surface area contributes to improved bacterial capture as a result of increased TSP immobilization. The bacterial captures of B, M, S, and SM surfaces suggest that there may be an optimum slope for the sides of the mountain-shaped bumps. There is insufficient data to determine what the ideal slope or orientation of the ridges should be to maximize bacterial capture. However, the data does suggest that an optimum orientation exists.

5

Conclusions & future work

There are many products for pathogen detection currently available on the market. These products have various challenges and issues. Some are unsuitable for applications where speed is critical, while others are expensive, oversensitive to impurities, or require labelling which slows the procedure.

Biosensors offer the possibility of producing fast results with reduced sample preparation time. Broadly speaking, they are composed of two parts: the transduction mechanism, and the recognition element or probe. The type of probes, method used to immobilize them, and surface on which they are immobilized can significantly impact data gathered from a biosensor. Probes can be DNA or nucleotide-based, protein-based (antibodies, enzymes, etc), or bacteriophage-based. Recent findings show that the TSP from P22 bacteriophages can be used as a recognition element in biosensors. Research work conducted in this project agrees with and verifies these findings.

The purpose of this research project was to characterize TSP adsorption behaviour on gold and silicon surfaces and to find a method to improve bacterial capture beyond the work conducted by Singh *et al*.

Methods for immobilization of TSP were explored, and TSP surface density counts were taken for genetically modified TSP immobilized on surfaces such as gold thin-films using thiol linkage and silicon using amide

linkage schemes. The TSP counts taken support data indicating that adsorption is dependent on the immobilization scheme used.

Methods to improve bacterial immobilization were explored, roughened mountain-shaped ridge-covered silicon (MSRCS) surfaces were created, and their bacterial capture densities (BCDs) were measured. BCD measurements showed that MSRCS surfaces attained bacterial capture close to or better than that of TSP immobilized on gold using thiol linkage. This work shows a very clear trend that surfaces with increased surface area, such as MSRCS surfaces, produce bacterial capture densities similar to or better than that of thiol linkage immobilization schemes on gold. The objective of this project was successfully met with the novel application of MSRCS surfaces to the purpose of capturing bacteria.

Although this thesis contributes data towards the development of a commercializable biosensor, more work is needed to encourage widespread TSP use. To allow use of TSP as a probe in QCM-based biosensors, fabrication methods to produce MSRCS surfaces need to be translated over to quartz surfaces. A demonstration that this technology is viable with commercially available portable sensor systems would also make an excellent marketing tool to publicize this technology. One such product is the Spreeta SPR sensor system, a small, disposable SPR sensor manufactured by Sensata Technologies. Development of ELISA-like or high-throughput screening (HTS) TSP-based assays for detection of bacteria followed by comparative testing (to existing products) would also convey the value of using TSP as a molecular probe/ recognition element.

Other areas to explore include cleaving the poly-his tag from the TSP protein (followed by retesting its functionality) to see how this affects oriented bacterial binding. Such work requires studying the modified protein's orientation and bacterial capture on various surfaces.

6

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